An epidemiological investigation of *Coxiella burnetii* and *Chlamydia* spp. as infectious agents causing abortion in dairy cattle in Uruguay

Submitted by

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Abstract

Infectious diseases are the leading aetiological cause of bovine abortion globally as well as in Uruguayan dairy farms. Studies conducted in this country showed that a proportion of aborted fetuses without diagnosis present histological lesions compatible with the action of an infectious agent. These undiagnosed abortion cases could be due to infectious agents not currently included in the battery of diagnostic tests routinely applied. Infectious agents such as *Coxiella burnetii* and *Chlamydia* spp., typically considered to have a minor or relative marginal role in causing abortion in cattle, have never been systematically investigated in Uruguay. This thesis challenged the current diagnostic protocols, seeking evidence supporting the inclusion of these agents to dismiss the number of undiagnosed cases. This work investigated the association of these pathogens with cattle abortion in commercial dairy herds and the potential as zoonotic threats in the local context.
The thesis will first introduce the main characteristics of dairy production in Uruguay (**Chapter One**) and thoroughly review *Coxiella burnetii* and *Chlamydia abortus* (**Chapter Two**). After this, two main sections will be presented. The first section including a systematic review and meta-analysis (**Chapter Three**), and the second section comprising the observational fieldwork studies conducted out on dairy farms in Uruguay, which includes a cross-sectional study of pooled milk (PM) samples from commercial herds (**Chapter Four**), a case-series sampling of placentas (**Chapter Five**), a case-control sampling of aborted and non-aborted dairy animals (**Chapter Six**), and finally a retrospective cohort evaluation of workers exposed to a local bovine Q fever outbreak (**Chapter Seven**). A final discussion (**Chapter Eight**) will consider the main findings' implications and integrate these results into the general body of knowledge.
The systematic review and meta-analysis evidenced bulk tank milk (BTM) sampling as a broadly used epidemiological methodology for large-scale investigations of *C. burnetii* and provided a global framework of the prevalence of *C. burnetii* in collective milk samples from commercial farms. This study showed a widespread herd-level circulation of *C. burnetii* in bovine dairy farms by reporting a high overall global prevalence of 37.0% ($CI_{95\%}$ 25.2-49.5%). A meta-regression showed the herd size as the most relevant moderator, with the odds of a BTM sample testing positive doubling with every unit increase. This *C. burnetii* meta-prevalence roles as a benchmark for comparison with the findings of the molecular investigation on PM samples obtained from local dairy farms. The mass-scale molecular evaluation on PM samples evidenced a low incidence (1.7%) of *C. burnetii* DNA. The findings showed that clinically healthy (asymptomatic) cows might shed the bacterium, raising awareness of Q fever as potential food safety and public health concern considering the *C. burnetii* survival as a milk-borne pathogen in unpasteurised milk and raw dairy products. Due to the exceptionally high infectivity, low infective doses, and aerosol transmission, the culture of *C. burnetii* and Chlamydiales should be done on BSL-3 laboratories not currently operating in Uruguay. Bacterial cultivation was not attempted in any of the studies conducted in this thesis. Hitherto the lack of diagnostic tools and the impossibility of doing culture have restricted local epidemiological investigation of these agents. Therefore, developing diagnostic tests to be used routinely in domestic laboratories is imperative to save costs and optimise currently available facilities and work with higher autonomy. For this purpose, a published protocol targeting well-evaluated genes was adapted to provide an available tool for local laboratories. *Coxiella burnetii, C. abortus, and C. pecorum* were investigated for the first time using an m-PCR in placentas from aborted dairy cows. *Coxiella burnetii*-DNA was detected and quantified in those samples, which supported this bacterium as an abortifacient agent in Uruguay. No co-infections of these pathogens were found. Evidence supporting Chlamydiales as a source of cattle abortion remain blurred. *Coxiella burnetii* was detected on the aborted placenta from a cow from an artisanal cheese-producing farm. Consumption of raw milk and dairy products represent a potential source for human infection. This finding underlined that the public health risk posed by *C. burnetii* should not be neglected and should be emphasised the need for on-farm milk pasteurisation by local artisanal cheesemakers. Molecular investigation of *C. abortus* in vulvo-vaginal swabs samples showed no evidence of this bacterium neither in aborted nor in control animals. Difficulties in identifying low-grade infection and evaluating a single sample per animal would have constrained the detection. The first attempted studies conducted so far support *C. abortus* as a no substantial abortifacient agent in cattle from Uruguay. Serological evidence confirmed the local bovine population as a potential reservoir for *C. burnetii* infection in humans. Anti-*C. burnetii* phase II IgM and IgG immunoglobulins were detected in a group of farmworkers and laboratory technicians exposed to aborted dairy cattle or aborted materials (fetuses and placenta) by indirect immunofluorescence.
Molecular approaches were assessed, optimised and validated on veterinary clinical samples such as aborted placentas, vulvo-vaginal swabs or collective milk samples, providing valuable alternatives beyond the bacterial culture and isolation. The thesis presents original research studies that utilise different epidemiological strategies to search for evidence of an association between the infection by the pathogens and the occurrence of bovine abortion.

To my lovely sister Victoria because by saving Juan, she saved us all a bit.

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Covid-19 statement

Unfortunately, due to Covid-19 restrictions, some planned research activities were disrupted, cancelled or not completed. A proportion of pooled milk samples analysed in Chapter Four were not analysed due to disruption of access to laboratory facilities. The results and discussion included in this thesis will focus on samples wholly analysed. The fluorescent *in situ* hybridisation (FISH) evaluation planned to be performed on aborted placentas samples, initially processed in Uruguay and transported to the UK, at the diagnostics laboratory of the Bristol Veterinary School at Langford, was not conducted. Facing this limitation, some samples were analysed by immunohistochemistry (IHC) to investigate the presence of these pathogens within the lesion. Samples were sent for IHC evaluation abroad, as the required antibodies were not locally available.

Author's Declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's *Regulations and Code of Practice for Research Degree Programmes* and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such.
Any views expressed in the dissertation are those of the author.

SIGNED: .... DATE: ....

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Chapter 1: **Reproductive infectious diseases in Uruguayan dairy farms**

This chapter will first describe some of the major characteristics of the dairy industry in Uruguay and how this industry has changed over the last ten years (2009-2020). I will review nationwide milk production, commercial and sociological aspects, as well as the role of institutions in local dairies. **Chapter One** provides background information about the Uruguayan dairy industry to identify research gaps and outline the main aims and objectives of this thesis.

1.1 Characteristics of local dairy production and its evolution over the past decade

Uruguay is one of the leading producers and exporters of milk and dairy products in South America. This country has a total area of 17.4 million ha (173,620 km2), temperate climate conditions (annual mean temperature of 17.5°C) and average yearly precipitation around 1,300 – 1,400 mm. Though not a large country in terms of land area, Uruguay has a great variety of types of soil. The deepest soils with the highest potential and fertility are located in the southern and south-western regions of the country, mainly along the littoral of the Uruguay river (Figure 1.1), where dairy production is mostly concentrated, although it is developed throughout the country. During 2018, Uruguay achieved a total milk production output of 2,173 million litres, one of the highest production totals recorded over the last decade (DIEA, 2019).

The local dairy industry is characterised by the following aspects related to animal nutrition, dairy animals and milking routine. It features outdoors, pasture-based production with an intermediate level of concentrate inclusion. It is based fundamentally on a rotation of mixed legume-grass pastures and annual crops, with the addition of a varying proportion of silage, hay and concentrates depending on the management schemes applied (Chilibroste *et al*., 2010; Dini *et al*., 2012). Due to the typical seasonal pasture growth, farmers often make some use of bought-in supplements. With respect to the dairy animals, according to the last available data, Holstein-Friesian as the predominant breed (90%) in the national dairy herd that is composed of a total of 766,000 animals and of those 319,000 are milking cows (DIEA, 2019). The average herd size is 88 milking cows/herd, calculated based on the most recent reported number of milking cows and dairy herds. Commonly, cows are mechanically milked twice a day and silage and concentrate feed supplementation is given during milking. The milking process is performed in buildings popularly known as “tambos”, and most of them have their own cooled milk tanks.

1.1.1 *Productive traits*

I consider two crucial determinants of the profitability of the dairy industry: productive and reproductive traits. In terms of productive parameters at the individual and herd level, cows produce an average of 18 litres/day, and the annual production is around 5,039 litres/total number of cows (milking and dry cows). The analysis of the monthly distribution of the milk production over the past decades has revealed a noticeable peak of production during Spring (September, October and

November) (DIEA, 2019). According to recent data, milk production per hectare climbed to 8,500 litres in 2018 (DIEA, 2019).

1.1.2 *Reproductive traits*

With regard to reproductive aspects, farmers with different scales of production tend to utilise different breeding approaches. Whereas most small-scale farms have natural breeding by bulls, most of the large-scale farms use either artificial insemination and breeding by bulls; or only artificial insemination breeding (INALE, 2014). Calving takes place all year round, but Autumn and Winter are the predominant seasons. Regarding the generation of reproductive records and how this data is stored, there is a wide range of approaches, with the approach chosen depending on the scale of production. Most of the small-scale producers have no records of reproductive performance. As production scale increases, several tools such as paper records, general electronic sheets, and specific reproductive software, are incorporated (INALE, 2014).

1.1.3 *Milk consumption in Uruguay*

Milk consumption by local people is unusually high, in Uruguay, the per capita consumption of dairy products is estimated to be 277 litres/year, representing a daily intake of around 600 ml (DIEA, 2019). This milk consumption is higher than most countries' recommendations in the last report of FAO about milk and dairy products in people nutrition (Muehlhoff *et al*., 2013). Uruguayan milk consumption seems to be based on well-established traditional food habits, although no formal investigation into this behaviour has been conducted.

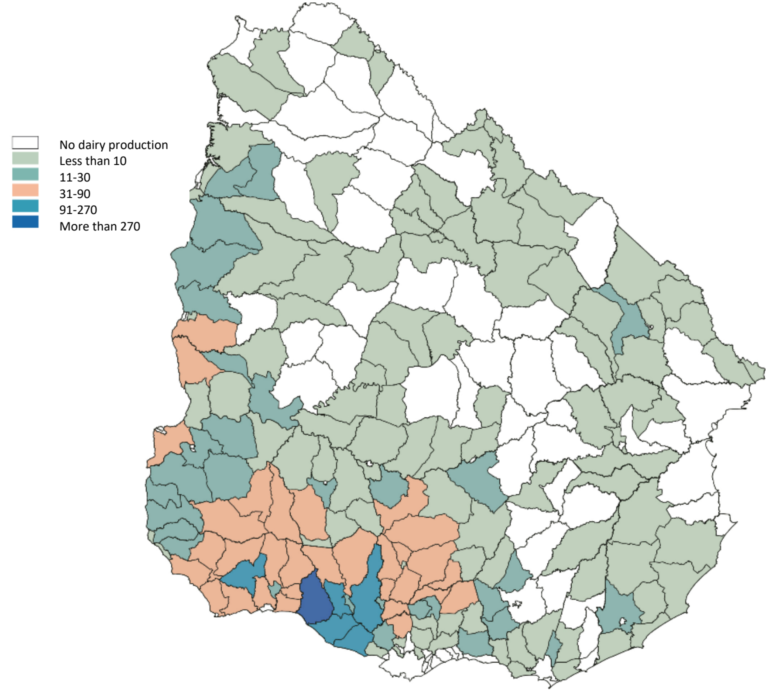


Figure 2.1: Number of commercial dairy farms per each police section (smaller land division) during 2017-2018.

1.1.4 *General characteristics of the dairy industry in Uruguay*

Turning now to trade features, Uruguay has a solid farming tradition with an agricultural sector strongly oriented towards exports. In this scenario, the dairy industry has great economic significance. Uruguayan exports reached 7,495 million dollars during 2018 and of those 5,547 million dollars, 74% came from the agricultural subsector. Within this subsector, dairy industry exports rose to 647 million dollars (11.7%), just behind other goods such as meat and live animals, agricultural, and forest products (DIEA, 2019). The majority of the local production (70%) is exported, mainly as milk powder, cheese and butter to regional and extra-regional destinations. For exports during the last year, Algeria (29%), Brazil (20%), Russia (17%), China (7%), and Cuba (6%) were the first five final destinations of Uruguayan dairy products (DIEA, 2019). Due to most of the local production being export-oriented, the Uruguayan dairy industry is highly dependent on its competitiveness against other exporting nations (Fariña & Chilibroste, 2019) and it is susceptible to price changes in the world market.

Local dairy has experienced perceptible structural changes. According to the national agricultural database, Uruguayan dairy production has grown from 1,694 million to 2,173 million litres during the last decade (2009 to 2018) (DIEA, 2019) with an average mean growth rate of 2.98% per year (Figure 1.2). Throughout this period the area allocated to milk production diminished from 800 thousand ha to 754 (−5.8%), and the total number of dairy farmers, taking into account both milk sellers and milk farm-processing farmers, dropped from 3,367 to 2,662 (−21%) (DIEA, 2019). When analysing changes in the number of farms in terms of farms size, we find that those classified as small (< 50 ha) and medium-sized (50 to 500 ha) suffered from a significant reduction (-30.5% and -4.3% respectively), in contrast to big farms (> 500 ha) that increased 17.5% (DIEA, 2019) (Figure 1.3). Based on this data, we can say that local dairy production seems to follow global trends characterised by farms size increment correlating to reductions in the number of farmers (Clark *et al.,* 2007; Klerkx & Nettle, 2013).

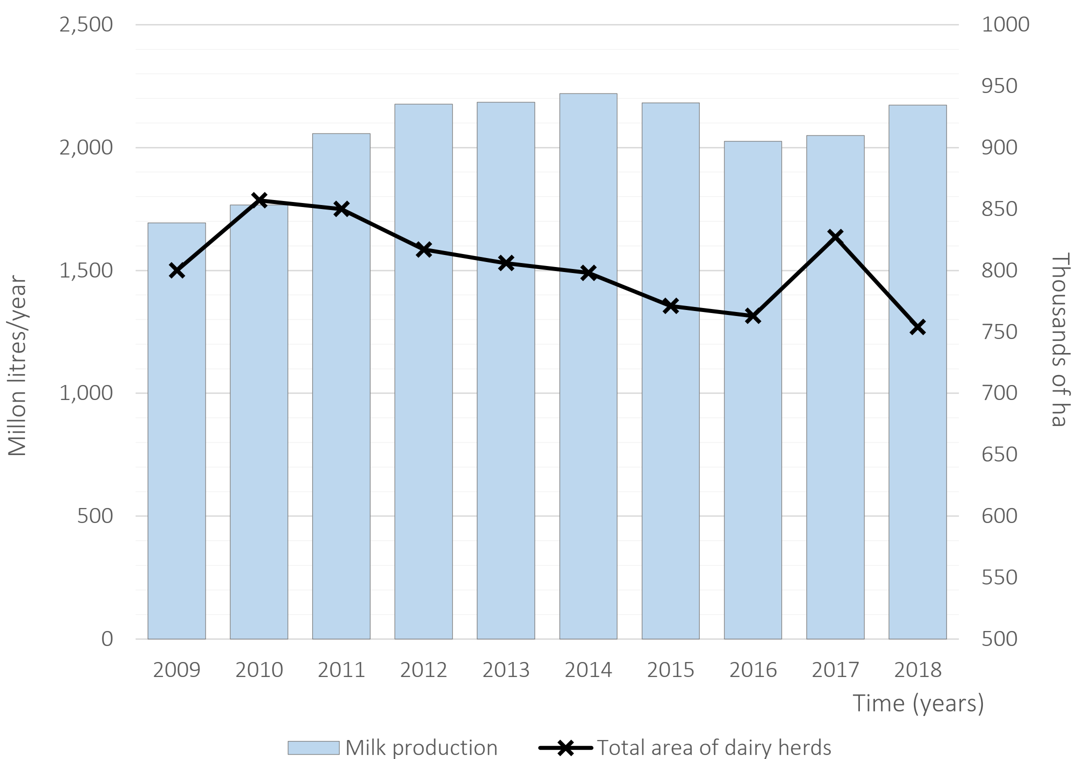


Figure 2.2: Evolution of milk production and dairy farming's total area over the past decade (2009-2018). Constructed based on statistics of DIEA, 2019.

Although not the focus of this thesis, some sociological aspects deserve to be mentioned. As stated above, there is a trend of decreasing numbers of small-medium farms; however, these types of farms are still the most prevalent. Almost 30% of current dairy farms that send milk to commercial supply chains comprise 50 hectares or fewer (DIEA, 2019). Most of these smallholders are family/familiar production systems where the primary workforce is made up of the members of the family. The majority of small-scale farms (83.4%) utilise only family labour, while this type of labour is not present in the larger farms where only hired labour is employed (Quesada, 2017). Milk production as a cultural phenomenon in Uruguay is characterised by idiosyncrasy, in as much as dairy activities are frequently taught by being passed from generation to generation. In this vein, local dairy is the agricultural sector with the highest female participation rate (Chiappe, 2001). All these aspects put the accent on the strong socio-economic significance of dairy in Uruguay.

Local dairy is affected by demographic changes brought about by people moving from rural to urban areas. Although people working on dairy farms have a robust feeling of identification with this activity, recent generations have started feeling the necessity of exploring alternatives, often due to the financial needs of social desire; thus, migration from the countryside to cities is a common phenomenon. This transformation is a current source of concern. Future systems should embrace technologies that reduce the effort and time of farming routines to positively impact the

farming lifestyle and make the dairy industry more attractive as a source of work for coming generations (Tarrant & Armstrong, 2012).

Turning now to other sociological aspects, the literacy of farmers has also been recently investigated. The majority of farmers from small-scale farms (55.6%) with less than 50 ha only complete primary education. By contrast, most of the primary farmer decision-makers (40.2%) from big farms (more than 500 ha) attain a university level of education (Quesada, 2017).

As previously stated, small-medium farms, though reduced in number, are still the most numerous. Usually, in small-scale systems, the farmworker and the entire family lives on the farm (Quesada, 2017). This suggests close contact between people and dairy cattle. The cohabitation of humans with livestock, as well as with companion animals, is thought to pose a heightened zoonotic risk of transmission. This aspect, frequently underestimated in the local context, will be a subject of consideration in the following chapters.

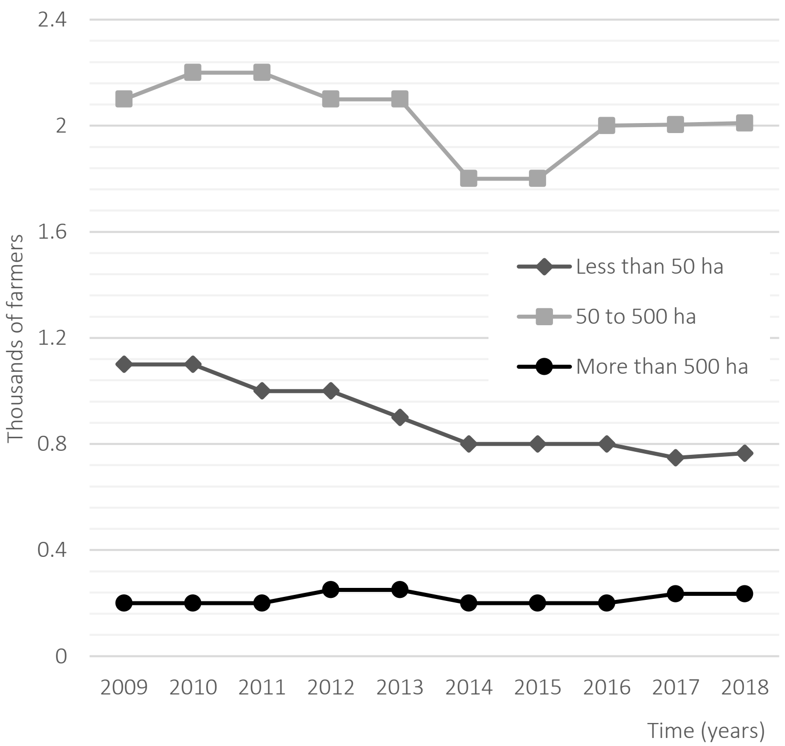


Figure 2.3: Evolution of dairy farm size over the past last decade (2009-2018). Constructed based on statistics of DIEA, 2019.

One noteworthy feature that characterises the dairy industry in Uruguay is the role of cooperatives. Cooperatives dominate the Uruguayan dairy chain. The vast majority of milk production (71%) is industrially processed by a single cooperative named The National Cooperative of Producers of Milk, “Cooperativa Nacional de Productores de Leche” (CONAPROLE), while the rest of the

production is delivered to another 13 small dairy plants and cooperatives (Viera *et al*., 2013; INALE, 2016). CONAPROLE has a leading role in the dairy sector with a payment system that presents a strong economic incentive for milk production with low cell count and provides advisory technical assistance for herds facing problems attaining this goal. Additionally, there is a strong degree of co-operation among dairy farmers designed to help them cope with land and resource limitations. For instance, the use of collective farms designated for heifers mating, hire technical assistance and cooperative use of expensive equipment, are frequent practices.

Other organisations besides the cooperatives, play a central role in the agricultural subsector. For instance, the Ministry of Livestock, Agriculture and Fishing of Uruguay, "Ministerio de Ganadería, Agricultura y Pesca" (MGAP) is another crucial participant in the local dairy industry. MGAP is a governmental organisation that acts as a policymaker and is responsible for the quality control of milk and the phytosanitary control of animals. This governmental department is also engaged in the implementation of the national traceability platform for individual livestock identification. Each bovine born in the country carries a unique identifier that is recorded in a database enabling traceability that is a valuable tool for food security.

1.2 Problem identification

Thus far, this chapter has focused on a description in general terms of the dairy industry in Uruguay. The following section will look at the different factors influencing the local animal stocking rate. The most relevant factors will be briefly mentioned before we go on with the main factor that concerns this thesis.

Based on the latest published data, dairy herd growth in Uruguay has been close to zero in the last few years (DIEA, 2019). Also, local dairy farms have a relatively low animal stocking rate (around 0.72 cows/ha) when compared to other countries in the database of the International Farm Comparison Network (IFCN) (Hemme, 2017). Restricted herd growth is identified as a clear limitation, and specialists claim that increasing the stocking animal rate seems to be the most sustainable path to developing production for local dairy systems (Fariña & Chilibroste, 2019). The constrained national herd growth could be aided by reducing factors such as fairly elevated culling and mortality rates in cows (Pereira *et al*., 2017); late age of first calving in heifers (Sotelo, 2017); low reproductive performance (involving both early embryonic losses and abortions) (Meikle *et al.,* 2013; Pereira *et al.,* 2017; Sotelo, 2017); and high mortality rates in calves (Schild, 2017).

The main focus of this thesis is poor reproductive performance. Within the interpretation of factors affecting the animal stocking, restricted reproductive efficiencies seem to be of great importance. The last survey of dairy farms carried out during 2014 revealed that 30 to 55% of dairy farms lack adequate animal replacement, and this limiting factor was even worse on those farms with lower annual milk production ranging from 100 to 300 thousand litres (INALE, 2014), these being mostly

small and medium farms. Along these lines, the improvements in productivity registered during the last decade have not been accompanied by reproductive efficiency advances. As mentioned above, the local milk production showed a mean annual growth rate of 2.98% during 2009 – 2018, reaching one of the highest historical production levels ever (DIEA, 2019). By contrast, during the same period, the calf stock suffered a persistent decline with a mean annual growth rate of -1.4% (DIEA, 2019) (Figure 1.4).

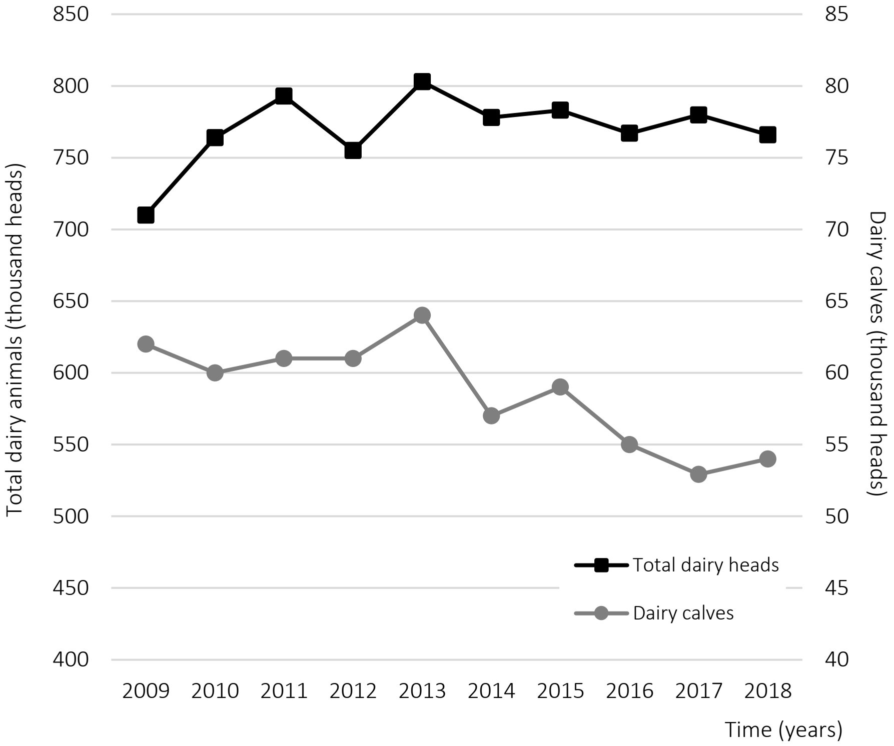


Figure 2.4: Evolution of dairy animals' national stock over the past decade (2009-2018), including total dairy animals and calve numbers. Constructed based on statistics of DIEA, 2019.

Only limited local information about reproductive efficiency in dairy cattle such as infertility, embryologic losses, and abortion incidence is currently available. Among the few existing studies, a technical 4-year report based on data from 200,000 lactations revealed that the calving-to-conception interval increased up to 172 days (INML- Mejoramiento Lechero 2014), 47 days longer than the last published data (Cavestany, 2000). This delay in achieving a successful pregnancy evidences an apparent alteration in reproductive efficiency. Additionally, a big internal database that includes records kept on 14,233 pregnant primiparous and multiparous cows from herds located in the densest dairy region of Uruguay, estimated pregnancy losses due to abortions to be near 7.5% and reaching 12% when all losses are considered (embryonic losses and abortions) (Dr Carlos Lemaire, personal communication, 2018). Although this rate of loss was estimated using a study

which was not a nationally representative sample, the rate revealed is consistent with regional published studies. For instance, a survey carried out on Argentinian dairy herds with continuous calving showed an abortion rate of 8.4% (Ricagni, 2006).

The impact of fetal losses can be estimated based on existing records. Since Uruguay has a current population of 319,000 dairy cows (DIEA, 2018), assuming a hypothetical rate of annual losses of around 7.5%, there could potentially be about 24,000 of fetal losses (embryonic mortality and fetal death annually). When considering the problem on a national scale, we see this possible massive loss of calves is significant. It not only negatively impacts the production of goods and leads to economic failures, but also has adverse effects on animal health (Holler, 2012). The economic costs will depend on when the loss occurs in relation to the gestation age but will include a reduction in milk production. An increase in production costs (repeat insemination, labour and veterinary treatments) may also lead to early culling of animals and certainly will lead to a shortage of future animal replacements (Thurmond & Picanso, 1990; Hovingh, 2002; de Vries, 2006). Farms with adequate reproductive, sanitary and nutritional conditions are expected to have a frequency of annual abortion rate of up to 5%, the point at which intervention should be undertaken (Kinsel, 1999; Laven, 2020). Considering this "normal" rate, a reduction of the current percentage by 2.5 points (from 7.5 to 5%), would represent the birth of almost 8000 calves each year. This estimate shows the potential impact of improvements in reproductive efficiency and, consequently, reduced pregnancy losses across the national animal stock.

1.3 The research gap in locally diagnosed reproductive diseases

This section will summarise existing research and highlight the research gap that needs to be addressed.

Infectious diseases are the major aetiological reason for bovine abortion globally (Thobokwe & Heuer, 2004; Carpenter *et al*., 2006), as well as in the USA (Kirkbride, 1992; Anderson, 2007) and in South American countries such as Brazil (Antoniassi *et al*., 2013), Chile (Paredes *et al.,* 2011), and Argentina (Campero *et al.,* 2003). Based on the few published studies, infectious diseases are also the most frequent cause of abortions in the Uruguayan dairy herd (Easton, 2006; Riet-Correa *et al*., 2014). A 4-year case series report (2002–2005) conducted by the Veterinary Laboratory Division of the MGAP analysed 431 bovine fetuses (54% from dairy farms) (Easton, 2006). Different aetiological agents were identified: *Leptospira* spp. (41%), *Neospora caninum* (36%), *Campylobacter fetus* (13%), *Brucella abortus* (3%), *Streptococcus* spp. (1.6%), *Salmonella* spp. (0.8%), *Bacillus licheniformis* (0.8%), Bovine viral diarrhoea virus (BVDv, 2%) and Bovine herpesvirus 1 (BoHV-1, 1.1%). Interestingly, 44% (190/431) of the analysed fetuses were undiagnosed, and of those 6.3% (27/431) had histological lesions compatible with the action of an infectious agent, although the specific cause could not be determined (Easton, 2006).

This scenario raises a question about what is happening to undiagnosed fetuses. The proportion of undiagnosed cases could be attributed either to a non-infectious cause or to an infectious abortion in which the determination of pathogen was not achieved. The latter would be possible due to the delay between fetal death and the expulsion of the aborted fetus. Diagnosticians often deal with different degrees of autolysis because the expulsion occurs several hours after fetal death. The autolysis makes complicated the isolation of agents and the interpretation of gross and histologic changes. Nevertheless, these cases could be due to infectious pathogens not currently included in the battery of diagnostic tests routinely applied.

This proportion of undiagnosed aborted fetuses with histological evidence supporting an infectious agent's action shows that there is still much to do in Uruguay in terms of understanding cattle abortion and the investigation of other infection pathogens must be addressed. Some infectious agents such as *Coxiella burnetii*, *Chlamydia abortus* and *Chlamydia pecorum* have never been systematically investigated in Uruguay before. The occurrence of these pathogens and their impact on reproductive performance is currently unknown. The proportion of undiagnosed abortions may be reduced with the further inclusion of these pathogens in the routine diagnostic protocol. Additionally, this study may have useful implications from the public health perspective as two of the current agents represent zoonotic threats.

Only limited information on reproductive efficiency is available; however, there is a high estimated incidence of abortion in cattle, possibly due to infectious diseases (7.5%, Dr Carlos Lemaire, personal communication), affecting reproductive productivity and the national animal stock. The estimated incidence from published evidence is likely an underestimate of the true burden of infectious disease considering that a high proportion of undiagnosed fetuses (44%) had histological lesions consistent with an infectious agent's action. Limited and not systematic testing had been undertaken for some abortifacient agents popularly known to have a relatively marginal role in cattle abortion, such as *C. burnetii* and *C. abortus*. In this thesis, I aim to address this knowledge gap by investigating these agents in commercial dairy herds (**Chapter Four**), their association with abortion (**Chapter Five** and **Chapter Six**), and their feasibility as a zoonotic threat in the local context (**Chapter Seven**).

2.1 *Coxiella burnetii*

2.1.1 *Coxiella burnetii* general characteristics

2.1.1.1 Nomenclature and Taxonomy

In 1933 Edward Derrick studied some outbreaks of a febrile illness affecting abattoir workers which occurred in Queensland, Australia. As the causative agent was unknown the illness was simply termed "Q Fever", with "Q" coming from "query", until a suitable name could be found once fuller knowledge of the disease was developed (Derrick, 1983). Derrick conducted several unsuccessful attempts to reproduce the disease. Subsequently, further studies performed by Mavis Freeman and Frank Macfarlane Burnet utilizing samples from Derrick's patients (blood or urine), experimentally replicated the febrile response in guinea pigs and also revealed the difficult filterable characteristic of the organism (Burnet & Freeman, 1983). To evidence that this organism was the agent responsible for the febrile illness in the abattoir workers, an agglutination test using worker serum and a suspension of clarified tissue from the infected guinea pigs was run (Burnet & Freeman, 1983; Hechemy, 2012).

During these same years, another research group in the USA led by Gordon Davis and Harold Cox, found a similar agent when studying the Rocky Mountain Spotted Fever. They tried to isolate the infectious agents from *Dermacentor andersonii* ticks gathered near Nine Mile creek in Montana (Davis *et al.*, 1938). The researchers ruled out Rocky Mountain Spotted fever due to the absence of the typically spotted rashes in guinea pigs with febrile illness. This promoted additional studies on this unknown agent. First, Davis and Cox evidenced relevant features about the agent such as its filterability capability through filters that retain ordinary bacteria, and then they showed the successful passage of the agent in guinea pigs (Davis *et al.*, 1938).

Additionally, the inclusion bodies, typically expected in viral infections, were not visualised using Giemsa staining (Cox, 1938). After a visit to Davis and Cox's laboratory, a researcher named Rolla Dyer developed fever and an influenza-like illness with symptoms similar to those previously found in the Australian abattoir workers. When researchers were confronted by this serendipitous finding, they inoculated blood obtained from Dyer in the guinea pig that later developed fever and clinical signs in line with those recorded in the previous studies. Further investigation was undertaken to gather more robust evidence supporting the hypothesis that the diseases affecting Dyer and that affecting the abattoir workers were due to the same agent. For this purpose, guinea pigs first infected by Burnet using blood samples from abattoir workers were cross-infected with organisms from Dyer's blood. These animals were shown to be immune to the second infection, which was highly indicative of a common organism found in *D. andersonii* ticks in the USA and the one responsible for the Australian "Q fever" cases.

As the agent exhibited similar morphology and staining responses as rickettsiae, it was first classified into the family *Rickettsiacea*. Cox proposed naming this organism *Rickettsia diaporica* because of its characteristic of filterability (McDade, 1990). It was renamed by the Australian research group *Rickettsia burnetii*, in honour of Burnet (McDade, 1990). Finally, Cornelius Phillip suggested renaming this organism *Coxiella burnetii*, for the significant contribution in discovering the Q fever agent made by both Harold Cox and Frank Burnet and thus to credit both groups of researchers (Phillip, 1948). Lately, phylogenetic studies based on 16S rRNA sequence analysis have evidenced that *Coxiella* is not closely related to the *Rickettsia* genus. *Coxiella burnetii* has been reclassified into the Coxiellaceae family, order *Legionellales* of the class *Gammaproteobacteria* of the Proteobacteria phylum (Drancourt & Raoult, 2005).

2.1.1.2 Coxiella burnetii *in the host cell*

Coxiella burnetii is an obligate intracellular Gram-negative bacterium, with a diameter of 0.2-0.4 µm and 0.4-1.0 µm in length (Drancourt & Raoult, 2005). This bacterium has two distinct types of cells that seem to be different stages of the developmental cycle of *C. burnetii*: a small cell variant (SCV) and large cell variant (LCV) (Norlander, 2000; Bielawska-Drózd *et al*., 2014). Both cell-types are characterised by different size, membrane structure, and proteome expression (Heinzen *et al.*, 1999). Additionally, LCV and SCV are metabolically different and present diverse resistance against environmental stressors, although with no apparent differences in terms of genome structure (McCaul *et al*., 1981). Host cells such as monocytes, macrophages, trophoblasts, adipocytes and epithelial cells are most frequently involved in the tropism of *C. burnetii* (Bechah *et al*., 2014; Boarbi *et al*., 2016; Sobotta *et al*., 2017).

When infection occurs, *C. burnetii* as the SCV cell type is typically engulfed by monocytes or macrophages, and after this first stage, SCV is enclosed in an acidified phagolysosome-like vacuole. Once the bacterium is inside the host cells, *C. burnetii* manipulates the parasitised cell's processes by disrupting cellular physiology and facilitating the instauration of the parasitophorous vacuoles. These vacuoles are generated from the normal phagosomes, following the normal canonical endocytic trafficking throughout endosomes to be converted into lysosomes (Romano *et al.*, 2007; Moffatt *et al.*, 2015). This bacterial strategy of surviving and replicating inside acidified vacuoles, and thus coping with the host cells' hostility, is unusual among intracellular pathogens (Moffatt *et al.*, 2015).

The vacuoles provide the optimal environment for the development of the LCV type, which is the metabolically and replicatively active cell form and has the active type 4B secretion system (T4BSS). This secretion system is of central importance for the intracellular niche formation and maintenance of the bacterium within the host (Beron *et al.*, 2002; Coleman *et al.*, 2004; Porter *et al.*, 2011). The differentiation of SCV into the LCV is estimated to occur within 1–2 days (Coleman *et al.*, 2004). Within the phagolysosomal parasitophorous vacuoles, the bacterium

appears like a mixture of the different-phase cell, including SCV in stationary-phase and LCV in the log-phase (Heinzen *et al*., 1999).

The LCV form changes back to the SCV variant throughout the stationary phase of replication (after roughly six days) (Coleman *et al*., 2004; Voth & Heinzen, 2007). The SCV type has the characteristics of a spore-like form of the bacterium, such as high resistance to an environmental stressor such as high temperature, osmotic pressure changes and UV radiation. The resistance of the SCV cell type allows its survival while maintaining infectivity (McCaul *et al*., 1981). Some proteins have differential expression between the two cell types; for instance, the ScvA protein appears in the SCV but not in the LCV (Heinzen *et al*., 1996). The alteration from one to the other cell type does not seem to involve genome changes (McCaul *et al*., 1981).

Coxiella burnetii presents several mechanisms to divert the parasitised host cell's normal metabolism and alter it into a container for bacterial survival and proliferation (Larson *et al*., 2016). The first step is the internalisation of *C. burnetii* into cells. This step is facilitated by binding to the receptors αvβ3 and αMβ2 integrins of the targeted cell also known as CR3, and monocyte/macrophages become activated after attaching to toll-like receptors (TLR) (Capo *et al*., 1999; Zamboni *et al*., 2004). The bacteria are passively taken up by an actin-dependent phagocytosis process involving several actin-interacting proteins (Rac1, mDia1, cortactin, ROCK, and RhoA) (Tujulin *et al*., 1998). During internalisation, the actin cytoskeleton is reshaped, and this process determines the modification of the host cell membrane. Along with bacterial infection the microfilaments play a crucial role (Baca *et al*., 1993a; Conti *et al*., 2014).

Once internalisation into the host cell is completed, *C. burnetii* initially reside in a membrane-bound organelle named *Coxiella*-containing vacuole (CCV) with phagolysosomal characteristics. In fact, the formation of large CCV greatly depends on the actin cytoskeleton of the cells that are being infected (Aguilera *et al*., 2009) and involves protein secretion by the bacterium as well (Howe *et al*., 2003). Numerous interactions between *C. burnetii* and endosomal-autophagosomal compartments occur during the itinerary of intracellular trafficking. This structure undergoes gradual maturation, which will first acquire, and then subsequently progressively lose, different endosome markers (known as early and later markers). Firstly, CCV acquires the endocytic marker Rab5 and microtubule-associated protein light-chain 3 (LC3) an autophagosomal marker. The CCV suffer from a gradual maturation that involves the loss of Rab5 and the acquirement of Rab7, lysosome-associated membrane glycoprotein 1 (LAMP1) and lysosomal enzymes, especially cathepsin D (CatD). This gradual maturation is speculated to be a biological strategy favouring the differentiation with the CCV niche from the metabolically inactive *C. burnetii* SCV towards the replicative bacterial form LCV. The latter, as a metabolically active form, can resist the deleterious impacts of the degradative lysosomal mechanism (Larson *et al*., 2016). During the intracellular journey, CCV uses several compartments as membrane sources (endosomes, lysosomes,

autophagosomes) (Campoy *et al*., 2011; Campoy *et al*., 2013). Finally, the CCV becomes capacious, occupying most of the cytoplasm.

2.1.1.3 Coxiella burnetii *lipopolysaccharide*

The main virulent factor identified for *C. burnetii* is the presence of a full-length lipopolysaccharide (LPS) in the membrane. *Coxiella burnetii* exhibits two different LPS phenotypes that determine different phase variations. One of the phenotypes is known as "phase 1" and presents the full length of LPS, like the smooth LPS of other bacteria. Phase 1 bacteria present a high degree of virulence, can be isolated from natural sources and can replicate even in immunocompetent hosts. The other *C. burnetii* phenotype, named "phase 2" is avirulent and incapable of replicating, lacks the O-antigenic region and resembles a Gram-negative rough LPS bacterium (Moos *et al*., 1987; Andoh *et al*., 2007; Toman *et al*., 2009). A phase 1 phenotype can switch to a phase 2 phenotype after serial passage in cell culture (Hotta *et al*., 2002). The phase 2 phenotype does not occur in the natural environment. The difference between these phenotypes lies in the O-antigenic, and this LPS modification is apparently caused by genomic deletion (Maurin & Raoult, 1999).

During infection, these two different LPS phenotypes generate phase-specific immunoglobulins. While phase 1 antibodies are generated against the full-length LPS of the phase 1 phenotype, phase 2 antibodies are produced against the common proteins located on the surface (Marrie & Raoult, 1997). The production of phase-specific immunoglobulin has great importance for the differentiation based on the differences in serology in acute and chronic infection. Immunoglobulins against phase I *C. burnetii* strain are commonly abundant developed in chronic Q fever cases, whereas antibodies against phase II are generated in higher levels in patients with acute Q fever (Fournier *et al.*, 1998).

2.1.1.4 The Coxiella burnetii *genome*

The complete genome sequence of the Nine Mile strain was first reported in 2003 and disclosed a circular genome composed by 1,995,275 base pairs. This study revealed numerous insertion sequence (IS) elements (29) that were dispersedly identified without any evident grouping pattern (Seshadri *et al*., 2003). Different copies of this family's elements, such as the *IS110*-like element, *IS1111*, *IS30* and *ISAs1*, were recognised. These variable insertion sequences like the *IS1111* is a central genetic characteristic of the *C. burnetii* genome. Further research has shown that the number of IS elements varies greatly among strains; for instance, the number of copies of the *IS1111* element can fluctuate between 7 and 110 (Klee *et al*., 2006).

Most strains of *C. burnetii* have a single extra-chromosomal plasmid. Diverse types of plasmid have been found: QpDV (33 kb), QpDG (42 kb), QpH1 (36 kb), and QpRS (39 kb) (Roest *et al*., 2013a; Chisnall, 2018). The establishment of acute or chronic Q fever was thought to be linked to the type of extra-chromosomal plasmid presented in the strain; however, further investigations have

identified and probed inconsistencies in this theory (Thiele & Willems, 1994). The plasmids share conserved regions principally involving proteins such as integrase and recombinase, and those proteins associated with replication (Lautenschlager *et al*., 2000). Additionally, some conserved regions among plasmid comprise the Dot/Icm type IV secretion system that plays a crucial role in the parasitophorous vacuole development that works as a replicative niche (Maturana *et al*., 2013). This system of secretion releases proteins with effector purposes that facilitate the reorganisation of cell compartments to achieve infection (Beare *et al.*, 2011; Qiu & Luo, 2017).

As stated above, the theory of genomic variation of plasmids as a relevant factor for the infection outcome was discarded, whereas host characteristics such as genetics and pre-existent health status have been identified as factors of great importance for the development of either acute or chronic infection (Porter *et al*., 2011; Eldin *et al*., 2017). While the genetic variation of plasmids appears to be linked to the geographic source where the isolate comes from (Glazunova *et al*., 2005), different animal reservoirs may hold strains that differ in virulence.

2.1.1.5 Environmental persistence

Coxiella burnetii can survive outside of a eukaryotic host cell because of its cell types variation. For instance, the SCV type has shown high resistance to osmotic shock, elevated temperatures, UV light, desiccation, and a variety of chemicals (disinfectants) (Coleman *et al*., 2004). The hardiness displayed under environmental stress conditions is critical for transmission as *C. burnetii,* mainly spread via aerosols (Hawker *et al*., 1998; Tissot-Dupont *et al*., 1999; Tissot-Dupont *et al*., 2004; van der Hoek *et al.,* 2011). The SCV cell type is considered to have a spore-like structure due to its similarities to endospores. However, the *C. burnetii* genome lacks the genes needed for endospore formation (McCaul *et al.,* 1981; Seshadri *et al.,* 2003; Coleman *et al.,* 2004). Thus, the use of the term ‘spore’ is simply descriptive, and this form is not comparable to clostridium or bacillus spores beyond its environmental resistance. *Coxiella burnetii* can remain viable in the stable non-replicating SCV form for several years, even in unfavourable conditions (Reimer, 1993; Kersh *et al.,* 2013a). Once in the spore stage, *C. burnetii* has a long persistence in numerous materials under diverse temperature conditions, for instance: for 7 to 10 months in wool at 20 °C, for at least a month in meat at 4 °C, and more than three years in milk powder at about 20 – 22 °C (Welsh *et al.*, 1959; Kumar *et al.,* 1981).

In addition to the fact that *C. burnetii* remains in the environment after its release, this bacterium is potentially spread over long distances, even reaching places with no presence of livestock. The bacterium is presumed to travel on the wind, small mammals, birds, vehicles, and human feet.

2.1.2 The effect of *Coxiella burnetii* in cattle

2.2.1.1 Pathogenesis of Coxiella burnetii

Coxiella burnetii infection in cattle is thought to occur after the oral uptake or the inhalation of the bacterium from the environment. When *C. burnetii* infection occurs through the respiratory tract, it is assumed that mononuclear phagocytes and alveolar macrophages are the target cells (Shannon & Heinzen, 2009). The phase 1 *C. burnetii* phenotype seems to aid in inhibiting the immune response although not producing a complete suppression of the process of phagocytosis, allowing in this way the bacterial incorporation into macrophages for replication (Bauer *et al.,* 2015). The study of bacterial dissemination among different organs identified the trophoblasts of the allantochorion as the main target cells after infection in pregnant ruminants (Roest, 2013). When infection occurs in a non-pregnant individual, *C. burnetii* replication takes place in several organs, including spleen (Roest, 2013). It is essential to consider that the trophoblasts cells are part of the fetal placenta and to some extent, are out of reach of the maternal immune system (Roest, 2013). This helps explain why even when immune response is activated after *C. burnetii* infection, it cannot prevent the replication of the bacteria by which they develop into trophoblasts.

When phagocytosis occurs, *C. burnetii* seem to stall the maturation process of phagosome (Bauer *et al.,* 2015). Several virulence factors are expressed in order to control infection development and replication. Also, any of numerous cellular effectors localised along different organelles and, in the nucleus, may develop central roles in how *C. burnetii* restraints secretory pathways, transcription process and the cell host apoptosis (van Schaik *et al.,* 2013).

The SCV transform into LCV after a “lag period” of 1-2 days, in this stage the initial phagosome converts into a parasitophorous vacuole (PV) characterised by an internal acid pH (4.5 - 5) and a membrane rich in cholesterol (Howe & Heinzen, 2005; Minnick & Raghavan, 2012; Gilk, 2012). LCV then begins its binary fission during a “log” growth period, that is a period extended until it achieves a stationary phase of replication around 4 - 6 days after the infection (Ghigo *et al.,* 2012). After this phase, the LCV can begin the transformation back to a SCV cell type (Voth & Heinzen, 2007).

Frequently nulliparous heifers from endemically infected dairy herds are reported as non-infected animals (Taurel *et al.,* 2011; Taurel *et al.,* 2012). The seroconversion mostly occurs then during the first 90 days in milk in primiparous animals (Nogareda *et al.,* 2012), indicating that infection arises in the periparturient period. In herds with endemic infection, dairy calves present maternal immunoglobulins against *C. burnetii* until 3 months of life (Tutusaus *et al.,* 2015).

Additionally, seroconversion followed by seronegativization was demonstrated in primiparous cows within the first 150 days in milk (Freick *et al.,* 2017). The precise moment when seroconversion takes place after the infection is still undetermined (Kennerman *et al.,* 2010); however, results suggest that *C. burnetii* infection in heifers (primiparous) possibly occurs during the last phase of gestation or during the beginning of the post-partum period (Freick *et al.,* 2017).

Bacterial shedding through vaginal material at parturition was demonstrated to occur before seroconversion in primiparous animals (Freick *et al.*, 2017). Another aspect that is not well elucidated in relation to *C. burnetii* infection is the potential occurrence of a late reactivation following infection during an early stage of life. This possible reactivation is thought to occur potentially during parturition, but this remains unresolved. A study postulated the reactivation of the bacterium to be likely associated with a peak in cortisol levels of pregnant dams around day 171 - 177 of pregnancy (García-Ispierto *et al.*, 2010).

2.2.1.2 Shedding routes from cows

The complete understanding of the shedding route of *C. burnetii* is crucial to create plans to restrict the risk of transmission and establish control plans. The main routes of bacterial excretion differ among ruminants (Rodolakis *et al.*, 2007). While parturition products (vaginal mucus and birth products) are the main source of shedding in goats and sheep, milk is the primary elimination route of *C. burnetii* in bovines (Adesiyun *et al.*, 1985; Schaal, 1982; Willems *et al.*, 1994). Recent *in-vitro* investigations revealed that udder cells permitted the highest replication levels of *C. burnetii* contrasted with placental, lung and intestinal cells (Sobotta *et al.*, 2017). Cattle may also present shedding of *C. burnetii* via vaginal mucus (Bildfell *et al.*, 2000; Berri *et al.*, 2002), faeces (Guatteo *et al.*, 2006), semen (Kruszewska *et al.*, 1997) and urine (Heinzen *et al.*, 1999). Even asymptomatic or seronegative cattle have been detected as *C. burnetii* milk shedders (Guatteo *et al.*, 2007). *Coxiella burnetii* can be extensively eliminated through milk for up to 13 months (Roest *et al.*, 2011a; Kargar *et al.*, 2013), although excretion may be intermittent (Rodolakis *et al.*, 2007). Two types of milk shedding forms had been identified, where cows can be persistent (also called heavy shedders) or sporadic shedders (Guatteo *et al.*, 2007). Compared with other shedding routes, birth fluids and faeces in bovines present a low number of *C. burnetii* (Guatteo *et al.*, 2007; Rodolakis *et al.*, 2007). Additionally, the persistent shedding pattern identified in milk is not shown in vaginal discharges or faeces (Guatteo *et al.*, 2007). This may suggest that the digestive environment or the reproductive tract are not as attractive for *C. burnetii* as the udder.

The heterogeneity of shedding routes, as well as the variety of shedding burden within these routes, strongly influence the intra-herd infection dynamics (Guatteo *et al.*, 2007; Courcoul *et al.*, 2011). Milk is considered a key source of further infections as *C. burnetii* DNA can be recovered in this biological material for up to 32 months (Angelakis & Raoult, 2010). The shedding of *C. burnetii* through milk differs in the level of infection and the infected animal's parity. For instance, multiparous cows, as those which potentially have faced more prolonged exposure to the pathogen, often present higher shedding compared to primiparous animals (Böttcher *et al.*, 2011). Also, possibly due to the enhanced chance of exposure to *C. burnetii* coming with age, multiparous cows presented higher seroprevalence in contrast to primiparous cows and heifers. This supports the

claim that horizontal transmission performs a central role in *C. burnetii* infection in bovines. Additionally, cows seem to present a modification of the main shedding route of *C. burnetii* over time, with the highest vaginal shedding level at parturition and predominant bacterial shedding through milk having occurred by the 100th day in the milk period (Freick *et al.*, 2017).
A corelation between seropositive levels and the intensity of bacterial shedding has been reported recently. Whereas weakly seropositive cows present a low shedding load, those cows with strong positive results are more likely to present heavy shedding of *C. burnetii* (Böttcher *et al.*, 2013). Despite this correlation, some milk shedder cows can remain seronegative (Barberio *et al.*, 2014). The remarkable tropism to udder appears to explain the high shedding of *C. burnetii* by milk in cows. Histological studies from infected cattle detected *C. burnetii* primarily from epithelial cells from mammary glands (Agerholm *et al.*, 2013). It has been recently revealed that both strains the virulent (Nine Mile I) and the avirulent (NM phase II) display a complete replication cycle in epithelial cells of udder (Sobotta *et al.*, 2017). Also, *C. burnetii* milk shedding has been connected with subclinical mastitis (Barlow *et al.*, 2008).

2.2.1.3 Bacterial transmission among cattle

The horizontal transmission of *C. burnetii*, direct animal to animal respiratory transmission, is the main route for infection among cattle. Animals acquire the infection through inhalation of the bacterium. High-density farms may contribute to an increased risk of *C. burnetii* infection. Dairy cattle housed under intensive conditions, typically involving high animal density, enhance the risk of infection. Under these conditions, contaminated material could potentially infect a higher number of individuals as they are in close proximity (McCaughey *et al.*, 2010; Boroduske *at el.*, 2017). Bearing in mind that Uruguayan dairy farms typically feature production under extensive production with regular grazing times, the risk of transmission could possibly be reduced. Extensive housed cattle may experience less exposure to infective aerosols considering that pastures typically have a high grade of soil moisture, except in extraordinarily dry seasons.

The spread of *C. burnetii* can be facilitated by the transport of infected cows between herds; however, environmental transmission is the primary factor in endemic areas (Nusinovici *et al.*, 2015a; Pandit *et al.*, 2016). Thus, environmental factors appears to have a critical role in the transmission of *C. burnetii* and merit attention. For instance, infective aerosols can produce infection even in places located at 5 km (or more) distance from an infected farm and the risk of infection increases in dairy herds placed in windy areas (Schimmer *et al.*, 2010; Nusinovici *et al.*, 2014).

The wind seems to perform a central role in the airborne transmission of *C. burnetii* between animals located on different farms as well as from animals to humans (van der Hoek *et al.*, 2011; O'Connor *et al.*, 2014). The dispersal of contaminated particles explains the wind-borne spread of

C. burnetii from infected herds to surrounding susceptible populations. Wetter soils seem to contrarrest the effect of wind on environmental transmission. Areas with high humidity soils have higher and more dense vegetation cover and appear less prone to erosion caused by wind implying certain degree of protection against particles spread by air (van der Hoek *et al*., 2011).

Regarding vertical transmission, a study done on 45 calves from multiparous and primiparous dams revealed no measurable precolostral immunoglobulin response in newborns born from dams with positive-*C. burnetii* cotyledons analysed by qPCR (Tutusaus *et al*., 2013). This suggests that new-borns have no contact with the pathogen during the intrauterine period, thus diminishing vertical transmission of *C. burnetii*. To further support this, calves born from seropositive dams are only found to be seroconverted after colostrum ingestion (Tutusaus *et al*., 2013). Findings from previous work studying mice also support the theory that the union between fetus and placenta prevents the vertical transmission of *C. burnetii* (Baumgärtner & Bachmanns, 1992). A recent case-control study done in animals from an endemically infected herd has shown the presence of *C. burnetii* DNA and anti-*C. burnetii* IgG immunoglobulins in precolostral blood from stillbirths, but not in calves born alive (Freick *et al*., 2018). However, the low sample size of this study prevented the statistical validity of the results. Except for this publication, no extensive research has been done on the levels of antibodies against *C. burnetii* in the ruminant neonates. This seems to be a potential field of future study. Further research should be done on the vertical transmission of *C. burnetii*, especially on the mechanism that prevents bacteria from crossing the placenta, to have enough evidence to dismiss it.

Different infected species present dissimilar times of *C. burnetii* excretion (Arricau-Bouvery & Rodolakis, 2005). The duration of the infection in domestic ruminants is not well established. In goats, sheep and cattle, the infection is perhaps life-long or may continue for many years. Domestic ruminants are mostly carriers with a subclinical manifestation of the disease but with bacterial shedding. The transmission probability rises with the rising number of cows in a herd (Paul *et al*., 2012). The pathogen contact rate is likely to rise with the animals' age because of the enhanced possibility of having come into interaction with *C. burnetii*; this has been well-verified in goats and sheep (Ruiz-Fons *et al*., 2010; García-Ispierto *et al*., 2011). The reproduction ratio (R_0) quantifies the number of individuals susceptible to infection by an infected individual. The management of animals under intensive conditions probably leads to increased interaction rates among animals (susceptible and infected), facilitating transmission. There is no available information about the R_0 of coxiellosis, only some modelling studies; thus, more transmission experiments are required (Courcoul, 2010).

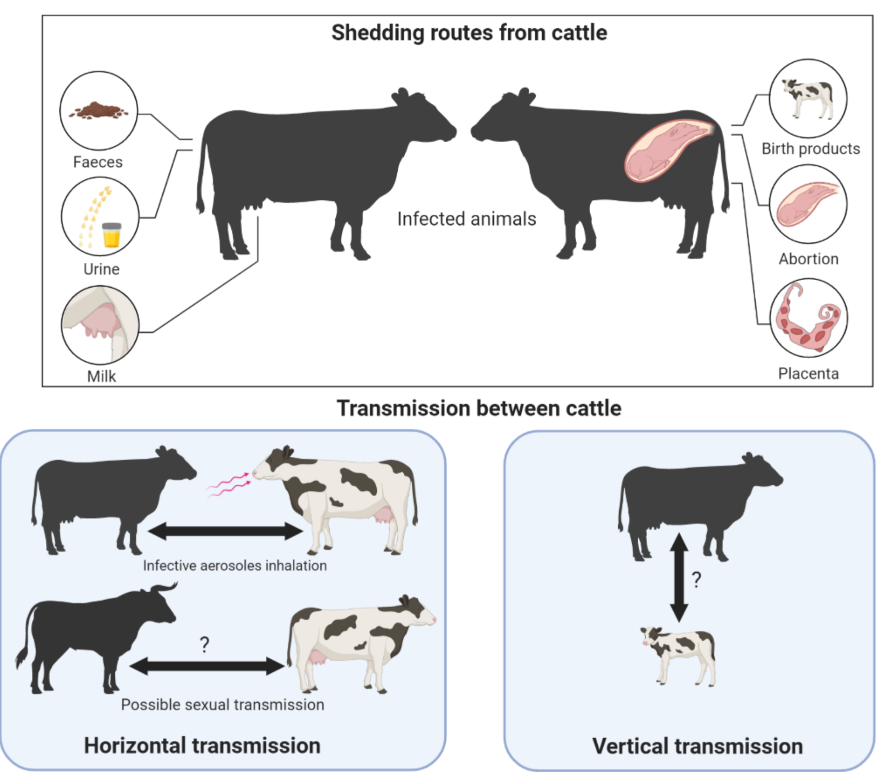


Figure 2.1: Transmission modes of *Coxiella burnetii* among cattle.

2.2.1.4 Consequences of infection in cattle

The *C. burnetii* infection is predominately asymptomatic; however, when pregnant animals are affected, it can cause a broad spectrum of clinical signs. Among the clinical manifestation, metritis, abortion, stillbirth, and weak offspring delivery are the most common signs (Arricau-Bouvery *et al.*, 2005). Controversial results have been found about *C. burnetii* infection and its possible clinical manifestation, as the bacterium can even be found in healthy and normal parturitions (Agerholm *et al.*, 2013; García-Ispierto *et al.*, 2014). The fact that *C. burnetii* can present in many healthy animals makes its study challenging when setting parameters such as a criteria "case" definition and controls selection. It is important to avoid misleading results.

A study conducted on cattle in Japan has revealed high *C. burnetii* seroprevalence in animals with reproductive problems (To *et al.*, 1998). By contrast, other investigations have detected high levels of *C. burnetii* infection in animals with no clinical disturbances, suggesting that the risk of reproductive alterations may not be strongly affected by the presence of maternal immunoglobulins (Guatteo *et al.*, 2006; Guatteo *et al.*, 2007; Muskens *et al.*, 2011).

As stated above, there is a broad spectrum of outcomes after *C. burnetii* infection, ranging from an inapparent course of the disease to normal calving or abortion. It is possible for any of the APSW complex (Abortion, Premature delivery, Stillbirth, and Weak offspring) to occur. Scientific

evidence of this complex in small ruminants is well documented but lacking for cattle (Agerholm, 2013). In this scenario, it could be the case, however, that a different bacterial load of *C. burnetii* in the placenta could produce a different degree of change in the placental tissue, and this could be the fundamental explanation for the wide range of states of the progeny. For example, trophoblasts with a low copy number of *C. burnetii* that remain inactive may produce “normal” pregnancies, where the fetus is not infected, and thus no fetal immune response is detected. Those calves will birth as healthy calves with a no measurable precolostral immunoglobulin response even when coming from a dam with *C. burnetii*-positive-PCR cotyledons. Cows diagnosed as *C. burnetii*-seropositive have been shown to give birth to seronegative calves, even with *C. burnetii*-positive cotyledons analysed by PCR (Tutusaus *et al.*, 2013).

Conversely, a placenta with trophoblast which is much more heavily loaded with *C. burnetii*, would produce an acute fetal infection leading to detectable antibodies as has been shown in the precolostral serum of stillborn (Freick *et al*., 2018). More research is needed to draw a clear link between placental bacterial load, placental changes, and the offspring's outcome. The tendency of more significant bacterial quantities in placenta samples from abortions due to *C. burnetii* have been shown in small ruminants by qPCR technique and compared with asymptomatic animals (Hazlett *et al*., 2013).

The occurrence of *C. burnetii* infection in cows has been implicated in placentitis, as well as much milder placental lesions with no histochemical evidence of inflammation (Luoto *et al*., 1950; Hansen *et al.*, 2011; Muskens *et al*., 2012). While placental inflammation is considered as the underlying cause of abortion, other mild tissular changes that damage the conceptus would produce other calving outcomes even those which may be clinically inapparent, although this thesis is speculative as strong scientific evidence is still wanting.

A study that has analysed fetal membranes from heifers and cows, with no specifications about the course of calving or state of progeny, has shown that placental inflammation is infrequently associated with *C. burnetii* infection (Hansen *et al*., 2011). This finding may explain why bovine coxiellosis is often clinically inapparent. This research has intended to investigate the association of *C. burnetii* infection and placental lesions but has not focused on abortion cases where placentitis may have been more frequently found. This report of infrequent evidence of inflammation in bovine placentas agrees with other findings that consider *C. burnetii* as an infrequent agent of abortion in this specie (Bildfell *et al*., 2000; Jensen *et al*., 2007; Muskens *et al*. 2011). Lately, other investigations have identified no relationship between the infection by *C. burnetii* in dams and pregnancy losses (Tramuta *et al*., 2011; Yang *et al*., 2012; López-Gatius *et al*., 2012). This body of study has been unable to arrive at conclusive insight about bovine coxiellosis as a risk factor for abortion.

Thus, the significance of *C. burnetii* infection will be indicated by placental inflammation and the subsequent dysfunction of the placenta, which is the primary reason for negative impacts on the offspring. The sole detection of bacterial DNA in the placenta is not informative enough. The confirmation of lesions, especially those suggestive of on-going acute inflammation, and the quantification of bacterial load is highly recommended as the presence of placental lesion would correlate with the number of copies of *C. burnetii* DNA in the tissues. The bacterial load in placentas with histological evidence of inflammation during *C. burnetii* infection appears to be different according to the ruminant species. The *C. burnetii* IHC-positive placenta samples from cattle showed a much lower number of infected trophoblasts contrasted with samples obtained from goats (Wouda & Dercksen 2007; Muskens *et al*. 2012). This suggests that a different level of *C. burnetii* infection actually reach the placenta in bovines. This supports the value of quantitative studies to corroborating this.

To sum up, different *C. burnetii* infection levels may produce diverse histological changes in the placenta, but these are not always inflammation, determining the different degree of placental dysfunction, which is often not as severe as losing all functionality. This alteration in the function determines the subsequent impact on the offspring, where a spectrum of consequences may be produced.

Bearing in mind the calves mentioned above born as seronegative or just seroconverted after the ingestion of colostrum (Tutusaus *et al*., 2013), it can be concluded that infection does not occur in the uterus, and not in the moment of birth. Conversely to what is seen in small ruminants, in cows birth products may not be the most relevant shedding route of *C. burnetii*. In this scenario, other queries can be raised. For instance, colostrum seems to have great relevance in *C. burnetii* infection in cattle, and possibly pasteurisation before consumption could be an effective strategy to avoid infection in calves. This field of investigation appears promising, although many aspects need evaluation as no experimental proof pointing to clear links for many aspects is currently available.

2.2.1.5 The zoonotic threat of Coxiella burnetii

Coxiella burnetii raises serious zoonotic concerns and has characteristics that facilitate its use as a potential bioterrorism agent. For instance, *C. burnetii* can be easily aerosolised, facilitating dispersion in aerosols, primarily in the form of its small spore-like structure. Also, this bacterium has a small infectious dose so that even a single organism could result in determine human disease, and it is extremely resistant to external stressors as well (Franz *et al*., 1997).

Ruminants are widely known as the main source of people Q fever infection, but in addition to sheep, goats and cattle, companion animals, particularly dogs and cats, also shed the bacterium and possibly pose a zoonotic risk (Marrie *et al*., 1988; Buhariwalla *et al*., 1996).

Most of the people affected by Q fever become infected through the aerosolization of contaminated aerosols and dust. During the parturition of an infected dam, a massive number of bacteria are shed

in the birth products, regardless of whether it is a normal birth or an abortion. *Coxiella burnetii* is spread in the surrounding environment, and after drying, bacteria can be aerosolised and easily inhaled by people, leading to infection (Marrie, 1990a; Maurin & Raoult, 1999; Schimmer *et al.*, 2010).

The intake of raw and contaminated milk, as well as raw milk-derived products, have been proposed as possible routes of *C. burnetii* infection (Raoult *et al*., 2005), although other researchers cast doubt on this theory, arguing that the digestive route seems to be an ineffective mode of transmission (Fishbein & Raoult, 1992; Rodolakis, 2009; Eldin *et al*., 2017). When we analyse the literature, we find studies from different countries supporting unpasteurised milk as a relevant route. For instance, during a small Q fever cluster that affected five individuals documented in Michigan (US) the epidemiological evidence pointed to the regular consumption of raw milk and unpasteurised milk products as the most probable infection source (Signs *et al*., 2012). Older reports suggesting raw milk consumption as being a source of Q fever in the UK, specifically in London (Marmion & Harvey 1956) and Staffordshire (Brown *et al*., 1968) have also been published, and studies have also documented such evidence in France (Fishbein & Raoult, 1992). Recently, the occurrence of DNA from *C. burnetii* was shown to be present in bovine raw milk commercially available for human consumption in the central region of Brazil (de Souza Ribeiro Mioni *et al.*, 2019), and the presence of viable *C. burnetii* was also demonstrated in raw milk hard cheeses after at least eight months of ripening (Barandika *et al*., 2019). Despite these findings, the role of raw milk and unpasteurised dairy products as a source of *C. burnetii* infection for humans remains controversial with many discrepancies and much uncertainty in the studies.

For the European Food Safety Authority, although cases have arisen from time to time, they are sporadic, and milk is regarded as a minor route of Q fever acquisition (EFSA, 2010). However, there is a modern trend of the consumption of unpasteurised milk products in many countries, built on the belief that raw dairy products possess properties with positive health impacts. This trend raises a warning about drinking raw milk as a risky practice. The consumption of raw milk should be discouraged since it can contain pathogens, other than *C. burnetii*, and milk must be pasteurised at 72 ◦C for 15 minutes or made safe by means of an equal heat process (Cerf & Condron, 2006; AFSSA, 2007).

2.2.1.6 Possible control strategies against Coxiella burnetii

Currently, Uruguay has no vaccines available to use against *C. burnetii* to use in humans or in animals. Considering this, the prevention of infection should focus on two main aspects: minimising potential exposure to the bacterium, and when exposure cannot be avoided, the likelihood of infection must be reduced by the use of personal protective equipment and the application of hygiene measures (Plummer *et al*., 2018). Some precautions commonly suggested

for preventing exposure to pathogens are difficult to implement in field conditions; however, other strategies are part of general prevention methods and will be mentioned in the following paragraphs.

Owing to the fact that domestic ruminants are the most common source of Q fever for people, the efforts highlighting controlling the disease incidence in these animals is mostly justified as a strategy to reduce the exposure in people. Control measures to avoid infection in domestic animals would decrease the probability of human exposure and thus, infection. Control measures should chiefly focus on the following aspects: identifying infected farms, minimizing the excretion of the bacterium into the environment and reducing human exposure (Roest *et al*., 2011a; Georgiev *et al*., 2013). The measures involved in each of the three strategies mentioned include keeping closed herds or implementing quarantine of newly purchased animals from external herds, disinfecting birthing areas, eliminating birth materials adequately, and undertaking *C. burnetii* vaccination in those countries or regions where its use is reasonable. These strategies will be briefly described in the following paragraphs.

2.2.1.6.1 Test and cull strategy

To begin with the strategies, the reliable identification of infected farms is a key measure. The use of composed milk samples, such as bulk tank milk (BTM), maybe an appropriate first step in studying the exposure of a herd to *C. burnetii*. The BTM samples are often the target sample for the investigation of *C. burnetii* at the population level, fundamentally because these samples are straightforward to obtained and are representative of the milking cows at the moment of the assessment. This strategy is founded on access to reliable diagnostic tests. Testing BTM samples has become a popular method for *C. burnetii* investigation at the population level. The herds status in relation to *C. burnetii* can be studied through BTM samples either by the detection of antibodies against the bacterium or by evidencing the presence of bacterial DNA, notwithstanding both approaches have interpretative limitations.

The levels of anti-*C. burnetii* BTM antibodies provide valuable information about herd exposure to the pathogen, reflecting the rate at which the bacterium circulates at the herd level. Additionally, PCR testing of BTM samples is utilised to determine the herd status concerning the excretion of the bacterium. When the analysis of BTM samples evidences both anti-*C. burnetii* immunoglobulins and *C. burnetii* DNA, this may be suggestive of active infection.

Regarding the interpretative limitations mentioned above, some aspects must be considered. The difficulty of interpretation lies in the plausible occurrence of infected animals that actually shed *C. burnetii* but not seroconvert (Guatteo *et al*., 2007; Rousset *et al*., 2009; Hansen *et al*., 2011). Another limitation is due to the persistence of antibodies, which is actually not precisely known. Cows infected in the past may produce a positive herd result with ELISA, even when they have cleared the infection and overcome the disease. Along these lines, an infected herd might be

misclassified as negative when PCR tested because of the lack of *C. burnetii* excretion at the moment of milk sampling due to some cows' intermittent shedding pattern when shedder animals are dried-off. The *C. burnetii* epidemiological behaviour of seronegative animals shedding *C. burnetii* and seropositive cows not shedding the bacterium (Guatteo *et al*., 2007; Rousset *et al*., 2009; Hansen *et al*., 2011) would seem to represent a limitation of the investigation at the individual level. Some of these limitations could possibly be offset, at least partially, when the investigation was using BTM, as many animals would be evaluated simultaneously being representative of the spectrum of epidemiological behaviours that can coexist in endemically infected herds.

It is also essential to note that the misidentification of an active herd infection could occur because of chronically infected cows. These animals, known as heavy shedders, excrete the bacterium during prolonged periods and present high antibody levels, although not suffering from an active infection (Guatteo *et al*., 2007; Guatteo *et al*., 2012).

Even though a positive overall correlation has been shown between bacterial shedding and serology (Guatteo *et al*., 2007; Courcoul *et al*., 2010), molecular and serological evaluations of BTM samples should be combined in order to obtain a better picture of what is going on at the population level, although the simultaneous application of these two approaches is not commonly used.
Some arguments against the use of BTM samples claim that, although they may be a useful epidemiological tool at the herd level, they are not informative enough about whether the bacterium represents a substantial problem in the herd. These critiques argue that further evaluation must be conducted. Despite this, the identification of infected farms was a central strategy among the measurements taken by the sanitary authority in the Netherlands after the huge Q fever outbreak registered in that country. The authorities conducted BTM tests on a fortnightly basis to identify infected farms and to follow up *C. burnetii*–negative herds. Once infected farms were certainty identified with certainty, all pregnant dams were culled (Hogerwerf *et al*., 2011).

2.2.1.6.2 Reduce environmental contamination

The reduction of the shedding of viable microorganism in the environment limits bacteria's threat to animals and people not infected. Environmental contamination is aided by the drying out of placental material, which is extensively infected, after incorrect disposal (Arricau-Bouvery *et al*., 2003; Roest *et al*., 2012). Contaminated dust may be spread as infectious aerosol particles that can be then inhaled and produce infection; environment comprises an important source of human and animal contamination. *Coxiella burnetii* is highly resistant in the field, especially in those contexts with favourable bacterial dispersion conditions.

All measures intended to reduce environmental contamination should be stressed. Removing birth materials in accordance with cleaning protocols is likely to cause a positive impact by lowering the bacterial levels in surrounding areas. The use of detergents followed by rinsing with water should

be done whenever possible. All individual preventative measures should be taken and in particular appropriate personal protective equipment should be worn.

Herds with *C. burnetii* active infection typically generate elevated bacterial levels circulating in the environment, leading as well to increased infection pressure. The investigation of environmental samples, such as dust samples by PCRs, can aid in monitoring.

2.2.1.6.3 Hygiene measures/Infection prevention control to avoid cross-contamination on farms
Finally, when exposure cannot be avoided, attempts to reduce infection risk should be pursued as much as possible. The use of personal protective equipment should be mandatory during work with animals, especially for farmers, veterinarians, and abattoir workers, which are the people most frequently affected. Measures such as workers changing their clothes before leaving the workplace and returning home, using rigorous hand hygiene practices, and avoiding hand-to-face contact when working with livestock should reduce the likelihood of infection.

2.1.3 Infection in humans

2.3.1.1 The epidemiological linkage of Coxiella burnetii *from bovine to human infection*

Domestic ruminants are deemed the main reservoir species for human *C. burnetii* infection; however, small ruminants (sheep and goats) are more frequently involved in human outbreaks worldwide (Rodolakis *et al*., 2006a). A high number of *C. burnetii* particles is typically spread into the environment at parturition or abortion. People assisting parturient dams are at significant risk of *C. burnetii* infection considering their massive exposure to the bacterium coupled with the low infective dose required for infection. The respiratory tract is the most frequent route for human infection during handling birth material, especially placentas (Arricau-Bouvery & Rodolakis, 2005). The ingestion of unpasteurized milk and dairy products has also been allied to Q fever outbreaks (Fishbein & Raoult, 1992; Signs *et al*., 2012).

2.3.1.2 Symptomatology

The manifestation of Q fever infection generally has three clinical presentations known as acute Q fever, chronic Q fever and post-Q fever fatigue syndrome. Most humans that get infected remain asymptomatic (up to 60%) or have mild symptoms (Raoult *et al*., 2005). Acute manifestation can occur after a two-three-week incubation period depending on the inoculum size (Eldin *et al*., 2017). Amongst those that develop symptoms, the clinic reported is often not specific, including a flu-like self-limiting illness (Raoult *et al*., 2005; Anderson *et al*., 2013a; Million & Raoult, 2015; Eldin *et al*., 2017). The predominant symptom is a persistent fever, that may last 15 days with temperatures up-to 40°C. Myalgia and retro-orbital headache are frequently simultaneous symptoms (Eldin *et al*., 2017; Million & Raoult, 2015). Due to its symptomatology being similar to influenza cases, Q fever is likely to go underdiagnosed and consequently underreported. The majority of people

affected fully recover after its self-limiting course, though some people may suffer from any of several severe complications such as hepatitis, pneumonia, endocarditis, osteoarticular infection, neurological symptoms, or spontaneous abortion (Parker *et al*., 2006). Pneumonia is frequently typified by dyspnoea, cough, and sputum (Million & Raoult, 2015; Eldin *et al*., 2017). Symptoms of hepatic insufficiency are also reported as a relevant symptom of Q fever with a frequent occurrence in endemic regions, contrasted with symptoms of pneumonia which are often reported in sporadic outbreaks (Eldin *et al.*, 2017). Granulomatous hepatitis and "doughnut" granulomas have been reported after biopsy studies (Pellegrin *et al*., 1980; Maurin & Raoult, 1999; Galache *et al*., 2004). Neurological alterations include severe headache (Raoult *et al*., 2005; Eldin *et al.*, 2017), meningitis, confusion, and disorientation as well as, olfactory, auditory, and visual hallucinations (Smith *et al*., 1993; Kofteridis *et al*., 2004).

Following an asymptomatic or symptomatic acute Q fever case, a small proportion (less than 5%) of cases can evolve into chronic disease even years after the initial infection. Despite this, chronic Q-fever has also been reported in people with no acute disease record (Anderson *et al*., 2013a). This chronic Q fever is also known as "persistent *C. burnetii* infection" (Million & Raoult, 2015; Million & Raoult, 2017). The chronic disease is mostly characterized by endocarditis and other vascular infections, but chronic pneumonia, chronic hepatitis, septic arthritis, and osteomyelitis can also occur (Anderson *et al*., 2013b). This progression to chronic disease mainly occurs in patients with pre-existent conditions such as being immunocompromised, or suffering from cardiac valve abnormalities, endocarditis, vascular infections, aneurysm, persistent lymphadenitis, or osteoarticular infections (Raoult *et al.*, 2005; Eldin *et al.*, 2017).

Most patients affected by post-Q fever fatigue syndrome recover after 6–12 months, but up-to 20% remain chronically fatigued (Morroy *et al*., 2016). Interestingly, chronic fatigue and persistent infection seem to be related to the persistence of bacterial DNA and antigens such as cellular components, though further investigation is needed to fully understand this process (Marmion *et al*., 2009).

Chapter Seven of the current thesis will present a serology investigation in laboratory and field workers exposed to positive-*C. burnetii* abortions which occurred on a dairy farm.

2.3.1.3 Treatment of Q fever

As most acute Q fever cases are self-limiting and have a spontaneous resolution, they are often not reported, and no treatments are applied. In cases that need hospitalization due to severity, a treatment strategy of antibiotics therapy is utilized. A tetracycline treatment is recommended, prescribing doxycycline at 200mg/day for two weeks (Million *et al*., 2009; Dijkstra *et al.,* 2011; Anderson *et al*., 2013a; Eldin *et al.*, 2017). Treatment applied to acute infection may avoid the progression to chronic Q fever (Kampschreur *et al.*, 2012); however, the application of medication is discouraged in asymptomatic cases and in symptomatology after the resolution (Eldin *et al.*,

2017). The use of antibiotics treatments, frequently tetracyclines, leads to reduced incidence of abortions but does not inhibit *C. burnetii* shedding (Woernle *et al*., 1985; Maurin & Raoult, 1999).

2.1.4 Virulence factors

There has been an attempt to link the genetic variability of the bacterium and its virulence to specific plasmid regions. *Coxiella burnetii* hold one of four possible plasmids, known as QpDV, QpH1, QpDG, QpRS, or QpRS-like plasmid (Beare *et al.*, 2006). Controversial results have been found regarding the theory about specific plasmids and their related genome encoding particular factors of virulence of pathotypes (Thiele & Willems, 1994). The expression of the lipopolysaccharide (LPS) molecules is closely linked to genetic diversity and virulence. The complete genome sequence of phase I Nine Mile RSA493 of *C. burnetii* was available in 2003 (Seshadri *et al.*, 2003), allowing the identification of missing genes, specifically those responsible for encoding adhesion to cells structures (pili and non-pilous adhesins) (Ghigo *et al.*, 2009). Other identified genes encode proteins implicated in the process of the uptake of microorganisms by host cells (note that macrophages present different degrees of uptake of phase I and phase II) and subsequent cytoskeletal reorganization is necessary for this (Meconi *et al.*, 1998; Capo *et al.*, 1999). Other genes are required to encode certain enzymes (superoxide dismutase, acid phosphatase, and catalase), thus avoiding the antimicrobial role of macrophages via the inhibition of reactive oxygen intermediates (Baca *et al.*, 1993b; Cianciotto, 2001). A gene responsible for the encoding of a peptidyl-poly- cis-trans-isomerase possibly influences cytokines' production and thus affects the *C. burnetii* replication process (Baca & Mallavia, 1997). A group of genes that hold the encoding of the type IV secretion system components (IcmT, IcmS, IcmK), could be determinant for establishing the phagosomes that contain *C. burnetii* (Zamboni *et al.*, 2003; Zusman *et al.*, 2003).

2.4.1.1 The immune response against Coxiella burnetii

The immune system involves two distinct responses, the innate immune response, and the adaptive immune response. The innate immune response becomes active shortly after exposure to a pathogen and phagocytes (macrophages, neutrophils, and monocytes) play a central role. Contrastingly, the adaptative immune response embodies a later responding mechanism which is very specific. This response provides long-lasting protection due to the memory activated by lymphocytes (B and T cells), antigens-presenting cells and effector cells.

Following infection, *C. burnetii* resides in the macrophages where it replicates inside a phagosome, thus attempting to escape from the host's immunological response. Although this strategy is displayed by *C. burnetii*, the bacterium does not entirely elude the immune system‘s reaction and both the cell-mediated and humoral immune response to infection result. In this intracellular stage, *C. burnetii* tries to survive and displays a strategy of resisting the macrophages by interfering with their intrinsic microbicidal properties (Capo & Mege, 2012). Beyond this first interaction with the innate immune system by the macrophages, the adaptive immune response is also necessary to fight

off the *C. burnetii* infection. The host immune system is not entirely misled by *C. burnetii's* strategy of residing in this sort of extracellular space created by the phagosome, and both cell-mediated and humoral immunological responses occur. The pattern of response that the host will develop is finally established by the pathogen and the innate immune receptors it activates. These events produce a local environment with specific cytokine and chemokine that varies and ultimately can influence the direction of the acquired cytokine response (Basset *et al.*, 2003). The adaptive immune response performs a relevant role by limiting the *C. burnetii* infection with a more significant contribution of Th1-type compared to antibody production. Animals presenting an effective Th1 response, can deal with infection and eliminate *C. burnetii*, possibly without seroconversion (Freick *et al.*, 2017). A complete understanding of the underlying processes behind cows shedding *C. burnetii* but without seroconversion, thus remaining seronegative animals, would give insight into bacterial pathogenesis. As mentioned in sections above in this chapter, the possible late seroconversion, or even cows that fail in seroconverting, can be feasibly explained by the stimulation mainly of the Th1-type immune response against an intracellular bacterium like *C. burnetii* (Shannon & Heinzen, 2009). Some theories behind the cows that do not present seroconversion, nor shed *C. burnetii* have been proposed, such as a sort of genetic resistance against the infection (Freick *et al.*, 2017), or the presence of an immune condition due to high interferon-gamma (IFN-γ) immunoreactivity (Motsch *et al.*, 2016). Further studies of cows' immunological response patterns against *C. burnetii* are desired.

Returning to the issue of innate immune response, *C. burnetii* generates specific mechanisms that facilitate its residence within myeloid cells. *Coxiella burnetii* uses a specific survival strategy based on subverted receptor-mediated phagocytosis (Capo *et al.*, 1999). These mechanisms try to disrupt the microbicidal action by means of the destabilization of the receptors engaged with phagocytosis and by affecting the maturation of the phagosomes (Baca *et al.*, 1994; Ghigo *et al.*, 2009). This battery of mechanisms displayed by *C. burnetii* is conditioned by the functional state of the myeloid cell, which affects the intracellular fate of *Coxiella* (Ghigo *et al.,* 2009).

The myeloid host cells hold a restricted number of phagocytic receptors able to uptake the microorganism during phagocytosis. There are roughly five types of receptors able to identify diverse bacterial structures. For instance, Fc (opsonized organisms), complement receptor CR3 (CD11b and CD18, integrin αMβ2) (opsonized and un-opsonized microorganisms), mannose receptors, scavenger receptors (diacyl lipids from bacterial surface), and Toll-like receptors (TLRs) (flagellin, peptidoglycan or LPS) (Taylor *et al.*, 2005). Among these, the phagocytosis that gets activated during *C. burnetii* infection seems to be integrin-dependent.

The mode of entry into host cells will condition the intracellular fate of *C. burnetii* (I and II). Monocytes take up the different bacterial phases with diverse rates of efficiency. Phase I *C. burnetii* variants (virulent organisms) are weakly internalized, so the survival of the bacterium is facilitated,

dissimilarly phase II (avirulent organisms) are competently internalized and thus eliminated. Different molecular mechanisms may explain these differences in the efficiencies of *C. burnetii* uptake shown by monocytes. The internalization of phase II cell variants is facilitated by αvβ3 integrin receptors and CR3 receptors, while the uptake of phase I is mediated by just αvβ3 integrin as binding receptors (Capo *et al*., 1999). The receptor CR3 is normally activated by αvβ3 integrin and CD47 (an integrin-associated protein). Phase I variant interferes the lectin sites producing conformational modifications of the domain and affecting the exposure of epitopes (Vetvicka *et al*., 1996), inhibiting CR3 activation (Capo *et al*., 1999). No evident inhibitory effect of phase I bacteria over CD47 has yet been explored (Capo *et al*., 1999).

Additionally, the spatial distribution of the CR3 receptors is relevant to determining the efficiency of *C. burnetii* phagocytosis (Capo *et al*., 2003). The bacterium seems to display a strategy for survival involving reduced engagement of CR3 by the alteration of the actin cytoskeleton (Ghigo *et al*., 2009). During infection, only the *C. burnetii* phase I, not so phase II organisms, produces a remodelling of the cellular cytoskeleton of monocytes comprising morphological rearmaments, membrane protrusions and polarized projections, with an increment and re-arrangement of the filamentous actin (F-actin) content (Meconi *et al*., 1998). This further supports the implication of actin cytoskeleton in the control of the bacterium phagocytosis. The macrophages phagocytosis relies on the restructuring of the actin cytoskeleton underlying the region of the membrane near the particle. The signals of the ligand and phagocyte receptors‘ interaction mediate the F-actin activation in this region (Greenberg, 1995). The phagocytosis facilitated by immunoglobulin Fc receptors, mentioned above, is conditioned by the activation of the protein tyrosine kinases (PTK). The PTK activation gives insight into the virulence of the bacteria, since phase I *C. burnetii* promotes early PTK stimulation, whereas the phase II variant does not affect PTK (Meconi *et al*., 2001). The persistence of pathogens in macrophages has been related to the restriction of PTK stimulation because the stimulation of PTK can generate a hostile environment for microorganisms (Bliska *et al.,* 1992; Nandan *et al*., 2000). PTK activation by the bacterium interferes with actin cytoskeleton reorganization, downregulating bacterial uptake (Meconi *et al*., 2001). PTK activation in monocytes leads to membrane ruffling, limiting in this way the colocalization of CR3 with αvβ3 integrin, thus inhibiting cell protrusion growth and the F-actin remodelling caused by the phase I bacterium. The PTK can pursue αvβ3 integrin, obstructing the crosstalk between the CR3 receptor and the cytoskeleton (Meconi *et al*., 1998; Patil *et al*., 1999).

The bacterial surface presents unique pathogen-associated molecular patterns (PAMPs) that are recognized by host immune cell receptors referred to as pattern recognition receptors (PRRs), in order to activate the defence response that involves cytokine signalling and phagocytosis (Medzhitov, 2001). Toll-like receptors (TLRs) are one of the main PRRs on the innate immune cells which are implicated in bacterial recognition. The polymorphism among TLR receptors may

be associated with the less pronounce responsiveness of macrophages during infection, and the diminished production of cytokines (Ammerdorffer *et al*., 2015). Macrophage identification of bacteria includes numerous members of the TLR group, but among those, two specifically (TLR4 and TLR2) are essential for the recognition of surface bacterial structures. When *C. burnetii* infection occurs, the TLR4 receptors in macrophages, which are generally engaged in the identification of Gram-negative bacteria during the recognition of LPS, regulate bacterial uptake (Honstettre *et al*., 2004). TLR4 restrains the protective immune response against *C. burnetii* infection via the development of granuloma and cytokine generation (Honstettre *et al*., 2004). Also, TLR2 receptors are implicated in the immune and inflammatory reactions to *C. burnetii*, but apparently do not play an essential role to the elimination of bacteria (Meghari *et al*., 2005).

In the face of acute infection, the protective strategy against *C. burnetii* entails a systemic cell-mediated immune reaction and the development of granuloma with IFN-γ (Capo & Mege, 2012). Despite not accomplishing complete eradication, T-cells seem fundamentally to control acute *C. burnetii* infection, although the specific type of T-cell involved is still undefined (Honstettre *et al*., 2004; Shannon & Heinzen, 2009).

The regulation led by T-cells appears to have some implications for the persistence of *C. burnetii* within the host and is therefore closely linked to the chronic presentation of the illness (Amara *et al.*, 2010). When chronic Q fever happens the protective response facing *C. burnetii* infection seems ineffective, indicating that the host‘s immune status play a decisive role in determining the illness's severity. Evidence suggests that chronically infected patients have deficient T-cell response, hindering the clearance of the bacteria (Waag & Williams, 1988). In this and similar scenarios, defective cell-mediated immunity response occurs typified by a reduced, or even absent, granulomas formation, often substituted by lymphocyte infiltration, necrosis foci, exacerbate cytokine (mostly interleukin-10) production, and elevated immunoglobulins levels (Ghigo *et al*., 2009; Capo & Mege, 2012).

This evidence supports the thesis that *C. burnetii* persistence in myeloid cells is due to an alteration in the conversion of phagosomes (Ghigo *et al.*, 2009). Once internalization of phase I and phase II bacteria occurs, *C. burnetii* cells are limited within the phagosomes. This structure is exposed to a series of fusion and fission stages with endocytic organelles until they reach the last stadium (phagolysosome), where bacteria are finally destroyed (Ghigo *et al.*, 2009). Early phagosomes have a pH around 6.0, but during their evolution, the intra pH gradually decreases, reaching 4.0 due to the acquisition of a vacuole proton pump ATPase (Scott *et al.,* 2003). *Coxiella burnetii* survival is achieved in this acidic environment (Hackstadt *et al.*, 1981; Akporiaye *et al.*, 1983; Chen *et al.*,1990) in which treatments with antibiotics are reported as being relatively ineffective (Raoult *et al*., 2005). This evolution process also comprises other changes, for example, the acquisition of a small

GTPase (rab7) and the incorporation of hydrolytic enzymes (cathepsin D), that is facilitated by the interaction with lysosomes (Scott *et al.,* 2003).

Regarding the former, a different acquisition of rab7 during phagosome conversion has been evidenced and may be related to bacterial virulence (Desjardins *et al*., 1994). For instance, phagosomes containing the phase II variant usually acquire rab7, while those containing phase I bacteria acquire rab7 only partially (Ghigo *et al.*, 2002). Since the amount of Rab proteins is crucial to fusion, this may explicate the impaired fusion of phase I-comprising phagosomes with lysosomes (Henry *et al*., 2004; Rink *et al*., 2005). The poor rab7 enrolment at the phagosome' surface may determine the creation of a phagosome incapable of interacting with the lysosomes. Along these lines, no protease cathepsin D is not accumulated in phagosomes comprising phase I *C. burnetii* because a defective phagosome-lysosome fusion occurs. Based on the points stated above, the disease's clinical presentation could be interpreted by successful phagosome conversion. In people suffering from chronic Q fever, monocytes are not capable to destroy *C. burnetii* and present an inadequate conversion of phagosome (Ghigo *et al*., 2004). By contrast, patients that recover from acute Q fever have monocytes that successfully deal with *C. burnetii* elimination by typical phagosome conversion. To sum up, the pathogenicity of *C. burnetii* is related to its capacity for hijacking phagosome conversion and limiting the immune defence‘s responses, enabling their persistence within host myeloid cells.

IFN-γ is generated by immune cells and modifies the balance of their cytokine production (Basset *et al*., 2003). IFN-γ is critical for controlling bacterial infection by triggering the host cells of such naive monocytes and determining the killing of the bacterium via the mechanism of apoptosis of macrophages (Koster *et al*., 1985; Izzo *et al*., 1993; Dellacasagrande *et al*., 1999). Dissimilar to other intracellular microorganisms, *C. burnetii* does not affect the viability of infected host cells, and bacterial death only occurs in those cells with IFN-γ-induced apoptosis, and this apoptotic mechanism seems to be mediated partially by TNF (Dellacasagrande *et al*., 1999). IFN-γ is also necessary for the generation of the Th1 protective immune reaction (Boehm *et al*., 1997). IFN-γ affects *C. burnetii* vacuoles' maturation by promoting the fusion between phagosome and lysosome and stimulating the alkalinisation of the vacuole without substantially affecting vacuole pH (Ghigo *et al.*, 2002). The production of IFNγ is similarly induced by all strains (Ammerdorffer *et al.*, 2017).

IFN-γ generates reductive macrophages which create insufficient production of interleukins IL-6 and IL-10 (Basset *et al.*, 2003). In contrast to the microbicidal activity stimulated by IFN-γ, the IL-10 helps bacterial replication through the stimulus of *C. burnetii* multiplication within monocytes (Blauer *et al.*, 1995; Park & Skerrett, 1996; Ghigo *et al.*, 2001). Furthermore, IL-10 is associated with the faulty destruction of *C. burnetii* by monocytes in patients with endocarditis due to Q fever (Ghigo *et al.*, 2001). An inadequate immune response typifies chronic Q fever presentation, which has been related to enhanced levels of IL-10 (Raoult *et al*., 2005; Shannon & Heinzen, 2009). An

increased level of IL-10 may be the underlying explanation for the frequent immunosuppression in chronic Q fever (Capo & Mege, 2012). During chronic Q fever, bacterial replication is not inhibited, despite elevated amounts of immunoglobulins (IgG, IgM, and IgA) against both cell variants (phase I and II). Furthermore, monocytes are unable to kill the bacterium (Dellacasagrande *et al.*, 2000a) and their endothelium migration seems to be IL-10-dependent (Willems *et al.*, 1994; Meghari *et al.*, 2006a; Meghari *et al.*, 2006b; Benoit *et al.*, 2008). The chronification of the disease, when, in fact, *C. burnetii* primary infection is usually asymptomatic, occurs most frequently in those patients with pre-existent conditions. This negative evolution of the *C. burnetii* primary infection seems to be IL-10 dependent, where the risk of chronicity is associated with monocytes that present overgeneration of IL-10 (Capo *et al.*, 1996; Honstettre *et al.*, 2003). The IL-10 determines alterations in the trafficking process in leukocytes, by affecting the formation of granulomas, which are structures essential for protection against the bacterium (Meghari *et al.*, 2006a; Meghari *et al.*, 2006b). Additionally, the higher frequency of Q fever in men than in women would also seem to be influenced by the role of IL-10. A study done in *C. burnetii* infected mice that evaluated gene modulation showed that about 60% of the modulations were linked to sex hormones with a greater degree of modulation expressed in males, and among the genes upregulated in males are those coding for IL-10 (Capo & Mege, 2012).

Recent research has shown that not all isolates which have gathered diverse host species stimulate the human immune system in the same way, demonstrating different patterns of pro-inflammatory cytokines (Ammerdorffer *et al.*, 2017). Interestingly, humans' peripheral mononuclear blood cells generate substantially higher quantities of TNF-α, IL-22, and IL-1β after a *C. burnetii* bovine strain stimulus than they do when affected by a goat or sheep strain infection (Ammerdorffer *et al.*, 2017).

So far, T cell-mediated immunity seems crucial for the defence against infection by *C. burnetii*, but the role display by B cells in a host's defence is not completely clear. Less is understood about the humoral immune response when *C. burnetii* infection occurs. As being an intracellular pathogen it is believed that B cell antibody production is not central. The role of B cells and protective antibodies needs to be explored further. The B lymphocytes can be divided into two types the B1 and the B2. B1 cells act as effectors of innate-like immunity and are skilled in answering the stimulus without T cells' help (Montecino-Rodriguez & Dorshkind, 2006). This cell type provides IgA, but in addition to antibody production, they can also phagocyte and kill bacteria upon uptake (Parra *et al.*, 2012). Based on the expression of the receptor CD5, B1 cells may also be further classified into B1a (CD5+) and B1b (CD5-) (Fillatreau *et al.*, 2002). B1a B cells showed multiple functions during bacterial clearance following primary *C. burnetii* phase I infection such as phagocytosis and cytokine and antibody production (Schoenlaub, 2016). B2 cells are formed in the bone marrow and need to be T cell stimulated to achieve a highest activation, and characteristically produce complex antibodies such as IgG (Montecino-Rodriguez & Dorshkind, 2006; Abbas *et al.*,

2012). Humoral response against infection occurs between seven and fifteen days after symptomatology occurrence and is typified by elevated levels of immunoglobulins reacting to phase II LPS (Maurin & Raoult, 1999; Shannon & Heinzen, 2009; Anderson *et al*., 2013ab).

2.1.5 Investigation of *Coxiella burnetii* in Uruguay

2.5.1.1 Human cases

The first case of Q fever in Uruguay was registered in 1956 and involved an abattoir worker (Salveraglio *et al*., 1956). A case of Q fever in a child was reported almost a decade later, but the epidemiological investigation could not identify the source of infection (Peluffo *et al*., 1966). Between 1975 and 1985, a total of 14 local outbreaks of Q fever occurred, all of them involving people with activities in meat-processing plants. Out of 1358 clinically suspected cases, only 60% were serologically confirmed (Somma-Moreira *et al*., 1987; Ortiz-Molina *et al*., 1987).

The first report of *C. burnetii* on a dairy farm was a Q fever outbreak involving five mature members of a family (Braselli *et al*., 1989). All the cases presented an acute onset of the symptomatology, and the disease was confirmed by indirect immunofluorescence (IFI) investigation on sera samples. The infection source was assumed to be contaminated dust inhaled by the patients, since none of the patients had consumed unpasteurized milk.

The first report of human endocarditis after a *C. burnetii* infection was described in a 36-year-old male patient exposed to the bacterium while working as a veterinary assistant in an abattoir. The patient reported having a heart murmur since the age of seven and chronic rheumatic valvulopathy; this pre-existent illness may have aided the evolution of the infection into a more severe clinical condition (Moreira-Braselli *et al*., 1994).

Most of the human Q fever outbreaks locally recorded have been linked to exposure during high-risk occupational activities, and most of them were epidemiologically traced to cattle (Somma-Moreira *et al*., 1987). However, during the past decade (2003-2004), a Q fever outbreak linked with wildlife and comprising 25 cases occurred (Hernández *et al*., 2007). All the cases were workers from an experimental wildlife breeding station located in the southeast of Uruguay. The epidemiological investigation revealed that workers became infected through the inhalation of contaminated dust during grass mowing. A group of pampas deer (*Ozotoceros bezoarticus*) was postulated as the presumed reservoir for the infection.

None of the human Q fever outbreaks reported in Uruguay has been clearly linked to bovine abortions caused by this agent. **Chapter Seven** of this thesis presents a retrospective cohort study of laboratory and farm workers exposed to a *C. burnetii*-positive cluster of abortions on a dairy farm in Uruguay.

2.5.1.2 *Ruminants' investigation*

The majority of investigations done in Uruguay concerning productive animals only includes serologic studies. Studies carried out on cattle revealed many different results, with seroprevalences ranging from 4.7% to 24.1% (Salveraglio *et al.*, 1956; Bacigalupi *et al.*, 1958**;** Caffarena *et al.*, 1965). Serological evaluation of anti-*C. burnetii* antibodies in sheep showed a 10.3% seroprevalence (Bacigalupi *et al.*, 1958). Recently, the causative association between *C. burnetii* infection and abortion in dairy cows has been locally described. The bovine Q fever outbreak involved four cases of bovine abortion. The presumptive diagnosis was made based on gross examination and histopathology of aborted material (placentas and aborted fetuses), and IHC and DNA conventional PCR accomplished the confirmation. Other abortifacient agents were systematically ruled out (Macías-Rioseco *et al*., 2019). The introduction of heifers from abroad into the main herd appeared to be the possible infection source. However, the evidence supporting this purchase as responsible for introducing *C. burnetii* was not conclusive, and neither were other infection sources ruled out.

2.2 *Chlamydia* spp.

2.2.1 *Chlamydia* spp. general characteristics

2.2.1.1 *Nomenclature and Taxonomy*

The taxonomy of the bacterium known as *Chlamydia* has been quite controversial and has suffered several changes throughout the years. Before 1980 the order *Chlamydiales* included the family *Chlamydiaceae* which contained just one genus *Chlamydia* with two species, one comprising the *chlamydial* isolates from humans and the other including the *chlamydial* isolates from animals (Gunn & Lofstedt, 2016). In 1999 the order *Chlamydiales* was reclassified using new DNA-based methods that enabled a more specific distinction. This reclassification was mainly based on the 16S and 23S rRNA homology (Everett *et al*., 1999). The genus *Chlamydia* was modified and split into two genera: *Chlamydia* and Chlamydophila; however, this modification was not universally accepted (Everett, 1999). Years later, in 2010, *Chlamydia* was proposed as a single genus within the family *Chlamydiaceae* (Greub, 2010; Greub, 2013).

Thus, currently the phylum *Chlamydiae* contains the order *Chlamydiales*, which comprises four families: *Chlamydiaceae*, *Parachlamydiaceae*, *Simkaniaceae*, and *Waddliaceae* (Everett *et al*., 1999a; Rurangirwa *et al*., 1999). As stated above, the family *Chlamydiaceae* comprises *Chlamydia* as the single genus. *Chlamydia* genus contains the following 13 species: *C. trachomatis, C. pneumoniae, C. abortus, C. caviae, C. felis, C. muridarum, C. pecorum, C. psittaci, C. suis, C. avium, C. gallinacea, C. serpentis,* and *C. poikilothermis* (Bommana *et al*., 2019). The term "*chlamydia*" is frequently utilised to denote members of the genus *Chlamydia*, but it is also more widely used to refer to all the members of the *Chlamydiae* phylum and lead to misunderstanding

(Gunn & Lofstedt, 2016). Recently, a group of researchers proposed the reclassification of the phylum *Chlamydiae* into two orders, the *Chlamydiales* and *Parachlamydiales* (Gupta *et al*., 2015). However, the Subcommittee on the taxonomy of *Chlamydiae* of the International Committee on Systematics of Prokaryotes rejected this proposal because of insufficient evidence based on the 16S rRNA trees (Greub & Bavoil, 2018). The diversity of *chlamydia* seems to have been underestimated, and future new isolates of the bacteria seem likely to trigger further discussions about their taxonomy classification.

The *Chlamydiae* phylum includes Gram-negative obligate intracellular bacteria that can use a wide range of host cells ranging from human to amoebae (Bachmann *et al*., 2014). These bacteria act as pathogens that cause diseases in humans and many animals, including cattle, goats, sheep, pigs, horses, cats, birds, koalas and rodents. Within the several *chlamydial* species, the most frequently involved in ruminant disorders are *C. abortus* (previously known as *C. psittaci* serotype 1) and *C. pecorum* (formerly known as *C. psittaci* serotype 2) (Fukushi & Hirai, 1992; Anderson, 1996; DeGraves *et al*., 2004).

All the members of the family *Chlamydiaceae* have a complex and conserved biphasic developmental cycle that alternates between replication and infection stages. For bacterial survival, this biphasic cycle relies on the parasitism of a eukaryotic cell. The biphasic life cycle comprises two bacterial forms which have morphological and functional differences. One of the forms is the elementary body (EB) which is the infectious stage. The EB particles cannot replicate or divide, nor would they be capable to persist for an prolonged period outside a host cell. Thus, EB must enter into a new host cell to perpetuate the cycle as the next form, the reticulate/reticular body (RB). The RB is non-infectious but is capable of dividing and metabolically active (Guerra *et al*., 2015). Briefly, the growth cycle is composed of three phases. First, EB attaches to and penetrates susceptible cells by using specific receptor sites. Second, once inside the cell, the EB particle changes into a RB particle. Binary fission multiplication and reorganisation turning the particle back into EB takes place. Finally, the host cells expel EB particles. The EB invade new susceptible cells and initiate another round of infection (Guerra *et al*., 2015). Bacteria can turn into a persistent phase as a tactic to survive facing adverse conditions and allow for long-term survival of the bacterium inside the parasite-host cell (Hogan *et al*., 2004).

2.2.1.2 Chlamydia spp. in the host cell

The mucosal surfaces are the first places for chlamydial infection, and some species tend to produce a pathology limited to these sites. Despite this, other species, and even biovars within a particular species, can invade further and produce pathology at distal organs (Meeusen *et al*., 2004).

First, bacteria attach to the host cell, and complex interactions between the cell and the pathogen occur to achieve internalisation (Dautry-Varsat *et al*., 2005). Numerous bacterial ligands and host receptors are involved in the process of binding (Hackstadt, 2012; Hegemann & Moelleken, 2012; Mehlitz & Rudel, 2013). The range of mechanisms during binding and the subsequent internalisation vary from species to species and may explain the tropism shown in specific hosts and even in different tissues (Elwell *et al*., 2016). The EB's particles may either be up taken by an actin-dependent or by an actin-independent process. The actin-dependent uptake mechanism is based on actin-rich filopodia, macropinosome, or phagocytic cups (Nans *et al*., 2014). When *Chlamydia* spp. makes contact with the host cells an actin remodelling occurs promoting bacterial internalisation (Hackstadt, 2012; Nans *et al*., 2014). This actin-dependent internalisation is started by injection into the cytoplasm of the host cell of previously synthesised effectors through the type 3 secretion system (T3SS) (Saka *et al*., 2011). This affects the actin filaments and thus, facilitates the uptake of bacteria. The bacteria deal with the unfriendly host intracellular environment by using a big battery of secreted effectors. The T3SS resembles a syringe needle and is a molecular system that allows bacterial effector molecules to be directly injected across host membranes (Mueller *et al*., 2014). These effectors facilitate the cytoskeletal reorganisation that promotes the bacterial invasion and triggers signalling among the host cells (Dai & Li, 2014). The resultant intracellular vesicle, comprising the EBs, is termed inclusion. Many EBs may bind and join into the same cell of host, generating multiple inclusions inside the host cell. Replication of *Chlamydiales*, as obligate intracellular pathogens, occurs in this specialised compartment bounded by a membrane.

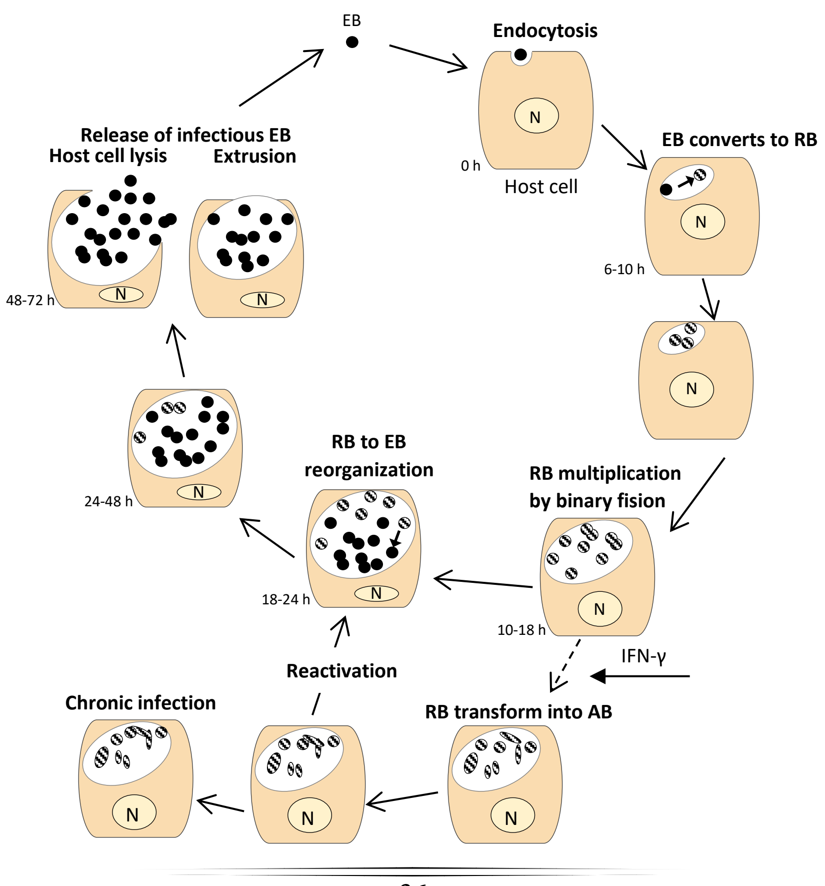


Figure 2.2: Chlamydial developmental cycle. Two cell types characterize the life cycle: the small (0.3 to 0.6 µm in diameter) extracellular infectious cell, called the Elementary Body (EB), and the larger (0.6 to 1.5 µm in diameter) vegetative form, the Reticulate Body (RB). First, the EB adheres to and invades the eukaryotic cell. Into RB, this cell type matures, presenting a binary fission multiplication and establishing an intravacuolar microcolony, named an inclusion. Inclusion is removed from the phagolysosome pathway but is dependent on the Golgi apparatus. The RB can reorganize into EB, which will be released by lysis from the cell or extrusion, initiating a new infection. The cycle is usually completed in 36 - 96 hour. When stressful conditions occur, the microcolony may enter a cryptic form, sustained by aberrant bodies (ABs). Adapted from Longbottom and Coulter (2003) and Corsaro & Venditti (2004).

The internalisation process requires the action of the RHO-family GTPases acting as regulators of the actin polymerisation, and different species seem to entail a specific GTPase (Bastidas *et al*., 2013). Once internalisation is completed, the next relevant phase is to establish the intracellular niche. This phase starts with the transportation of the nascent inclusions containing *Chlamydia* spp. along microtubules directed towards the microtube organising centre (MTOC). The intracellular survival of *Chlamydia* spp. will be conditioned by its ability to achieve selective fusion for instance, by promoting fusion with some host cellular components such as exocytic vesicles with nutrients and preventing fusion with cellular elements, for example, lysosomes. The recruitment of specific fusion regulators makes this possible. *Chlamydia* spp. reaches this selective fusion mainly by recruiting members from three groups of fusion regulators: RAB GTPases, SNARE proteins and phosphoinositide lipid kinases (Elwell *et al*., 2016).

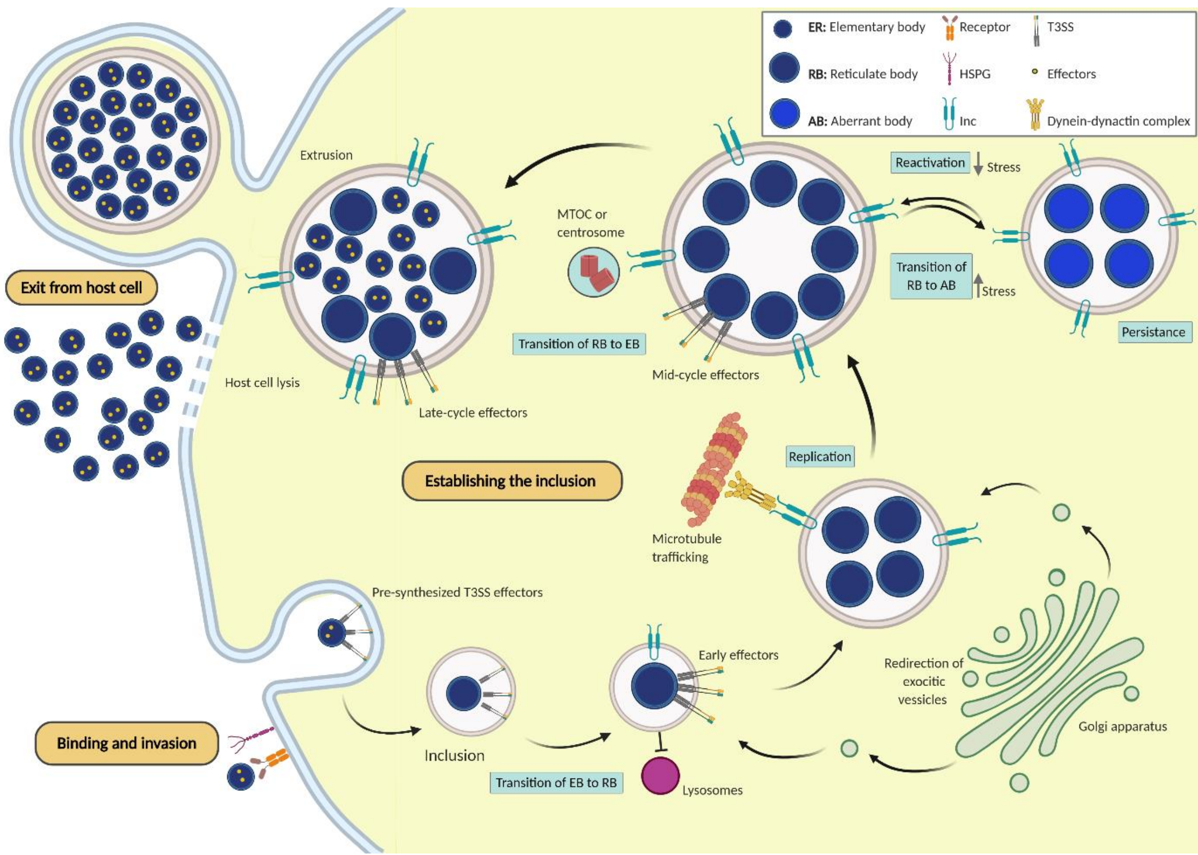


Figure 2.3: The life cycle of *Chlamydia* spp. Initially, the elementary bodies (EBs) bind to the host cells by forming a trimolecular bridge constituted by bacterial adhesins, host receptors and host heparan sulphate proteoglycans (HSPGs). The pre-synthesised type III secretion system (T3SS) effectors are injected into the cell. The T3SS facilitate internalisation through cytoskeletal rearrangements and/or establish an anti-apoptotic state by mitogenic signalling. The EBs are endocytosed into a membrane-bound compartment, the inclusion, which rapidly separates from the canonical endolysosomal pathway. The bacterial protein synthesis initiates, and EBs are converted to reticulate bodies (RBs). Inclusion membrane proteins (Incs) redirect exocytic vesicles that are in transit from the Golgi apparatus to the plasma membrane, thus facilitating the acquisition of nutrients. The emerging inclusion is moved, probably by an inclusion membrane proteins along microtubules to the microtubule-organising centre (MTOC) or centrosome. The RBs undergo exponential replication within the host cell's inclusions and control processes by means of the secretion of further effectors. If a stressful condition occurs, the RBs turn into a persistent state and transition to distended aberrant bodies. Once the stress has been overcome, the bacteria can be reactivated. Throughout the late phases of the infection, and before converting back to EBs, the RBs secrete late-cycle effectors and produce elementary-body-specific effectors. Finally, EBs exit the host cell by the process of either cellular lysis or extrusion. Modified from Elwell *et al.* (2016).

Along with this selective fusion, the RBs particles use strategies centred on altering the inclusion membrane to evade the fusion with lysosomes. *Chlamydiae* try to mimic the other cellular organelles by incorporating host-derived lipids into the inclusion membrane (Gitsels *et al*., 2020). Additionally, in the early phases after infection, *Chlamydiae* scape from the endocytic degradation pathway by modifying the inclusion membrane with gene products termed inclusion membrane proteins (Incs). These Incs lead the recruitment of the specific Rab GTPases mentioned above. The recruited Rab GTPases direct the endocytic process to begin either recycling or degradation (Gitsels *et al*., 2020). Both GTPases, Rab5 and Rab7 mainly promote degradation. The Rab5 is necessary for phagosome maturation and subsequent phagosomes-endosomes fusion (Gorvel *et al*., 1991; Bucci *et al*., 1992). As phagosomes mature, the protein is substituted by Rab7, which enables the bacterium to control the fusion between phagosomes and lysosomes and late endosomes (Meresse *et al*., 1995; Bucci *et al*., 2000).

The *Chlamydia* spp. nutrition deserves special attention. *Chlamydia* spp. lacks the biosynthetic enzymes necessary for the acquisition of lipids (Stephens *et al*., 1998). However, lipids such as phosphatidylcholine, sphingomyelin, phosphatidylinositol, and cholesterol, are important. They play an essential role in central bacterial mechanisms such as homotypic fusion, replication, and the stability and growth of the inclusion membrane, redifferentiation from RBs to EBs, and reactivation from the persistence stage (Elwell & Engel, 2012; Gurumurthy *et al*., 2014). *Chlamydia* spp. have generated specialised mechanisms, including vesicular and non-vesicular pathways, to acquire lipids as their biosynthetic enzymes are absent (Elwell & Engel, 2012).

The cytoplasmic vacuoles start growing and are surrounded by F-actin and intermediate filaments that make a dynamic structural framework, providing stability and restricting the passage of bacterial products to the cytosol of the host cell (Kokes & Valdivia, 2012; Bastidas *et al*., 2013). The transition from EBs to RBs occurs, and the transcription of early genes begins (Tan, 2012). Early effectors remodel the inclusion membrane; they also lead to the redirection of exocytic vesicles to the inclusion and enable interactions between host and bacteria (Moore & Ouellette, 2014). During the differentiation process, there is a reduction in the cross-linked complexes making membrane fluidity necessary for bacterial replication (Nelson, 2012). The RBs developmental form is fundamentally engaged in nutrient procurement and replication of bacteria (Bastidas *et al*., 2013). Accordingly, they possess a high expression of proteins engaged in the generation of ATP, the synthesis of protein and the transportation of nutrients. The bacterial replication is done by binary fission and leads to a significant expansion of the inclusion.

During a later stage, RBs asynchronously transform back into EBs, and their detachment possibly promotes this transformation from the membrane of the inclusion structures (Fields, 2012). The genes widely known as late-cycle genes, encode the outer membrane complex (COMC) of chlamydial organisms, which permit the spore-like comportment of EBs. This characteristic of EBs

is crucial for bacterial persistence in extracellular conditions. The exiting of EBs from the host cell is done by either the lysis of the host cell or the extrusion of the inclusion structure resembling an exocytosis process (Hybiske & Stephens, 2007). In the first way of exiting, the inclusion membrane suffers from changes in its permeability; and similarly, the nuclear membrane starts having increased permeabilisation. Finally, the plasma membrane is lysed by a calcium-dependent process resulting in the host cell's death (Hybiske & Stephens, 2007). When extrusion of the inclusion occurs, there is no release of inflammatory contents that keep the EBs protected from the host's immune response and possibly aid the bacterial persistence (Elwell *et al*., 2016).

The EBs have a spore-like cell wall called the outer membrane complex that enables their survival in the extracellular environment. This outer membrane is stabilised by a network of proteins that are cross-linked by disulfide bonds, confiding resistance against osmotic and physical stress (Nelson, 2012). The EBs were first thought to be metabolically inactive, although this has been disputed lately by some researchers arguing for changes in metabolic rates and biosynthetic activities (Omsland *et al*., 2014).

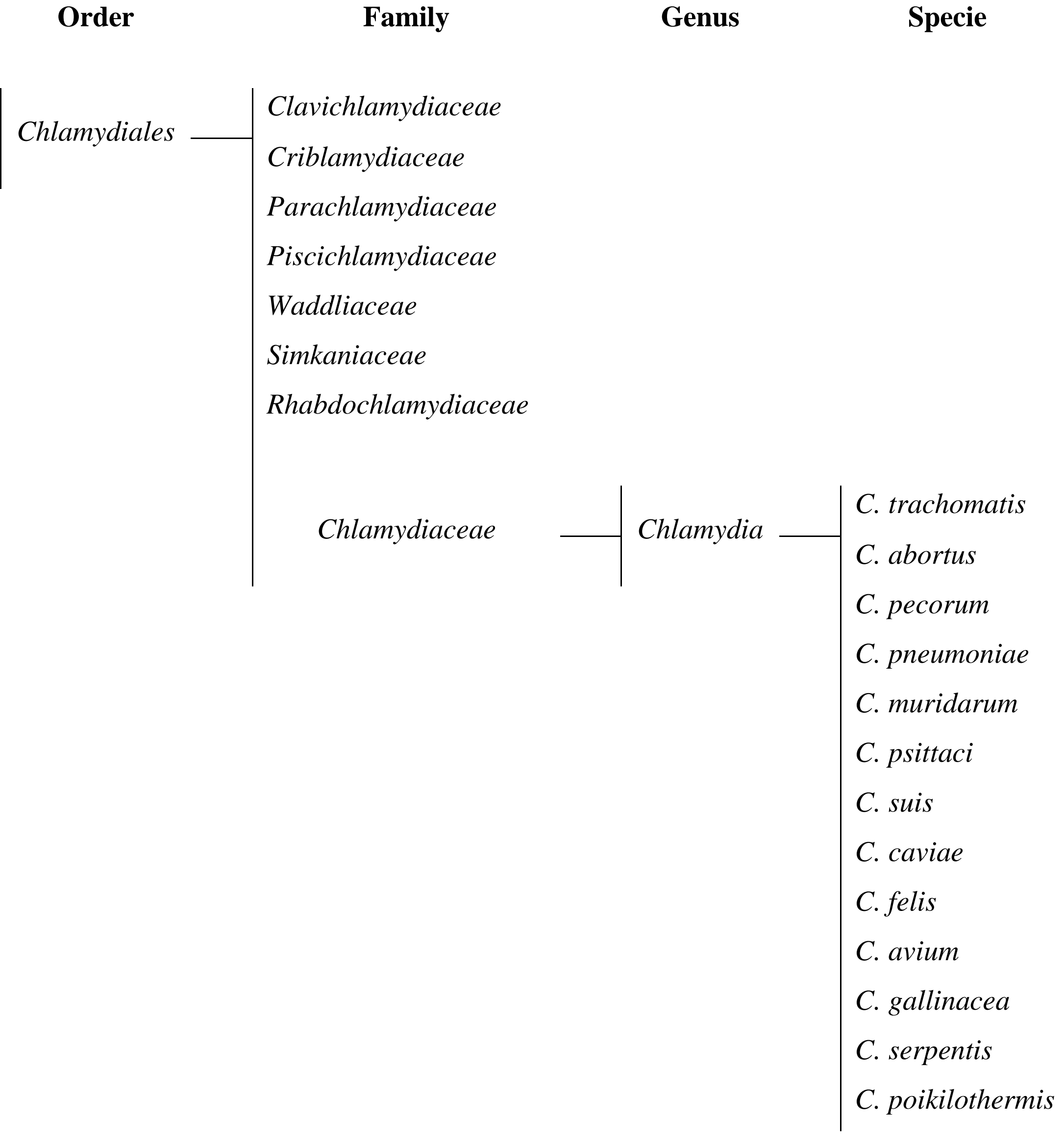


Figure 2.4: Taxonomy of order Chlamydiales.

2.2.1.3 Chlamydia spp. genome

Members of the *Chlamydiae* phylum present a considerably reduced genome size of around 1.04 Mb and lack many metabolic enzymes (Stephens *et al*., 1998). Despite this reduced size, comparative studies of the *Chlamydia* genomes have shown a high degree of conservation across species (Knittler *et al*., 2014). The *Chlamydia* genome's reduced size makes these bacteria dependent on a host cell to accomplish various metabolic requirements. As the nutrients are externally supplied, the development of *Chlamydiae* involves an obligate intracellular life cycle which is dependent on the metabolic state of host cells (Harper *et al*., 2000). The bacteria hijack important cellular routes and resources from the host to been used for their own needs. Supporting

this, the loss of many genes involved in metabolic pathways in the *Chlamydiaceae* family members may be responsible for scavenging some molecules from the host (Thomson *et al*., 2005).

A large proportion of the small *Chlamydiae* genome is assigned to synthesizing proteins that will be transported to the cytoplasm of the host cell. *Chlamydia* spp. utilises a battery of effectors to create the intracellular niche in order to survive and regulate the host immunological response. To date, the sequences of seven *C. abortus* genomes have been published (Thomson *et al*., 2005; Sait *et al*., 2011; Joseph *et al*., 2015). The *C. abortus* strain, known as S26/3, was the first reference genome used in the UK and contained a 1.1 Mb chromosome (Joseph *et al*., 2015). The majority of *Chlamydia* spp. strains have plasmids that are relevant to its virulence (Lei *et al*., 2014), unlike other members of the genus, *C. abortus* has had no virulence-associated plasmid has reported (Sachse *et al*., 2015; Joseph *et al*., 2015).

A single toxin gene tox was found in the *Chlamydiae* and resembled the cytotoxic gene from enterobacteria such as *E. coli* and *Clostridium*. The *C. abortus* genome sequence's evaluation has revealed the absence of 'niche-specific' tox orthologs, genes for the nucleotide-salvaging or tryptophan synthesis that typically distinguish other species (Reinhold *et al*., 2011). These differences would limit the cytotoxicity of *C. abortus* as well as explain to some extent some functional disparities with other members of the *Chlamydiae* phylum.

All of the *Chlamydiaceae* genomes share specific genes involved in critical functions such as the interaction with the host, including the first step of adhesion and antigen facilitated immune evasion (Nunes & Gomes, 2014). The inclusion membrane proteins (Incs) and the polymorphic membrane proteins (pmp) are examples of these common genes (Gupta & Griffiths, 2006). Also, as mentioned above, the T3SS is a virulence system that permits the pathogen to insert effectors into the host cell and is present in *Chlamydiaceae*. Among T3SS effectors, a type called translocated actin recruiting protein (TARP) is found in all genomes of *Chlamydiaceae* and also is presumed to facilitate infection by the remodelling of host cell actin (Nunes & Gomes, 2014). Additionally, all *Chlamydiaceae* genomes present hypervariable regions known as plasticity zones (PZ). Several genes have been identified within these PZs and contribute to the niche tropism and bacterial pathogenicity (Rockey, 2011; Thomson *et al*., 2005). Although all these similarities appear in *Chlamydiaceae* genomes, comparative analyses have disclosed differences (both inter and intraspecies) that could explain to some extent host and tissue tropisms (Bachmann *et al*., 2014). This field of study needs further investigation.

The variability observed within the PZ region and across the pmp genes between *Chlamydiaceae* species, seems to be determinant of the adaptation to different hosts (Bachmann *et al*., 2014). The PZ region encodes several genes involved in the interspecies variation. One of these encoded genes is the tryptophan (Trp) operon. This operon encodes the tryptophan synthase needed for the

synthesis of tryptophan which is critical for the persistence of the bacterium. Unlike *C. trachomatis, C. abortus* lacks the Trp operon which makes the latter dependent on the host cell tryptophan and possibly more exposed to the IFN-γ mediated indolamine 2,3-dioxygenase activation which facilitates tryptophan depletion. This genetic feature is translated into bacterial behaviour. Due to the absence of the Trp operon, *C. abortus* typically colonises niches where there is plenty of access to host tryptophan or where protection from IFN-γ mediated degradation is available (Thomson *et al*., 2005).

The evaluation of *chlamydial* genomes has revealed that different species acquired biotin in different ways. Some species present genes that regulate biotin synthesis (bioF_2ADB), others possess biotin transporter genes (bioY), or even both types of genes (for biotin synthesis and transport) (Fisher *et al*., 2012). *Chlamydia abortus*, *C. pecorum* and *C. psittaci* showed the existence of genes involved in biotin synthesis (Sait *et al*., 2014), and mainly*, C. abortus* and *C. psittaci*, keep both transport and biotin synthesis genes. This might permit these bacteria to switch between biotin metabolisms depending on the biotin availability at the infection site. Pregnant dams are known to suffer from transient biotin faults, and a correlation has been found between reduced biotin concentration in pregnant animals and decreased fetal biotin levels using a mouse model (Mock, 2005; Taniguchi & Watanabe, 2008). This can explain to some extent the reduced pathology in a pregnant mouse exhibited by the LLG *C. abortus* strains which were discovered to present a putative degraded biotin synthesis pathway (Bouakane *et al*., 2003; Sait *et al*., 2011). Evidence indicates that the presence of biotin synthesis genes may be crucial for bacterial survival in the placenta tissues (Thomson *et al*., 2005).

The genes pmp mentioned above were first found on the outer membrane of *C. abortus* (Longbottom *et al*., 1998). Since then, evidence supporting the presence of pmp genes in all of the *Chlamydia* species to varying degrees, has been reported (Knittler *et al*., 2014). The elevated number of these genes showed by some *Chlamydia* species may reflect their capacity to produce infection in multiple hosts (Knittler *et al*., 2014). Immunogenic properties had been attributed to pmp genes, which may facilitate the attachment of EBs to the host cell's surface. Additionally, pmp genes facilitate the bacterial evasion of the host's immunological response as they are involved in antigenic variation (Longbottom *et al*., 1998).

Regarding intraspecies variation, *Chlamydial* strains represent isolates of the same species, which are frequently classified by the genetic variation of the outer membrane protein cell surface antigen gene (*OmpA*) that is a single-copy gene (Lan & Igo, 1998). This *OmpA* encodes the major outer membrane protein (MOMP). The MOMP is a 40 kDa-size protein that is the most copious protein in both EBs and RBs, representing around 60% of the total protein mass (Caldwell *et al*., 1981). While *OmpA* is well known as the most polymorphic *Chlamydial* gene, MOMP is the central target of the immune response of host against *Chlamydiae* (Fitch *et al*., 1993). *Chlamydia trachomatis* is

the only species in the *Chlamydia* genus that comprises serovars. These serovars are different groups of strains differentiated by MOMP variations that present diverse host and tissue tropisms (Bachmann *et al*., 2014).

Chlamydia abortus is one of the most important aetiology of abortion in small domestic ruminants, although it is considered an infrequent cause of abortion in cows. The genomic investigation done in *C. abortus* strains isolated from sheep, goats and cattle identified few differences in the PZ region, suggesting tiny intraspecies variations (Thomson *et al*., 2005; Sait *et al*., 2011; Joseph *et al*., 2015). These minimal variations among different species' strains are unlikely to induce those noticeable differences in the disease pathogenesis regarding abortion, though this cannot be entirely discounted.

At present, a few sequences of *C. abortus* genomes are available (Thomson *et al*., 2005; Sait *et al*., 2011; Joseph *et al*., 2015). The first genome used as reference was the strain S26/3, initially isolated in the UK. Later, two Greek isolations known as LLG and POS, two French isolated strains AB7 and AB16; and strains denominated as Guangdong and B577, have also been identified. Unlike other *Chlamydial* species, most of the *C. abortus* are phenotypical, genomic and antigenically similar (Vretou *et al*., 1996; Laroucau *et al*., 2009). However, the Greek strain mentioned present antigenic diversity unique among pmp proteins (Siarkou *et al*., 2002).

2.2.1.4 Chlamydia spp. virulence factors

Roughly 10% of the *Chlamydiae* genome encodes virulence effectors (Betts-Hampikian & Fields, 2010), and three virulence factors seem largely to determine the *C. abortus* pathogenesis (Carter & Wise, 2004). Evidence suggests the genus-specific, complement fixation antigen, lipopolysaccharide antigen (LPS), is a virulence factor and the main promoter of inflammatory reaction after infection. Additionally, the *Chlamydial* Protease/proteasome-like Activity Factor (CPAF) also functions as a virulence factor of *Chlamydia*. The CPAF protease is an atypical serine protein (Huang *et al*., 2008), conserved to a high degree within the *Chlamydiales* order (Dong *et al*., 2005). Research has shown that CPAF promotes chlamydial pathogenesis by different paths, including the evasion of the host defence response and the enabling of the expansion of the chlamydial vacuole (Chen *et al*., 2009). The CPAF also facilitates the bacteria to escape from recognition by the T-cell by reducing the host transcription factors related with the generation of Major Histocompatibility Complex (MHC). Thus, the connections between the immune cells facilitated by MHC are affected, enabling *Chlamydia* replication.

Additionally, CPAF can degrade specific host proteins when this protease is secreted into the cellular host cytoplasm (Zhong *et al*., 2001). The above-mentioned T3SS, is another predominant virulence factor for chlamydial infection and pathogenesis. This system creates a hole in the vacuole membrane facilitating the transference of products (effectors) into the host cells' cytosol

(Rodolakis *et al*., 1998; Buxton *et al*., 2002). This translocation is done throughout the inner membrane, the periplasmic space, and the outer membrane by the injectosomes which are apparats that resemble syringes (Galan & Collmer, 1999; Galan and Wolf-Watz, 2006; Hayes *et al*., 2010). Additionally, most strains, unlike *C. abortus*, carry a plasmid which also contributes to virulence (Lei *et al*., 2014).

2.2.1.5 Environmental persistence and commensalism

The bacterial loads of the pathogen contained in normal births and aborted fetuses and birth products represent the largest source of contamination into the environment, and a potential risk for transmission to people and animals (Essig & Longbottom, 2015). The information available about *Chlamydiae*'s environmental persistence is limited. These organisms seem to be extremely resistant to conditions such as coolness, dryness, and darkness; however, evidence suggests that bacterial survival rates can be reduced, when bacteria are facing elevated temperatures. For instance, the EBs particles survive for almost 20 days in cool water and months in dry faeces, litter, dust, and feathers (Krauss & Schiefer, 1983; Perez-Martinez & Storz, 1985). The cryopreservation of semen does not effectively eliminate the bacterium (Storz *et al*., 1976). The high lipid content of *Chlamydiae* cell walls becomes them prone to organic solvents, detergents (Longbottom & Coulter, 2003).

Chlamydia can persist for long periods in the genital tract and can be difficult to be detected. This asymptomatic infection has resulted in ongoing discussion about whether *Chlamydia* plays a role as actual pathogens or commensals microorganisms. Currently, there is some uncertainty about whether these bacteria are commensals organisms or genuine pathogens (Reinhold *et al*. 2011). It has been quite challenging to find strong evidence supporting a link between the detection of DNA of Chlamydia in clinical specimens and the occurrence of illness. The persistence of Chlamydial infection during long periods while causing no disease and presenting no antigenic activity, hampers its clinical and serologic diagnosis.

2.2.2 The effect of *Chlamydia* spp. in cattle

The high prevalence of infection registered in cattle herds worldwide (varying from 45% to 100%), supports the ubiquitous distribution of Chlamydial infection (Kaltenboeck *et al*., 2005; Reinhold *et al*., 2011). Despite this high prevalence, the typical absence of a robust association between infection and clinical disease has prompted disagreements about the significance of *Chlamydia* members as pathogen agents.

From the two species associated most often with ruminant diseases, *C. abortus* has long been identified as a frequent cause of abortion during late-term pregnancy in sheep, with significant economic and welfare consequences. This bacterium is the etiologic agent of the illness popularly identified as ovine enzootic abortion (OEA) o enzootic abortion of ewes (EAE) that leads to significant economic losses in the livestock industry worldwide (Moeller, 2001; Hazlett, 2013).

The enzootic abortion produced by *C. abortus* in goats is analogous to that occurring in sheep in terms of clinical manifestation and pathological findings (Schlafer & Miller, 2007). Besides abortion, *C. abortus* infection of goats and sheep during pregnancy also causes stillbirth, weak offspring that easily fall ill or have problems when standing up (Storz, 1971).

Although it has been reported as an abortifacient agent in cattle (Borel *et al*., 2006), *C. abortus* is considered as an infrequent cause of bovine abortion. This is very different from what happens in sheep, where *C. abortus* is the most widespread infectious cause of abortion. The *C. abortus* infections seem to lead to different pathological outcomes, depending on whether infection occurs in sheep or in cattle. Specific differences in bacterial recognition by the host's immune system could be behind this differential pathogenesis but are not well understood. More investigation into this aspect is necessary, especially considering that these animals are habitually farmed together. *Chlamydia abortus* abortion in cows arises during the 6th to 8th month of pregnancy, and chiefly in the first pregnancy of heifers. Given that *Chlamydiae* alter the functions of the placenta, in addition to abortions, other alterations such as perinatal losses and the birth of *Chlamydiae* infected, premature, weak calves have been described as an outcome of the infection (Perez-Martinez & Storz, 1985; Cavirani *et al*., 2001; Wang *et al*., 2001).

Evidence has implied that abortion is most likely to occur in cows that have tested positive to *Chlamydiae* infection (6.6-fold higher risk) (Wehrend *et al*., 2005). Likewise, a significant association has been revealed between the increased occurrence of perinatal offspring losses, premature calving, and abortion with chlamydial infection detected by PCR-based tests (Kemmerling *et al*., 2009).

Chlamydia abortus infection usually results in asymptomatic infection. Even though chlamydial infections do not habitually cause disease, the possibility cannot be discounted completely that the recurrent and chronic infection may eventually affect animal health and development. Bovines are prone to the infection even if they do not, often show signs of disease. When infection occurs in cattle, it may produce sub-clinical and chronic reproductive disturbances, as well as infertility and mastitis (DeGraves *et al*., 2004; Wehrend *et al*., 2005; Kaltenboeck *et al*., 2005; Appino *et al*., 2007; Reinhold *et al*., 2011). Additionally, there have been reports of chlamydial infection in cattle causing polyarthritis (Storz *et al*., 1966; Twomey *et al*., 2003), encephalomyelitis (McNutt & Waller, 1940), enteritis (Doughri *et al*., 1974), keratoconjunctivitis (Otter *et al*., 2003), hepatitis (Reggiardo *et al*., 1989), pneumonia (Wilson & Thomson, 1968), vaginitis and endometritis (Wittenbrink *et al*., 1993), and chronic mastitis (Biesenkamp-Uhe *et al*., 2007).

2.2.2.1 Pathogenesis of Chlamydia *spp.*

Primary infection is presumed to occur by way of the oro-nasal route, and the tonsils hold the initial infection from where blood or lymph dissemination facilitate the bacteria reaching other organs

(Essig & Longbottom, 2015). Aborted fetuses, placenta and discharges during parturition from infected animals can contaminate food and drinking water, and thus other animals can become infected after the ingestion of these materials (Longbottom & Coulter, 2003; DeGraves *et al*., 2004).

Infection is initiated when EB particles infect epithelial cells of mucosal surfaces. Inside the host epithelial cell, infectious EBs become enclosed in endocytic vesicles where the transformation into RBs occurs. These inclusion structures serve as an intracellular membrane-bound niche. Within the inclusion, RBs split by the process of binary fission and then convert back to EB particles. The release of EBs forms is done by extrusion of the inclusions structures or the host cells' lysis. The free EB particles infect neighbouring cells. Many of the events implicated in the development cycle by which EBs are converted to RBs and RBs transition to EBs are unexplained and remain important questions to be examined.

Chlamydiaceae generate non-toxic or minimally toxic products, and their growth is modulated by their access to cytoplasmic nutrients from the host cell. The *Chlamydiae*'s cytotoxic effect is minimal, and disease arises when the host's immune response is weakened when it is facing persistent bacterial infection (Wang *et al*., 2009).

The host and niche tropism between *Chlamydia* species likely depend on the combination of several genetic differences and not on a single gene. The existence of mixed chlamydial infections within a host and within an organ is often reported (Borel *et al*., 2006). It is still not completely clear if *Chlamydia* species affecting cattle have different tropisms among host organs. According to studies published involving adult animals, *C. abortus* is mainly related to the genital tract and mammary tissues, *C. psittaci* has been detected in respiratory and genital samples (Kauffold *et al*., 2007; Kemmerling *et al*., 2009), and *C. pecorum* has been related with pulmonary, intestinal, joint, ocular and central nervous system syndrome (Kaltenboeck *et al*., 2009). In naturally infected calves, *C. abortus* has been found in the respiratory tract and conjunctiva, while *C. pecorum* has been chiefly found in gastro-intestinal samples (Reinhold *et al*., 2008). Several issues regarding coinfecting *chlamydial* species in bovines are yet to be understood, and further studies on the permutations of the organism are needed.

The ability to set up chronic sub-clinical infections is a common feature of all *chlamydial* species. Typically, once animals are infected, they remain as carriers for their whole productive lives (Koehler *et al*., 1997). When infected animals face stressful situations, the RBs can turn into aberrant bodies (ABs), this is a mechanism that permits the bacteria to remain within the host cells until the stressor is overcome (Schoborg, 2011). The formation of ABs may be linked with the characteristic of producing sub-clinical infections, although ABs have only been evidenced in *in vitro* studies and further investigation in the field supporting this is needed (Bavoil, 2014).

Usually, *C. abortus* produces a sub-clinical infection in an animal until the animal becomes pregnant, the moment at which a recrudescent from the persistent stage occurs and may cause illness. Although the location where *C. abortus* persists until this point is unknown, some researchers suggest that the bacterium possibly resides in the tonsil as this is the place that holds the infection initially (Jones & Anderson, 1988; Longbottom *et al*., 2013). Similarly, the mechanisms behind *C. abortus*'s persistence as a sub-clinical infection are unknown, as are what prompts the recrudescence of infection when pregnancy occurs (Rocchi *et al*., 2009).

2.2.2.2 Chlamydia shedding routes from cows and bacterial transmission among cattle

It is typically accepted that transmission in cattle happens; likewise, it occurs in sheep and goats; however, this has not been confirmed. In flocks, an aborting sheep sheds a massive number of infectious EBs in the aborted fetus, uterine discharges, and placenta, causing infection in other flock mates (Shewen, 1980). Additionally, vaginal, ocular, and nasal *Chlamydiae* shedding have been reported, as well as the bacterial elimination through semen, and urine (Perez-Martinez & Storz, 1985; Longbottom and Coulter, 2003; Kauffold *et al*., 2007; Reinhold *et al*., 2008; Kemmerling *et al*., 2009). Also, the faecal elimination of *Chlamydiae* by a carrier animal within a herd is considered to be an important transmission route. The presence of the bacteria in the exhaled air remains controversial, and no substantial evidence has been reported. Based on these observations, horizontal bacterial transmission appears to be the most significant risk for naive animals.

Bacterial transmission can be achieved by the direct interaction between animals or by indirect routes such as faecal-oral infection or the inhalation of contaminated air (Longbottom and Coulter, 2003).

Infection may also be acquired by the venereal route, through both natural mating and artificial insemination (Storz *et al*., 1976; Amin *et al*., 1999). However, the utilisation of artificial insemination has recently been found as to be a factor for a diminished risk of exposure to *C. abortus* (Djellata *et al*., 2020). Similarly, a higher prevalence of chlamydiosis has been reported in those herds employing natural services (Kemmerling *et al*., 2009), due to bacterial presence in animals with vesiculitis (Storz *et al*., 1968), or even asymptomatic bulls (Kauffold *et al*., 2007).

The introduction of infected replacement animals into a previously clean farm (flock or herd) seems to be the primary route of *C. abortus* transmission (Milne *et al*., 2009). Infected individuals could shed a considerable number of bacteria into the environment (Essig & Longbottom, 2015). Once in the environment, the bacteria can remain viable for several days, and even longer in cold weather, thus enabling further bacterial transmission (Longbottom & Coulter, 2003).

Further research into horizontal transmission has identified carrier cows from chlamydia-positive herds as the most probable bacterial infection source for calves. Evidence suggests that calves acquire both *C. abortus* and *C. pecorum* within the first two weeks after birth (Jee *et al*., 2004). Enhanced intensity and prevalence of *Chlamydia* infection have been found in herds with high calf population densities (Jee *et al*., 2004).

No reports investigating vertical transmission have been published, although in-utero infection is deemed likely. The vertical transmission of *C. abortus* from sheep to lambs is possible, though there has been no conclusive evidence gather by experimental trials, neither results supporting if this has a significant epidemiologic role for the disease (Essig & Longbottom, 2015).

2.2.2.3 Consequences of infection in cattle

The clinical manifestation of *C. abortus* infection in cattle gives less evidence that the diseases caused by *C. abortus* in sheep or *C. psittaci* in birds where outbreaks affecting several animals are often produced. Consequently, bovine chlamydial infection has been usually incorrectly considered irrelevant. Though for the most part latent or even without clinical manifestation, the chlamydial infection in cattle has a quantifiable impact at herd-level health (Reinhold *et al*., 2011). Measuring the effect is challenging as often there is no clear cause and effect link.

However, numerous features of *Chlamydiae* result in a substantial and negative impact on fertility as well as on milk production and animal growth rates. A cross-sectional study of a random selection of dairy farms gathered evidence supporting the idea that chlamydial infection has a significant negative economic effect, even when sub-clinical presentation occurs (Kemmerling *et al*., 2009).

Some studies have explored the changes in the fertility of cattle exposed to *C. abortus* re-infection. Briefly, the results have demonstrated that fertility is influenced by *C. abortus* sub-clinical, non-venereally transmitted infection (DeGraves *et al*., 2004; Kaltenboeck *et al*., 2005). The findings have indicated that *C. abortus* re-infection may induce infertility, but the infection outcome would be significantly influenced by the bacterial infecting dose, and by the route and source of the infection, and by the immunological response developed against the former infection.

Results from experimental intranasal infection in sheep have shown that relatively low infectious doses prompt chlamydial latency in non-pregnant animals (Longbottom *et al*., 2013). This latent infection only becomes activated when animals get pregnant when the infection proliferates and reaches placenta generating gestational disturbances, and possibly abortion (Essig & Longbottom, 2015). This persistence showed by *C. abortus*, followed by recrudescence, may play an essential role in determining abortion. Some results have shown that this asymptomatic form of the disease in non-pregnant females, may result in abortion just in a reduce proportion of animals (Waldhalm *et al*., 1971).

After the bacterial invasion of the placenta, the bacterium multiplies within trophoblast and may result in inflammation facilitated abortion. The cascade of inflammation is characterised by the generation of pro-inflammatory TNF-α and CXCL8. These molecules operate as signalling molecules that stimulate the recruitment of inflammatory infiltrate composed by neutrophils, monocytes, macrophages, T cells, and a lower number of B cells (Rocchi *et al.*, 20091). This cellular infiltrate leads to lesions in the fetal membranes and disruption in the chorionic epithelium and vascular thrombosis, causing abortion (Rocchi *et al.*, 2009). The protective inflammation reaction that attempts to clean the bacterial infection may end due to cytokines' profuse expression, especially TNF-α (Buxton *et al.*, 2002). Therefore the activation of the immune response in the placenta after *C. abortus* infection appears relevant in triggering abortion. Placental lesions often arise after 90 days of gestation, leading to abortion typically in late pregnancy phases (Buxton *et al.*, 1990; Essig & Longbottom, 2015). Sporadically, vulvar discharges may be found for up to 48 hours before the occurrence of the abortion, although abortion is often the most straightforward sign detected (Essig & Longbottom, 2015).

The economic impact of *C. abortus* infection in small ruminants is very high. After the first exposure to *C. abortus* following the bacterium's introduction into the flock, one-third of the pregnant sheep and twice as many pregnant goats may abort (Rodolakis & Mohamad, 2010). The level of abortions remains high for 2 or 3 years until almost all the females have become affected. After this, the disease often exhibits a cyclic nature, causing only up to 5% of abortions during some years until a new outbreak occurs, when almost every primiparous female will abort (Rodolakis & Mohamad, 2010). This cyclic pattern of evolution is caused by the life-long immunity induced after abortion that defends against future following infection (Rodolakis *et al.*, 1998). Curiously, *C. abortus* causes infectious abortion through primary infection, but it does not alter following pregnancies (Cheong *et al.*, 2019).

Several investigations have been conducted in sheep, yielding a broad spectrum of clinical findings including weak lambs, normal-appearance, stillborn, and pot-bellied lambs (Essig & Longbottom, 2015). Gross changes in the placenta include thickened membranes with a diverse degree of inflammation and frequently present yellow exudate (Longbottom & Coulter, 2003).

2.2.2.4 Diagnostic tests for Chlamydia *spp.*

The record of pregnancy losses and the detection of placental changes appear to be aspects significant for diagnostic guidance; however, confirmatory diagnostic needs support the use of laboratory tests. As in the case of other bacteria, the isolation and propagation of *C. abortus* is the gold-standard test, although this is a time-consuming approach (Essig & Longbottom, 2015). Smears should be taken from placental membranes and stained to investigate a potential chlamydial infection. For *Chlamydia* investigation, different stains can be utilised, including Giemsa or Ziehl-

Neelsen (Essig & Longbottom, 2015). Additionally, chlamydial antigens (often MOMP or LPS) can be detected by immunohistochemistry (IHC) in tissue sections.

Some serologic tests are also employed for the recognition of *C. abortus* exposure. The complement fixation test (CFT) is the most commonly utilized test, although it may give cross-reactive results as antibodies bind to LPS expressed by all the *Chlamydiaceae* members. ELISA is an approach more specifically appropriate for *C. abortus* antibody detection. This test detects antibodies to particular antigens such as MOMP or PMPs. There are several commercialised serological tests available; however, a weakness is that they cannot differentiate between naturally-infected animals and vaccinated animals (Sachse *et al*., 2009). This limitation of the commercial kits does not represent a significant problem in the local context as in Uruguay no vaccination has ever been used. Unfortunately, different serological investigations conducted in sheep by whole antigen-based CFT and ELISA for both the detection of *C. abortus* and *C. pecorum* infections have demonstrated weak performance with low specificity and sensitivity (Vretou *et al*., 2007; McCauley *et al*., 2007; Wilson *et al*., 2009; Bommana *et al*., 2017).

The utilisation of molecular tests represents a rapid and sensitive approach. Reactions targeting the *OmpA* gene have been demonstrated to be particular for *C. abortus* detection (Sachse *et al*., 2009). However, the presence of the bacteria does not automatically mean disease and results need to be correctly interpreted. *Chlamydial* nucleic acid can be intermittently detected over an extended period in rectal, conjunctival, and nasal swabs, even in animals without any clinical manifestation (Reinhold *et al*., 2008). Evidence of no continuing bacterial shedding supports the idea that individuals' carrier status may change over time, and thus false-negative PCR results can arise (Jee *et al*., 2004; Reinhold *et al*., 2008). Recent investigations have demonstrated the effectiveness of PCR testing coupled with restriction fragment length polymorphism (PCR-RFLP) in differentiating vaccinated animals from naturally-infected animals (Laroucau *et al.*, 2010; Wheelhouse *et al*., 2010). This PCR-RFLP technique detects specific SNPs solely present in the vaccine strain.

2.2.2.5 Measurements of control against Chlamydia *spp.*

As stated above, the main route of introducing the bacterium into a farm is by incorporating infected replacement animals from abroad. Keeping closed farms and restricting the incorporation of animals to those coming only from farms accredited as negative for the pathogen, are effective ways of preventing the introduction of the disease. For instance, the ovine enzootic abortion accreditation scheme comprises annual blood tests, in which after two consecutive negative results an accredited free status is obtained (Premium sheep and goat health scheme, 2008). Maintaining closed farms is rarely plausible for diverse reasons; thus, vaccination may play a vital role. Although it is available in other countries, negative *Chlamydial* accreditation is not currently available in Uruguay, so this is not a feasible strategy to apply locally.

The use of antibiotics has been suggested as an approach to controlling the severity of the disease (Entrican *et al*., 2001). Two administrations of antibiotics (tetracycline at a dose of 20 mg/kg) through the late pregnancy had been proposed to decrease the rate of abortions and the bacterial excretion at birth (Rodolakis *et al*., 1980). Still, this approach does not overcome the infection, and the bacterial shedding continues alongside the danger to pregnant women. There is not enough evidence today about the use of antibiotics as a safe and reliable strategy for preventing abortions or reducing bacterial shedding. When possible, this sort of measure should be utilised merely to avoid a high abortion rate during the first outbreak of the disorder; then vaccination should be implemented (Rodolakis *et al*., 1980). The potential risk of developing antibiotic resistance cannot be wholly dismissed (Entrican *et al*., 2012).

After the occurrence of abortion, the environment receives a massive bacterial shedding, and therefore actions to limit contamination should be increased by the isolation of the aborted animal which has suffered the abortion. The place where abortion has occurred should be decontaminated, and all infective materials should be discarded (Essig & Longbottom, 2015).

2.2.2.6 Vaccination

Typically, the essential aim of a vaccine is preventing infection. In the case of *Chlamydia*, a more realistic objective of vaccination would be to diminish pathology and reduce bacterial shedding (Beagley *et al*., 2009). Live-attenuated and inactivated vaccines have been utilised for chlamydiosis in animals. Currently, the *chlamydial* vaccines commercially-available are *C. abortus* vaccines and an inactivated *C. felis* vaccine (Longbottom, 2003). Some live attenuated vaccines against *C. trachomatis* have been evaluated but have shown significant limitations; therefore, no current vaccines to prevent *chlamydial* infections in humans are available.

Regarding *C. abortus* vaccination to prevent OEA in sheep, there are three commercially available vaccines with efficient results. From these, two vaccines (Enzovax® and CEVAC *Chlamydia*®) employ the live-attenuated 1B strain of *C. abortus*, while the remaining one (Mydiavac®) is composed of the inactivated whole organism (Essig & Longbottom, 2015). The live attenuated vaccines need to be utilised with caution because should neither be used in pregnant animals, nor handled by pregnant women (Entrican *et al*., 2012).

As the transmission of *C. abortus* occurs by the oral-nasal route, mucosal immunity appears to play a role in defining the disease's outcome (Entrican *et al.*, 2012). Considering that the route of the administration of a vaccine is fundamental for determining the efficacy of *chlamydial* vaccination, the combined use of systemic and mucosal vaccination may enhance the effectiveness of the results (Schautteet *et al*., 2011). Inducing a robust protective mucosal immunity could restrict entry and dissemination from the infection site.

2.2.2.7 Chlamydia *spp. co-infections*

Co-infections appear as a frequent finding in *chlamydial* infections at least in bovine production systems. Farms where *C. abortus* is frequently detected present other pathogens responsible for abortion, such as *Toxoplasma*, *Campylobacter* and *Leptospira* spp. (Longbottom *et al*., 2013; Vidal *et al*., 2017). *Chlamydia* spp. and *C. burnetii* concomitant infections have also been evidenced (McGivern *et al*., 1988).

2.2.2.8 The underestimated Chlamydia pecorum

Livestock species, including cattle, goats, and sheep, can similarly be infected by another pathogen within the genus *Chlamydia*, the *C. pecorum*. This is a ubiquitous bacterium and is often underestimated as a cause of pathology (Walker *et al*., 2015). *Chlamydia pecorum* triggers a broad spectrum of clinical signs involving arthritis, conjunctivitis and sporadic abortion, as well as pneumonia and mastitis (Walker *et al*., 2015).

2.2.3 Infection in humans

2.2.3.1 The zoonotic threat of Chlamydia *spp.*

Besides the economic losses due to decreased animal production, *C. abortus* is particularly alarming due to its potential zoonotic hazards. Reports about *C. abortus* as a zoonotic pathogen are available, and its threat for humans, although rare, is well documented, and it has been found for the most part to affecting women during pregnancy. The pathogen is often acquired during exposure to infected tissues, and if the infection affects pregnant women, they are at considerable risk of stillbirth, gestational septicaemia and abortion (Roberts *et al*., 1967; Pospischil *et al*., 2002a; Walder *et al*., 2005; Essig & Longbottom, 2015). The first cases of a human abortion produced by *C. abortus* infection were reported in 1986 (Eddy & Martin, 1986). Once pregnant women become infected, the bacteria can produce septicaemia that leads to disseminated intravascular coagulation, which finally can lead to spontaneous abortion (Johnson *et al*., 1985; Forsbach-Birk *et al*., 2013). Pregnant women should thus be encouraged to stay away from birthing ruminants. The pelvic inflammatory disease (PID) has been reported when *C. abortus* infection happens during the extra gestational stage (Walder *et al*., 2003). A recent report from Spain about the occurrence of atypical pneumonia associated with *C. abortus* infection has been published (Ortega *et al*., 2016).

Other *Chlamydia* species had also been identified as responsible for severe zoonotic diseases. *Chlamydia psittaci* is the causative agent of extensive zoonotic psittacosis, popularly identified as parrot fever or ornithosis (Cheong *et al*., 2019). The bacteria are spread through faecal or nasal discharges of infected birds, and zoonotic transmission occurs through the inhalation of infectious dust particles or air droplets (Andersen, 1996; Harkinezhad *et al*., 2009). Symptomatology in people is characterised by fever, headache, myalgia, chills, and malaise, sometimes accompanied by respiratory symptoms (Beeckman & Vanrompay, 2009).

Chlamydia caviae infection in humans may occur due to close contact with the primary hosts of the bacterium which are: cat, dog, guinea pig, rabbits, and horses; and cause mild conjunctivitis and severe community-acquired pneumonia (Lutz-Wohlgroth *et al*., 2006; Ramakers *et al*., 2017; Van Grootveld *et al*., 2018).

Infection of humans by other species is also possible, but few reports are available. For instance, *Chlamydia felis*, which has felines and dogs as the primary hosts, may produce conjunctivitis (Browning, 2004). Domestic poultry is the primary host of *Chlamydia gallinacea*, and infection in humans leads to atypical pneumoniae (Laroucau *et al*., 2009; Sachse *et al*., 2014). Finally, human infection by *Chlamydia suis*, that has swine as the primary host, has also been reported, but without associated symptomatology (De Puysseleyr *et al*., 2014; De Puysseleyr *et al*., 2017; Kieckens *et al*., 2018). *Chlamydia trachomatis* and *C. pneumoniae* have humans as the primary host; however, in the case of *C. pneumoniae*, a wide variety of non-human mammals and reptiles also act as host species. In the case of the latter, evidence generated by genotype analysis also suggests the likelihood of its zoonotic transmission (Cochrane *et al*., 2005; Kutlin *et al*., 2007). *Chlamydia avium, C. muridarum, C. pecorum* and *C. serpentis* transmission to humans is currently unknown (Cheong *et al*., 2019).

2.2.3.2 Epidemiological linkage of Chlamydia *spp. between bovine and human infection*

Human infection occurs through contact with infected animals, mainly during birthing, and may also occur by the consumption of water/food contaminated by infected abortion products.

The inhalation of infected products from small ruminants may likewise cause in serious respiratory illness in people. The ingestion of raw milk has also been proposed as a potential risk factor for human infection (Dawson, 1988). The failure to detect the bacterium in milk rules out the possibility of humans infection by the consumption of dairy products elaborated from infected animals, however; the possible contamination of milk by EBs coming from vaginal discharge appears as a significant risk that should not be underestimated (Radostits *et al*., 1994). A recent study from Spain linked the occurrence of atypical pneumonia and *C. abortus* infection (Ortega *et al*., 2016).

2.2.3.3 Symptomatology

Chlamydia abortus infection during a non-pregnant phase may induce mild influenza-like illness or, more rarely, pneumonia (Rodolakis & Mohamad, 2010). *Chlamydia abortus* infection may of concern especially to women from rural areas, as such areas are more likely to be where humans encounter herds of small ruminant animals and infection in pregnant women may have disastrous consequences. Human abortion may arise weeks or even months after contact with an infected animal. Typically, women become infected by the respiratory route when handling fetus, placenta,

or birth discharges without any nose or eye protection or disposable gloves. The first symptoms after infection in pregnant women include headache, fever, malaise, nausea, and vomiting. Abortion usually occurs late in pregnancy, after this febrile flu-like syndrome, accompanied by disseminated intravascular coagulation and failure in renal and liver functions, and even septicaemia (Pospischil *et al*., 2002b). Human abortions triggered by *C. abortus* have been documented in USA (Jorgensen, 1997), and in many European countries, such as France (Villemonteix *et al*., 1990), the Netherlands (Kampinga *et al*., 2000), Switzerland (Pospischil *et al*., 2002b), and Italy (Walder *et al*., 2005). Antibiotic treatment is necessary for women recovery (Helm *et al*., 1989). *Chlamydia abortus* infection in humans without treatment could result in life-threatening illnesses (Essig & Longbottom, 2015). A report of an extra gestational *C. abortus* infection in a young woman with severe PID, raised the chance that *Chlamydiaceae* other than *C. trachomatis* can cause PID (Walder *et al*., 2003).

2.2.3.4 Treatment of Chlamydia

Early treatments with oxytetracycline (TET) and erythromycin (MAC) have shown adequate responses in *C. abortus* infection in humans (Caul & Sillis, 1998; Longbottom & Coulter, 2003). Treatment should continue for up to 10 to 14 days to prevent relapse and to eradicate the bacterium. Isolation of patients is no necessary as no strong evidence of person to person transmission have been documented. A recent study suggested an initial treatment based on tetracyclines for patients experimenting with *chlamydial* infections, followed by the utilisation of quinolones and macrolides (Smith *et al*., 2010). There is substantial evidence supporting recurrent *chlamydial* infections in patients even after antibiotic treatment (Bragina *et al*., 2001; de Vries *et al*., 2009). Although *chlamydial* infections are commonly underestimated problems, there is still investigation required regarding how to treat *chlamydial* infections.

2.2.3.5 The immune response against Chlamydia *spp.*

In order to control the death or survival of the host cell, *Chlamydia* spp., somehow, can trigger pro-survival pathways and delay apoptotic pathways (van Zandbergen *et al*., 2004; Frazer *et al*., 2011; Bastidas *et al*., 2013). As *Chlamydia* spp. are intracellular pathogens, this strategy aims to avoid the host cell's premature death that prevents bacterial replication. Evidence had suggested that *Chlamydia* spp. infected cells display resistance to intrinsic and extrinsic stimuli of apoptosis (Sharma & Rudel, 2009). The *chlamydial* antigens recognition by the epithelial cells is done through the surface and endosomal receptors as well as cytosolic innate immune sensors (Elwell *et al*., 2016). Once these receptors are activated, the recruitment of inflammatory cells is prompted by the releasement of pro-inflammatory cytokines and chemokines (Nagarajan, 2012; Bastidas *et al*., 2013). *Chlamydiae* attempt to avoid clearance, and for this, the bacteria have developed a way to modify immune responses. *Chlamydial* infection can moderate the production of IFN or frustrate the downstream gene products that are part of the cell-autonomous immunity (Nagarajan, 2012).

Like other intracellular microorganisms, *Chlamydia* spp. produce significant modifications in the expression genes and the production of proteins by the host (Elwell *et al*., 2016).

The development of the protective immune response against *C. abortus* encloses complexity. Experimental infection with *C. abortus* in sheep by the intranasal route found that a low infective dose ($5x10^3$ inclusion forming units (IFU)) was presumed to induce persistent infection and end in abortion. In contrast, a higher dose ($5x10^7$ IFU) presented reduced rates of abortion (Longbottom *et al*., 2013). These findings may indicate that high infective doses might develop protective immunity. When comparing the number of *C. abortus* found in vaginal swabs after abortion in ewes challenged with high or low infective doses, the former presented a reduced number of bacteria, suggesting that a high dose may produce enough level immune response that facilitates the bacterial clearance.

Like other bacterial infections, *C. abortus* infection seems to stimulate the immune system by both innate and adaptive responses. Whether immune responses to *C. abortus* infection is fundamentally cell-mediated, antibody-based, or a mixture of both responses, remains unclear, though protection studies against primary infection have shown cellular responses to have a greater role than antibodies, with IFN-γ playing a significant immunological role in protection (Entrican *et al*., 2012). As *C. abortus* is an obligate intracellular microorganism, the immunity mediated by the cell may play a much more crucial role than antibodies in protection against primary infection (Entrican *et al*., 2002). Considering this, cell-mediated immunity is likely key for resolving *chlamydial* infection.

After *chlamydial* infection, neutrophils are one of the innate immune cells that first arrive at the place of infection. Although neutrophils have a short life due to apoptosis, they play an essential role because of the generation of inflammatory mediators (Register *et al*., 1986; van Zandbergen *et al*., 2004; Frazer *et al*., 2011). Neutrophils also enable bacterial dissemination from the site of infection, and long-term survival is possible as the neutrophils can be taken up by macrophages (Rupp *et al*., 2009).

Natural killer (NK) cells are also recruited to the infection site where they are presumed to act as an early source of IFN-γ (Tseng & Rank, 1998). The NK cells are also implicated in the induction of *Chlamydia*-infected epithelial cells through lysis. *Chlamydia* displays a downregulation of the major histocompatibility complex class I (MHC I) of epithelial cells, and these molecules are engaged with the selection of peptides for presentation at the surface of cells, essential to the immune response in vertebrates, thus avoiding the process of presentation to cells of the adaptive immune system. The MHC I are needed by NK inhibitory receptors, and when their expression is down-regulated, cells are likely to be the targets for NK cells (Hook *et al*., 2004).

As stated above, the restrictive effect of IFN-γ over the bacterial growth on tryptophan depletion was evidenced under *in vitro* conditions (Entrican *et al*., 1998). An increased level of IFN-γ in

lymph also evidenced the protective role of this cytokine in sheep that were challenged by *C. abortus*, and that had a previous *C. abortus*-abortion (Graham *et al*., 1995). Collectively, these findings indicate that IFN-γ production may protect against abortion caused by *C. abortus* (Rocchi *et al*., 2009). The role of IFN-γ in the restriction of intracellular *chlamydial* growth has been shown under *in vitro* conditions, this limitation is important for the host's defence but may also provoke a persistent infection important for pathogenesis (Mascellino *et al*., 2011). As described above, the mechanism of IFN-γ action is essentially the depletion of tryptophan.

The phagocytes (macrophages and dendritic cells (DCs)) are vital cells for the nexus between innate and adaptative immune systems, as they process the antigens from Chlamydia and present MHC I and II complexes to T cells. Macrophages also allow the replication, though limited, of *Chlamydia* within them (Rey-Ladino *et al*., 2007). To achieve survival within the macrophages, *Chlamydia* requires to avoid the lysosomal enzymes and inhibit the fusion between the vacuoles holding *Chlamydia* and lysosomes (Eissenberg & Wyrick, 1981). The survival of *Chlamydia* within macrophages works as a strategy to accomplish the bacterial dissemination from primary sites of infection (Moazed *et al*., 1998; Gieffers *et al*., 2004). Macrophages may play a role in developing persistent infections as once they get infected with *Chlamydia*, they can stimulate apoptosis of T cell death (Jendro *et al*., 2000). Only low infective doses present bacterial survival within macrophages, while higher doses developed quick cytotoxicity (Wyrick & Brownridge, 1978; Wyrick *et al*., 1978; Manor & Sarov, 1986).

The innate immune system uses pathogens recognition receptors (PRRs) as sentinels. The PRRs are located in the surface of the innate immune cells or within them and detect specific molecular patterns like the damage/danger-associated molecular patterns (DAMPs) and the pathogen-associated microbial patterns (PAMPs) (Janeway, 1989; Seong & Matzinger, 2004). The several complements of PRRs expressed by cells get activated and prompt signalling pathways in the cell. The signalling pathways can lead to the generation of cytokines, stimulation of the adaptive immune response and the initiation of death of cells (Bortoluci & Medzhitov, 2010). Despite that PRRs are a host defence mechanism, specific pathogens have developed adaptative strategies to use these receptors for their benefit to facilitate survival (Matzinger, 2002). The PRRs comprises the following four different groups: Toll-like receptors (TLRs), nucleotide-binding and oligomerisation domain (NOD), like receptors (NLRs), RIG-I-like receptors (RLRs) and C-type lectin receptors (CLRs).

The pro-inflammatory cytokines and chemokines such as CXCL8, TNF-α, IL-6 and IL-1β, recruit and activate further innate immune cells and also stimulate the adaptive immune response. The innate immune cytokines have been demonstrated to present protective and/or pathogenic function facing a *chlamydial* infection depending fundamentally on production time and location.

The cytokine TNF-α produces vasodilation, kills the intracellular pathogens (Kamalakaran *et al.*, 2013) and stimulate the recruitment of leukocytes to the place of infection (Bradley, 2008). The inhibition of the local TNF-α produced by antibody decrease the apoptosis rate in the inflammatory cells but have no impact on the clearance of *chlamydia* from the genital organs (Darville *et al.*, 2000). The inflammatory cascade is stimulated by the innate immune cytokines by the stimulation of other pro-inflammatory cytokines, for instance, the release of TNF-α and IL-6 is stimulated after the binding of IL-1β to IL-1 receptors (Netea *et al.*, 2010).

Keeping with the phagocyte cells, DCs are also relevant in activating and polarising T cell immunity as they present antigens. After chlamydial infection, DCs arrive at the site of infection (Brunham & Rey-Ladino, 2005) and facilitate bacterial survival, persistent infection, and dissemination as bacteria are capable of living and persisting inside these cells (Rey-Ladino *et al.*, 2007). After *chlamydial* infection, the recruitment of CD4+ T cells to the genital tract also occurs (Johansson *et al.*, 1997; Gondek *et al.*, 2012).

Evidence gathered in small ruminants has revealed that once a female has aborted, it does not abort again after re-infection, suggesting the generation of immunity strong enough to protect against abortion (Littlejohn, 1950). However, other results proved that the protective immune response is not sufficient to clear the *C. abortus* completely and bacterial shedding may continue even after the abortion (Livingstone *et al.*, 2005), and may cause a chronic infection.

Regarding the antibody response when a ruminant is facing *Chlamydial* infection, similar levels of antibodies against *C. abortus* have been found in aborted and non-aborted sheep (Livingstone *et al.*, 2005). Although antibodies do not seem to be involved in protection against abortion during primary placental infection, they may be relevant for immunological protection after the abortion. Animal antibody response against *Chlamydia* has an inconsistent pattern or may even not be detectable (Reinhold *et al.*, 2008). This fact raises questions about the utility of serology tests for the identification of positive animals.

2.2.4 Investigation of *Chlamydia* spp. in Uruguay and South America

2.2.4.1 The investigation conducted in ruminants and humans

The presence of antibodies against *C. abortus* in Uruguayan dairy cattle has been investigated in a convenient sampling study by an ELISA commercial kit (Cattáneo *et al*., 2009). In this study, a total of 318 cows from 28 small farms located in the middle-western area of Uruguay (departments of Durazno and Florida) were evaluated, from those 28% presented seropositive results. There is not any local molecular investigation about *Chlamydia* spp.

A few studies conducted in neighbouring countries such as Argentina and Brazil and others across South America are available about the role of *C. abortus* in bovine abortion. The confirmation of the presence of DNA from *Chlamydiaceae* species in reproductive losses from cattle has been recently reported in Argentina (Rojas *et al*., 2018). *Chlamydia abortus* was detected in 5 out of a total of 251 aborted fetuses and stillborns by means of the ArrayTube approach. Later, a group of researchers also from Argentina presented the first description of caprine abortions due to *C. abortus* (Di Paolo *et al*., 2019). The diagnosis was based on the detection of *chlamydia*-like bodies in trophoblasts using modified Ziehl-Neelsen stain, consistent gross and microscopy placental changes, and strong positive IHC cytoplasm of the trophoblasts and the intercellular space, and positive 23S rRNA-PCR. *Toxoplasma gondii* and *C. burnetii* were ruled out. In Brazil, an anti-Chlamydophila prevalence of 51.9% was reported by CFT among 417 samples collected from dairy and beef cattle from the state of São Paulo (Igayara-Souza *et al*., 2004). Recently, a serological evaluation by ELISA done in animals from 24 randomly selected farms located in the northern regions of Costa Rica (the provinces of Alajuela and Heredia), showed *C. abortus* was not significantly present as a pathogen (Salazar *et al*., 2015).

2.3 Concluding remarks

Infective reproductive losses have been identified as one of the most relevant sources of economic failures in the dairy industry in Uruguay. Investigating and understanding the role of pathogens which have not yet been investigated can enhance disease-prevention strategies. At present, no systematic evaluation of *C. burnetii* or of *Chlamydia* spp. has been conducted in Uruguay. Different molecular-based techniques such as PCR and qPCR testing can be utilized to identify and quantify bacterial species rapidly, often with high specificity and sensitivity.

This previous review above has provided broad evidence that *C. burnetii* is a frequently neglected pathogen that threatens not only productive animals but also public health. Moreover, there is a huge question mark about *Chlamydia spp.* infection in cattle in Uruguay.

As both *Chlamydia* spp. and *C. burnetii* bacteria can have severe negative human health consequences, especially in pregnant women, a careful national approach of investigation within a one-health framework is urgently needed in a country where women play a key role in the agricultural context.

2.4 Hypothesis and aims

Coxiella burnetii and *Chlamydia* spp. are often implicated in abortion in ruminants worldwide; however, no systematic and rigorous investigations have been done in Uruguay. Given the percentage of un-diagnosed abortions that are locally registered in conjunction with the lack of investigation of these bacteria, it has been hypothesised that the investigation of these organisms would show their occurrence in abortions in dairy cattle.

The aims of the study of agricultural ruminants in Uruguay presented in this thesis are:

i) To investigate the presence of *C. burnetii* and *Chlamydia* spp. in aborted material from dairy cattle

ii) To study the presence and burden of *C. burnetii* in collective milk samples from commercial farms

iii) To contrast the frequency of *Chlamydia* detection in vulvo-vaginal swabs from aborted animals and non-aborted herd mates

iv) to conduct a retrospective study about the role of aborting cattle as source of human Q fever

*Chapter 3: **Molecular prevalence of** **Coxiella burnetii** **in bulk-tank milk from bovine dairy herds: systematic review and meta-analysis***

The work presented in this chapter had been already published on the journal *One Health.* This publication can be founded in the Appendix A.

Rabaza, A., Fraga, M., Corbellini, L. G., Turner, K. M., Riet-Correa, F., & Eisler, M. C. (2021). Molecular prevalence of *Coxiella burnetii* in bulk-tank milk from bovine dairy herds: Systematic review and meta-analysis. One Health, (2021) 100208.
https://pubmed.ncbi.nlm.nih.gov/33553561/

3.1 Introduction

Coxiella burnetii the intracellular Gram-negative bacterium responsible for the zoonotic disease Q fever (Porter *et al.*, 2011) has many reservoirs, including ruminants, that represent the primary source of environmental contamination and of infection in people (Woldehiwet, 2004). This agent causes fertility disorders and metritis in cattle and is implicated in bovine abortion (Lang, 1990; To *et al.,* Bildfell *et al.,* 2000). It often leads to abortion in small ruminants when a pregnant dam is infected, as *C. burnetii* exhibits a specific tropism for the trophoblast cells in placental cotyledons (Rodolakis *et al.,* 2007).
Coxiella burnetii has a complex epidemiological pattern and characteristics that make its control challenging. It is widely disseminated in nature and infects a large number of species, including mammals, birds, reptiles and fish (Sawyer *et al.,* 1987). There are two maintenance cycles in nature, one involving domestic species, and another including wild animal species and their ectoparasites. Ticks may be involved in the transmission of *C. burnetii* between wildlife and domestic species (Aitken *et al.,* 1987). Additionally, the agent is extremely resistant, remaining viable in the environment over extended periods (Aitken *et al.,* 1987). *Coxiella burnetii* can also undergo air-borne transmission by way of contaminated dust particles, which can be facilitated by hot and dry weather conditions (Roest *et al.,* 2011a; Nusinovici *et al.,* 2015a).

A large human outbreak of Q fever reported in the Netherlands (2007-2010), comprising more than 4000 cases, brought attention to the need for robust surveillance campaigns and highlighted its importance as a threat to public health authorities (Roest *et al.,* 2011a; Schneeberger *et al.,* 2014). Transmission to people is principally by the inhalation of aerosolised contaminated animal placenta and birth fluids during abortions or the birth of normal offspring (Roest *et al.,* 2012). Practices such as the assistance of calving, handling of birth products, and manure spreading may present a high risk for *C. burnetii* transmission to humans (Berri *et al.,* 2003; Bernard *et al.,* 2012; Sun *et al.,* 2016). Raw milk and dairy product consumption may also carry risk since it can enable infection, although there is no consensus about the importance or effectiveness of the digestive route (Rodolakis *et al.,* 2007; Lang, 1990; Nusinovici *et al.,* 2015b; Schneeberger *et al.,* 2014; Roest *et*

al., 2012). Nevertheless, the risk posed by respiratory exposure to aerosols produced during milking of animals should not be underestimated (Berri *et al.,* 2002).

The level of bacterial load carried along the different routes differs among ruminants (Rodolakis *et al.,* 2007). While parturition products are the primary source of shedding in small ruminants, milk is the main shedding route of *C. burnetii* in dairy cattle. Even asymptomatic animals (Sun *et al.,* 2016) or seronegative cattle (Bernard *et al.,* 2012) have been identified as *C. burnetii* milk shedders. *Coxiella burnetii* can be excreted in milk for up-to 13 months (Fishbein & Raoult, 1992; Rodolakis *et al.,* 2007) although this may be intermittent (Lang, 1990). Two patterns of shedding have been identified in dairy cows which can be persistent heavy shedders or sporadic shedders (Sun *et al.,* 2016).

Based on these heterogeneous shedding patterns, composite samples such as bulk-tank milk (BTM) constitute useful and easily accessible specimens for large scale epidemiological investigation. A positive result provides robust evidence for the identification of infected herds, BTM testing is the preferred diagnostic approach for disease identification in many countries (Rodolakis, 2009) and has epidemiological value for the monitoring of infection status over time in follow-up evaluations (Eldin *et al.,* 2017).

Recent large human Q fever outbreaks in the Netherlands, Spain, France and Germany have increasingly focused attention on Q fever in many European countries where strategies including mandatory notification/reporting of the disease have been implemented.

3.1.1 Objectives

This chapter's specific objectives were to systematically review studies of the herd prevalence of *C. burnetii* in dairy cattle using PCR testing on BTM samples, conduct a meta-analysis to determine the overall European and global prevalences and assess geographic regions, average herd size, local legislation for Q fever, and per capita income for each country where studies were conducted as potential moderators. A systematic review was conducted to identify publications reporting the investigation of *C. burnetii* on BTM samples analysed by molecular techniques. The purpose of this meta-analysis was to examine the prevalence of *C. burnetii* in composite milk samples from different countries and get an estimated global overall herd prevalence. The results obtained in this chapter will offer a useful point of comparison later in this thesis for the original work conducted in **Chapter Four,** in which the herd prevalence of *C. burnetii* was investigated in pooled milk samples in Uruguay‘s commercial herds.

3.2 Materials and methods

3.2.1 Literature search and study selection

This study's systematic review and meta-analysis followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (Moher *et al.,* 2009) (Figure 3.2). The search strategy identified publications reporting the prevalence of *C. burnetii* on BTM samples analysed by molecular studies. The following electronic databases were used to identify studies published from January 1973 to November 2018 (week 43 of 2018): CAB Abstracts, Medline, PubMed, Web of Science, Scopus, Science Direct and Google Scholar. The literature search comprised the terms: "*Coxiella burnetii"* or "Q fever" or "coxiellosis" and "PCR" or "qPCR" or "real-time PCR" or "molecular diagnosis" and "BTM" or "milk", with no language restriction. No constraint on study design was applied at this phase. Additional publications were identified by cross checking references included in the articles. Duplicates were identified by reference management software (Mendeley) and manually removed.

3.2.2 Eligibility- Inclusion criteria

Publications on studies fulfilling all the following a pre-established criteria were eligible for inclusion: (i) molecular investigation of *C. burnetii* by PCR techniques, (ii) studies with random sampling, (iii) composite single test-day samples obtained from the bulk storage tank located on a dairy cattle farm, (iv) primary studies but not reviews, (v) cross-sectional studies reporting prevalence. Authors of articles not stating the total number of dairy cattle herds from which the sample was drawn were contacted to provide this missing data. All those authors unable to provide all the missing information were excluded from the evaluation. Publications were examined by two independent reviewers (AR and MF) to ensure they met the inclusion criteria. Discrepancies between the two reviewers in views of eligibility were discussed with the rest of authors until agreement was reached.

3.2.3 Data extraction and Meta-analysis

Studies were screened by title, and abstracts and irrelevant publications were excluded. The remaining studies were full-text checked against the inclusion criteria described above. Articles that did not fulfil all these criteria were excluded. The number of publications excluded is shown in Figure 3.1. Data were systematically extracted from all the studies that satisfied the inclusion criteria, including name of the first author, year of publication, study title, name of the journal, country where the study was conducted, study methodology (duration of sampling, herd size, sample size, number of positive herds and/or prevalence, randomisation), molecular technique and the selected target gene used. When informed, findings about the association between *C. burnetii* infection, and diverse risk factors were also gathered for the discussion of putative risk factors related to *C. burnetii* found in the studies.

The *C. burnetii* herd prevalence determined in BTM samples the dependant variable was considered as the effect size for the studies included in the meta-analysis. This meta-analysis of proportions was performed as outlined by Wang (Wang, 2018). The heterogeneity among studies was first investigated by Cochran's Q (X^2) that tests the null hypothesis of homogeneity, and then quantified by using Higgins' I^2 statistics (Borenstein *et al.,* 2009). The heterogeneity was measured to select the model for the overall weighted *C. burnetii* herd prevalence estimation. As the level of heterogeneity was high, a random-effects model was first used to address within-study variance (the sampling error) and the between-studies variance (τ^2). Possible sources of heterogeneity were investigated by analysing moderators. The evaluated moderators included: i) geographic region: Europe vs non-Europe; ii) average herd size; iii) local legislation for Q fever: mandatory notification vs non-mandatory notification (Sanzo *et al.,* 1993; Moher *et al.,* 2009; Borenstein *et al.,* 2009; van der Hoek *et al.,* 2010; Sidi-Boumedine *et al.,* 2010; Dorsett-Martin, 2010; Muskens *et al.,* 2011; Kargar *et al.,* 2013; Sulyok *et al.,* 2014; Barberio *et al.,* 2014; Anon, 2017; Wang, 2018), and iv) gross national income (GNI) per capita classification from the year the study was conducted, based on the Atlas method (The World Bank Data and Statistics, 2019). A subgroup analysis was performed for the categorical moderators. Categorical moderators were analysed using a mixed-effects model. The statistical significance of the moderators was evaluated by an omnibus test (*QM*) within the mixed-effects model (Viechtbauer, 2010). The proportion of heterogeneity accounted for by each moderator was explored using the R^2 index. Meta-regression was also utilised to explore heterogeneity among the studies. All the moderators and their interactions were entered in the initial model and non-significant terms were then dropped stepwise (from lowest R^2 to highest R^2) (Li *et al.,* 2020). The odds ratio (OR) for $\log_e$ average herd size was additionally investigated. Association among moderators was assessed by means of the Pearson correlation coefficient (r). Results from the meta-analysis with the corresponding 95% confidence intervals were summarized using forest plots. Egger's test was used to test for the possibility of a publication bias for studies with low or high effect sizes (Egger *et al*., 1997). All the assessments were conducted using RStudio software with metafor package, mvmeta package and metaprop commands (RStudio, 2020).

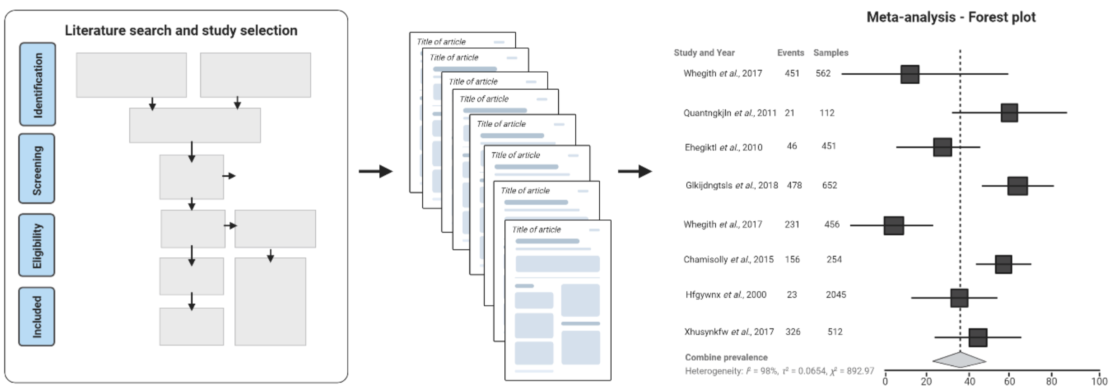


Figure 3.1: The systematic review and meta-analysis workflow.

3.3 Results

3.3.1 Description of the studies

After the removal of duplicates, a total of 179 studies was identified initially (Figure 3.2). Seventeen studies from twelve different countries (Belgium, Colombia, Hungary, Iran [2 studies], Italy [3 studies], Latvia, Netherlands [2 studies], Portugal, Spain, South Korea, UK and USA [2 studies]) were eligible for the meta-analysis based on the inclusion criteria. Six of those studies were conducted in non-European countries and 11 in European countries; 10 were conducted in countries where Q fever is a notifiable disease, while 7 were from countries where it is not. The study conducted in the Basque Country was included in the subgroup with mandatory notification, although this is the only Spanish province where the notification for Q fever is compulsory. Finally, 3 studies were conducted in upper-middle-income countries and 14 studies were in high income countries. The seventeen selected articles are summarised in Table 3.1. They included test results for a total of 4,031 BTM samples collected over 9 years (2006 to 2015). Studies employed either conventional PCR (n=5), quantitative PCR (n=9) or nested PCR (n=3) testing. The transposon-like repetitive region of the bacterial genome (*IS1111*) was the gene most frequently used as the target in these PCRs (n=14), followed by *com1* (n=2), icd (n=1) and 16S rRNA genes (n=1) (Table 3.1).

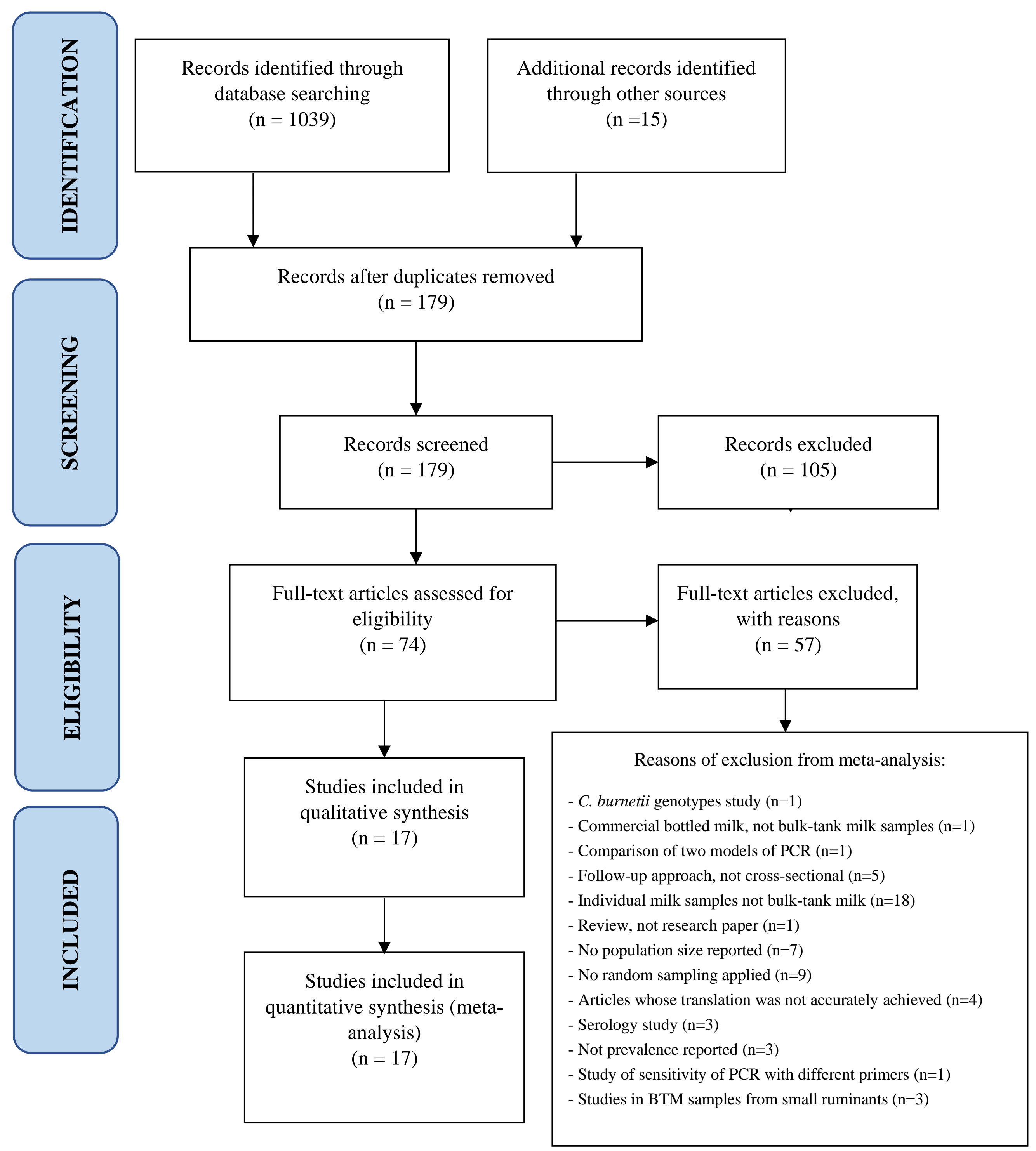


Figure 3.2: PRISMA flow diagram for the systematic review describing the study design process.

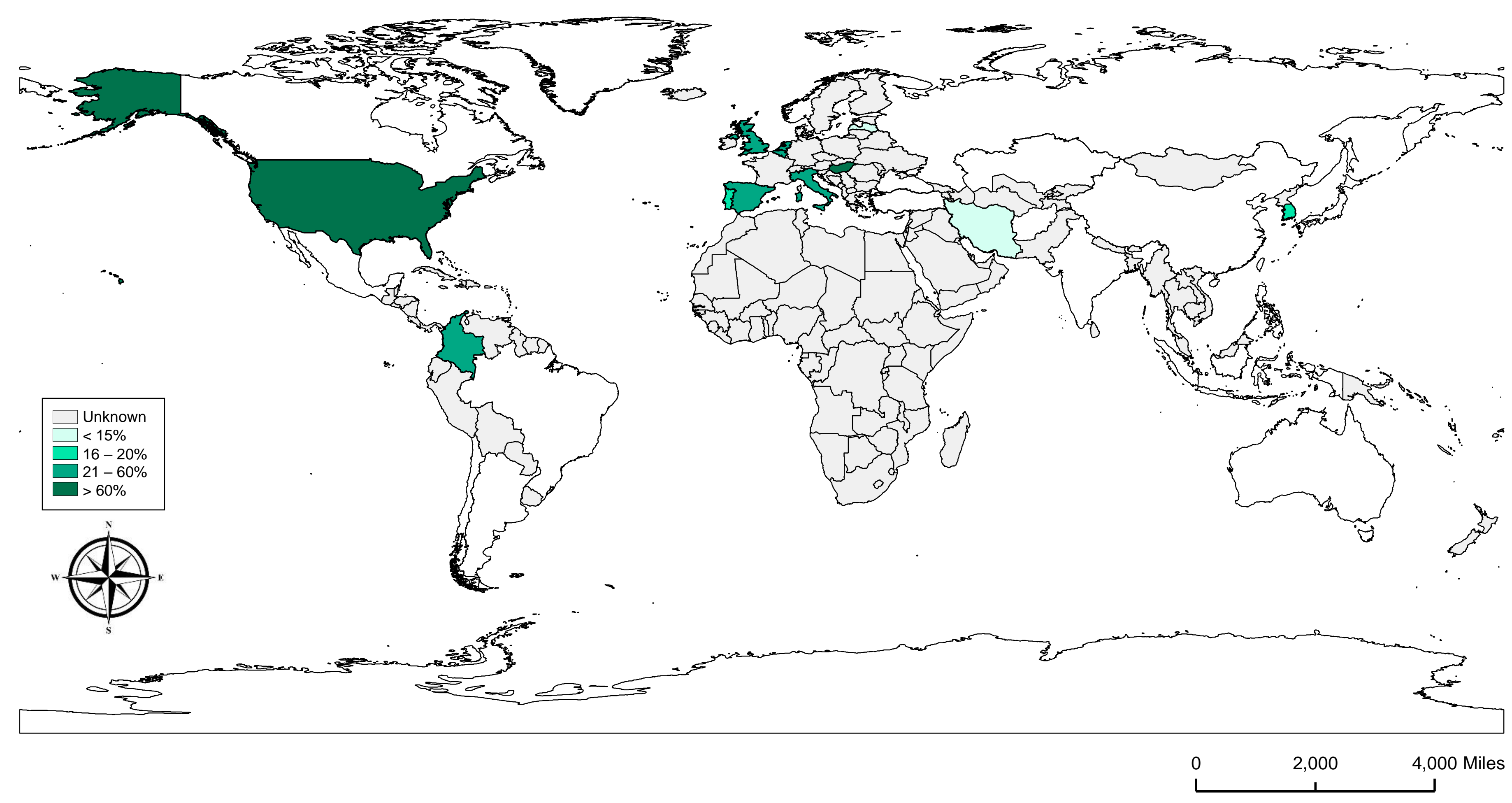


Figure 3.3: Prevalence of *Coxiella burnetii* in bulk-tank milk (BTM) samples analysed in countries with studies included in the meta-analysis. For those countries where several studies matched the inclusion criteria (Italy, Netherlands, Iran and USA), the prevalence reported by the most recent study is shown. For graphic purposes, non-nationwide studies were shown as whole country prevalence. Although Alaska is shown as part of the continental USA, no study of BTM *C. burnetii* in Alaska was included in the meta-analysis

Table 3.1: Characteristics and main results of the eligible studies ordered by molecular prevalence of *Coxiella burnetii* in composite milk samples.

Author	Year	Country	Study area	Average herd size	Period of study	Risk factor analysi	Gross national income per capita [*]	Is Q fever a mandator	Molecular approach	Target gene	N herds in	Percentag e of herds sampled	BTM(i) sample s	Positive BTM samples	Prevalenc e	95% CI	
Boroduske *et al. 2017*	2017	Latvia	Nationwide	8.6	2015	Yes	High-income	Yes	qPCR	*IS1111*	5,040	5	252	27	10.7	7.2	14.
Kargar *et al 2013*	2013	Iran	Johrom	3.7	-	Yes	Upper-middle-	Yes	nPCR	*com1*	3,000	3.3	100	11	11	5.5	18.
Seo *et al.* 2018	2018	South	Gyeongsang	74	2015	No	High-income	Yes	nPCR	16S	869	69.9	607	108	17.8	14.	20.
Rahimi *et al. 2010*	2010	Iran	Chaharmahal	48	2008	No	Upper-middle-	Yes	nPCR	*com1*	95	29.5	28	5	17.9	5.5	34.
van Engelen *et al.* 2014	2014	Netherlan	Nationwide	71.7	2009-	Yes	High-income	Yes	qPCR	*IS1111*	20,746	1.5	309	58	18.8	14.	23.
Anastácio *et al.* 2016	2016	Portugal	Nationwide	21.7	2009-	Yes	High-income	No	PCR	*IS1111*	1,712	2.6	45	9	20	10.	33.
Velasova *et al. 2017*	2017	UK	Nationwide	133	2014-	No	High-income	No	qPCR	*icd* /	10,491	2.1	220	57	25.9	20.	31.
Czaplicki *et al.* 2012	2012	Belgium	Wallonia	28.5	2006	Yes	High-income	No	qPCR	*IS1111*	5,086	1	50	15	30	8.7	51.
Magnino *et al*. 2009	2009	Italy	Cremona,	180	2007-	No	High-income	No	PCR	*IS1111*	3,550	11.2	400	161	40.2	35.	45.
Valla *et al. 2014*	2014	Italy	Nationwide	42.5	2011-	No	High-income	No	PCR	*IS1111*	30,000	1.1	344	138	40.1	35.	45.
Contreras *et al.* 2015	2015	Colombia	Monteria	150-600	2012	No	Upper-middle-	No	PCR	*IS1111*	3,341	0.3	11	5	45.5	16.	75.
Astobiza *et al.* 2012	2012	Spain	Bizkaia	46.1	2009-	No	High-income	No /	qPCR	*IS1111*	178	100	178	92	51.7	44.	59
Muskens *et al.* 2011	2011	Netherlan	Nationwide	65.7	2007	No	High-income	Yes	qPCR	*IS1111*	21,313	1.6	341	193	56.6	50.	61.
Vicari *et al*. 2013	2013	Italy	Lombardy	182	2011	No	High-income	No	PCR	*IS1111*	5,750	5	287	173	60.3	54.	65.
Bauer *et al.* 2015	2015	USA	Indiana	145.3	2011	No	High-income	Yes	qPCR	*IS1111*	1,225	25.8	316	193	61.1	55.	66.
Gyuranecz *et al.*2012	2012	Hungary	Nationwide	14.5	2010-	No	High-income	Yes	qPCR	*IS1111*	17,172	0.1	15	10	66.7	40.	88.
APHIS 2011	2007	USA	18 states(iii)	162.6	2007	No	High-income	Yes	qPCR	*IS1111*	54,100	1	528	406	76.9	73.	80.

(i): BTM: bulk-tank milk samples, one per herd; PCR: conventional PCR; qPCR: real-time PCR; nPCR: nested PCR. (ii) mandatory notification in Basque Country. (iii) California, Idaho, Indiana, Iowa, Kentucky, Michigan, Minnesota, Missouri, New Mexico, New York, Ohio, Pennsylvania, Texas, Vermont, Virginia, Washington, Wisconsin. [*] The World Bank Data and Statistics.

3.3.2 The estimated overall meta-prevalence of *Coxiella burnetii* in BTM samples

The median size of the eligible studies was 252 BTM samples. Of the total 4,031 BTM samples, 1,661 were diagnosed positive by molecular techniques. The percentages of positive BTM samples among the studies ranged from 10.7 to 76.9%. The overall weighted prevalence of *C. burnetii* in the random-effects meta-analysis was estimated at 37.0% ($CI_{95\%}$25.2-49.5%). The I^2 value of 98.0% ($CI_{95\%}$95.9–99.0) suggested high heterogeneity, with a τ^2 of 0.0654 ($CI_{95\%}$0.3296-1.4997), and an X^2 statistic of 892.97 (P<0.0001). The overall meta-analysis is shown in a forest plot (Figure 3.4). No evidence of apparent bias was detected in the publications that were identified and incorporated in the meta-analysis (Egger's test P=0.599).

3.3.3 The meta-prevalence of *Coxiella burnetii* and moderator analyses

The weighted average prevalence was similar within each of the two geographic subgroups (36.9% in European countries and 37.1% in non-European countries; (I^2=98%; X^2=870.29, P<0.01; *QM* (df=1)=0.002, P=0.98), albeit with differing 95% confidence intervals of 22.8%– 52.2% in the former and 18.0%–58.5% in the latter group of countries (Figure 3.4). Similarly, countries with mandatory and non-mandatory notification of Q fever had a prevalence around 37.0% ($CI_{95\%}$22.3–52.9% and $CI_{95\%}$19.4–56.4%, respectively; (I^2=98%; X^2=892.61, P<0.01; *QM* (df=1)=0.010, P=1.00) (Figure 3.5). In the subgroup analysis based on the GNI per capita (Figure 3.6), the prevalence was 40.1% ($CI_{95\%}$27.9–52.9%) in high-income countries and 21.2% ($CI_{95\%}$2.2–50.2%) in upper-middle-income countries (I^2=98%; R^2=3.10%; X^2=844.20, P<0.01; *QM* (df=1)=1.39, P=0.24) (Figure 3.7). None of the three factors above appeared to contribute meaningfully to the observed level of heterogeneity based on the subgroup analysis. The meta-regression revealed that average herd size accounted for a significant proportion of the heterogeneity (I^2=97%; R^2=33.01%; X^2=552.23, P<0.01; *QM* =4.55, P=0.03). As a significant moderator, high-size herds presented a higher herd-level *C. burnetii* BTM prevalence. The odds ratio for the $\log_e$ of herd size was 2.00 ($CI_{95\%}$1.24-3.52; P=0.02). A strong positive correlation was found between countries located in Europe and countries with high GNI per capita income (r=0.633, P<0.05), but between location in Europe and compulsory disease notification (r=-0.239, P=0.24), and between high GNI per capita and notification (r=-0.076, P=0.82) correlations were weak and negative. Herd size was not meaningfully correlated with the origin of the studies (r=-0.468, P=0.12), notification (r=-0.428, P=0.16), or with GNI per capita (r=-0.444, P=0.14).

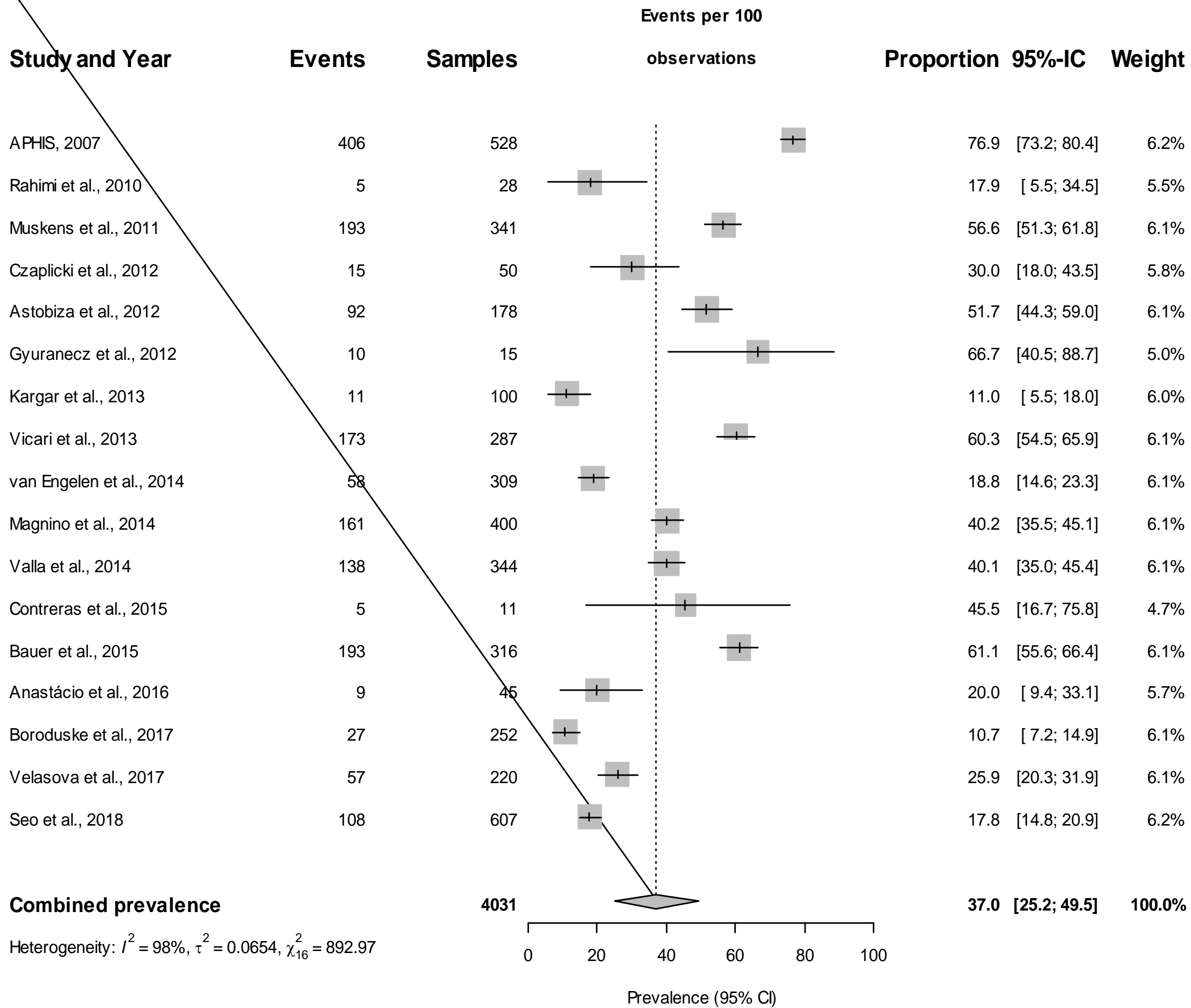


Figure 3.4: Forest plot for the meta-analysis of herd-level *Coxiella burnetii* prevalence based on bulk-tank milk samples from the seventeen studies that matched the inclusion criteria in the systematic review. All studies.

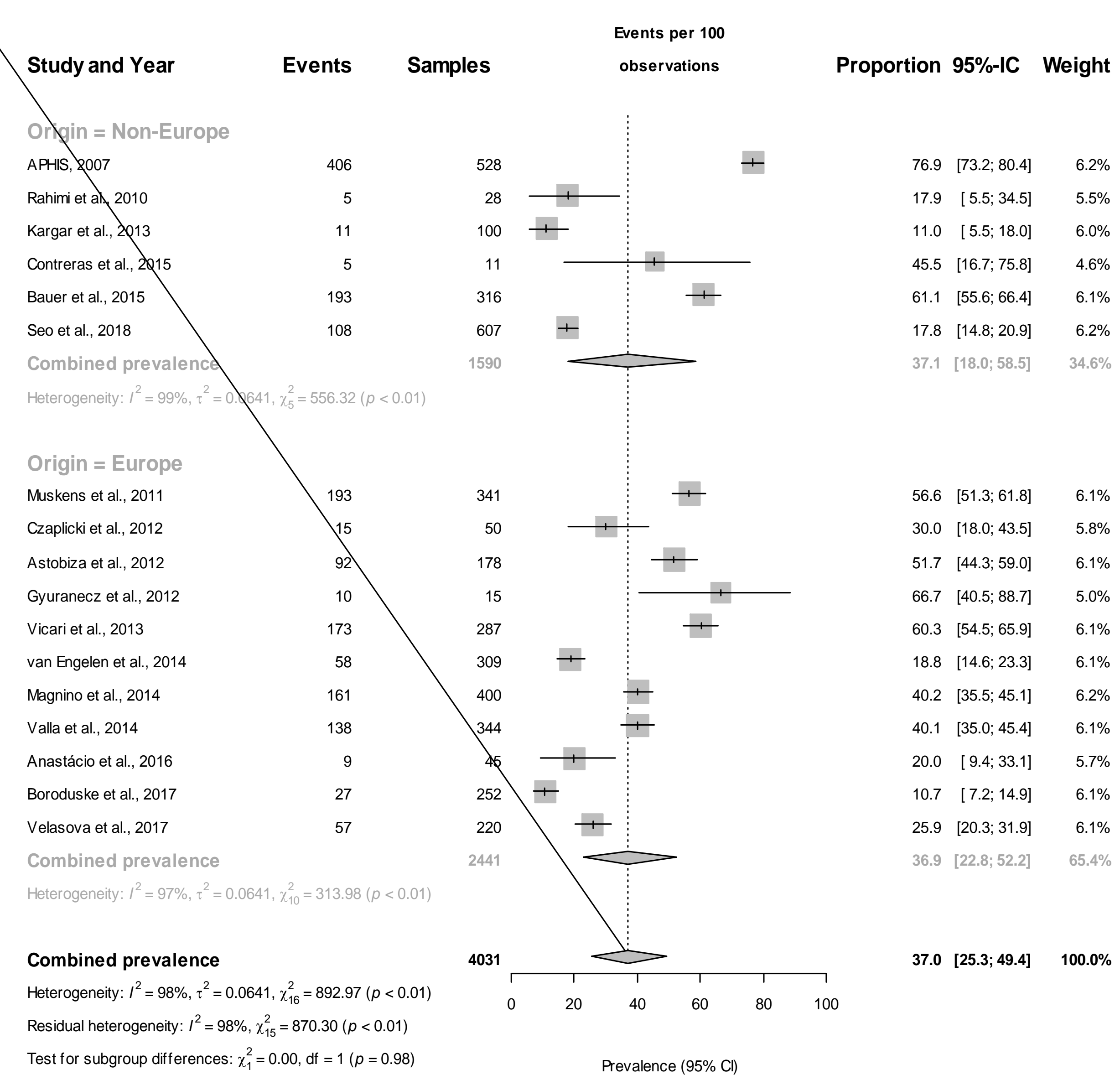


Figure 3.5: Forest plot for the meta-analysis of herd-level *Coxiella burnetii* prevalence based on bulk-tank milk samples from the seventeen studies that matched the inclusion criteria in the systematic review. European and non-European country subgroups.

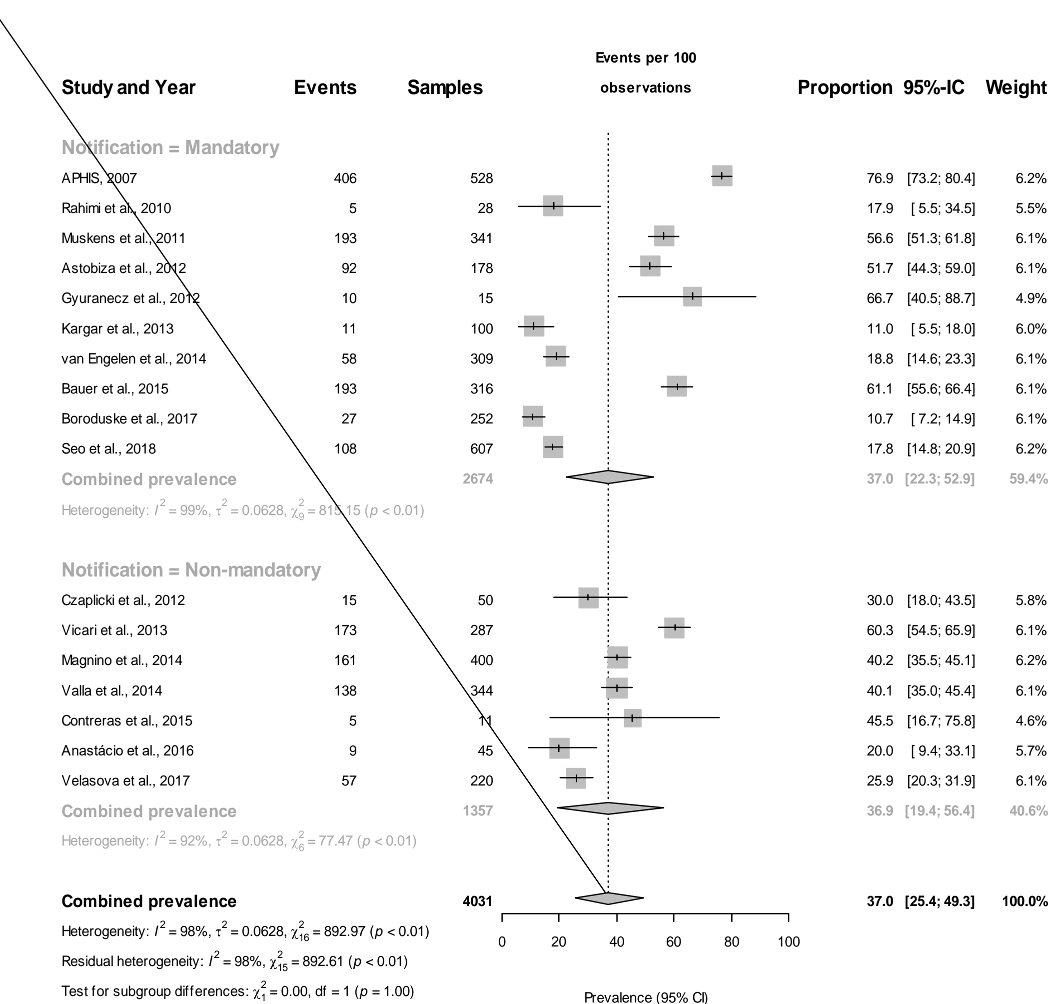


Figure 3.6: Forest plot for the meta-analysis of herd-level *Coxiella burnetii* prevalence based on bulk-tank milk samples from the seventeen studies that matched the inclusion criteria in the systematic review. Grouped by notification.

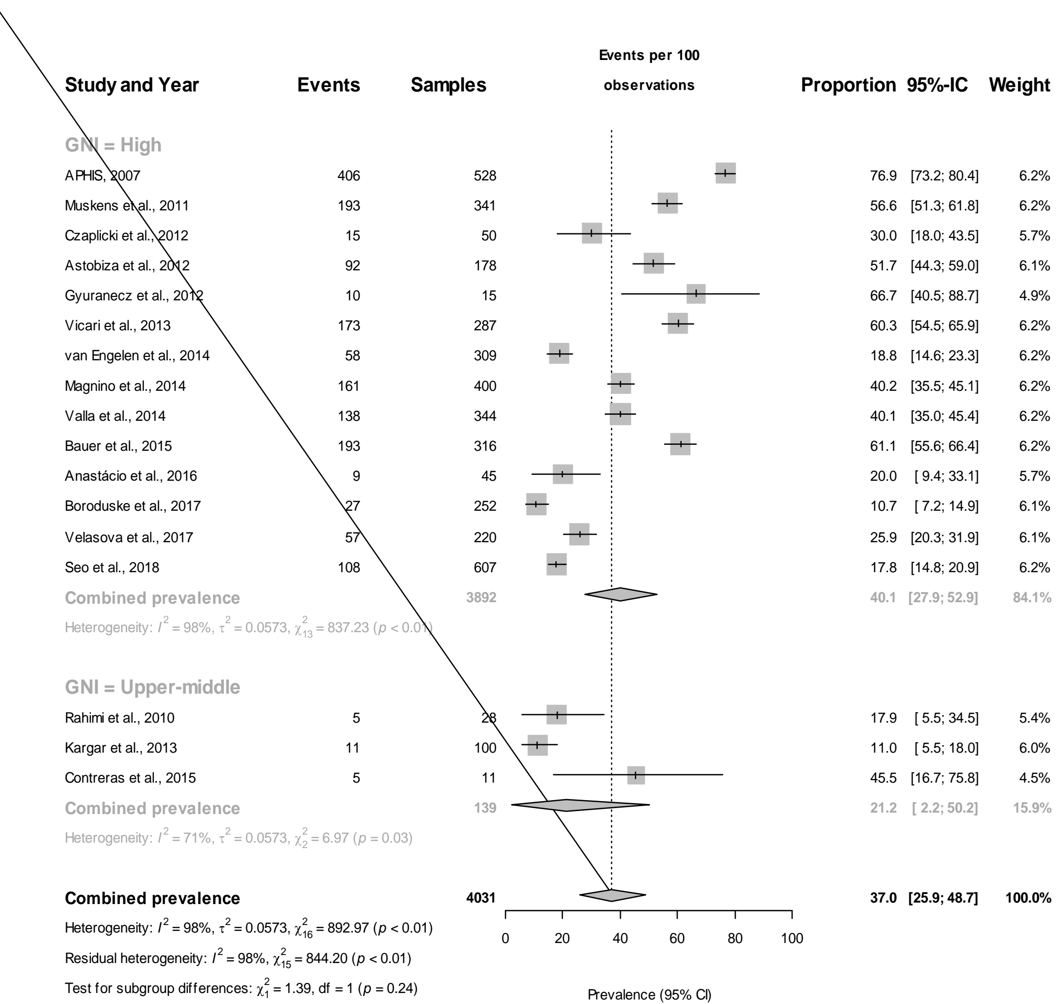


Figure 3.7: Forest plot for the meta-analysis of herd-level *Coxiella burnetii* prevalence based on bulk-tank milk samples from the seventeen studies that matched the inclusion criteria in the systematic review. Grouped by the per capita Gross National Income level.

3.4 Discussion

Global serological or molecular prevalences of pathogens as diverse as *Toxoplasma gondii* and *Helicobacter pylori* have been estimated by meta-analyses following a systematic review of the body of published studies (Hooi *et al.,* 2017; Montazeri *et al.,* 2020). We conducted a comprehensive keyword-based systematic review of the literature on the global molecular prevalence of *C. burnetii* in bovine BTM samples and data from those studies matching the inclusion criteria was extracted and included in a meta-analysis. For the purpose of this review, only adequately randomised studies with a cross-sectional design were included.

Heterogeneity among studies was first investigated by Higgins' I^2 statistic which indicates the proportion of heterogeneity not due to chance. A high level of heterogeneity (≥ 75%) indicates another source of variability besides random error. The high I^2 value (98%) led to the choice of a random-effects model for estimating the overall weighted *C. burnetii* herd-level prevalence among eligible articles, which makes no assumption that the prevalence is constant across the studies. The meta-analysis shows that *C. burnetii* is widely distributed in dairy farms in twelve countries from 3 continents (America, Europe, and Asia). The best estimate of global *C. burnetii* herd-level prevalence, based on the studies matching the current inclusion criteria, was 37.0%.

Bulk tank milk samples are a widely used approach for studying infectious diseases of dairy livestock at the population level, despite dry cows and unhealthy animals not being included and hence BTM only providing a partial representation of the herd sanitary status. The analysis of BTM samples represents a suitable and convenient approach for the investigation of *C. burnetii*, not only for initial farm-level screening in situations where their disease status is unknown, but also for repeated analyses during monitoring programmes or after sanitary interventions such as antibiotic administration (Taurel *et al.,* 2014) or vaccination (Astobiza *et al.,* 2013; Boarbi *et al.,* 2014). A positive BTM result confirms herd exposure to *C. burnetii*.

The molecular diagnostic methods of the studies included in this meta-analysis targeted different regions of the bacterial *C. burnetii* genome. The repetitive element *IS1111* was selected in most of the published studies, as this multiple copy gene is presumed to increase the sensitivity of the test (Kargar *et al.,* 2015). Other studies used PCRs targeting *com1*, icd and 16S rRNA genes. The *com1* element is frequently used for accurate quantification, as this is a single-copy gene (Kersh *et al.,* 2010). Additionally, the analysis of 16S RNAs may reveal the prevalence of Coxiella as a genus, by the identification of both *C. burnetii* and Coxiella-like organisms (Seo *et al.,* 2018).

The overall weighted *C. burnetii* prevalence found in bovine dairy herds was higher than the 5.1% to 22.1% range reported for BTM samples from dairy sheep flocks (García-Perez *et al.,* 2009; Marenzoni *et al.,* 2013; Anastácio *et al.,* 2016). This difference could be explained by the primary route of bacterial transmission in each species. A higher *C. burnetii* prevalence with a longer duration might be expected in bovine milk, which is the predominant route of shedding for cows, whereas milk is less significant to *C. burnetii* transmission from goats and sheep (Roest *et al.,* 2011a; Kargar *et al.,* 2013).
Two nationwide studies using the same molecular approach in a similar number of herds in Dutch dairy herds revealed markedly different prevalence levels in 2011 (56.6%) and 2014 (18.8%) (Muskens *et al.,* 2011; Van Engelen *et al.,* 2014). The lower prevalence in 2014 might be related to the compulsory control measures applied in dairy goat farms after the large human Q fever outbreak in 2007-2010 (Schimmer *et al.,* 2008; Schneeberger *et al.,* 2014). There is evidence that the same strains may affect both cattle and goats in the Netherlands (Roest *et al.,* 2013), which may explain why measures applied to goat farms indirectly helped to reduce prevalence in bovine herds. Similarly, three studies conducted in Italian herds in 2013 and 2014 also reported differences in *C. burnetii* prevalence. Valla *et al*. (2014) revealed a nationwide prevalence of 40.0%, while Vicari *et al*. (2013) found a higher prevalence of 60.0% in the northwest region of Lombardy, where almost half of Italian cows' milk is produced (Zucali *et al.,* 2017). The molecular prevalence of *C. burnetii* found in Lombardy represented a marked increase compared to a previous two-year study (2007-2008) conducted in the same region (40.0%) (Magnino *et al.,* 2009).
Differences in the bacterial shedding patterns among ruminants and uncertainty about the importance of milk-borne infection may result in stress on different control measures depending on the species. In small ruminants, the identification of high-risk dams before parturition is important in avoiding zoonotic risk (Lucchese *et al.,* 2015). In cattle where milk is the primary shedding route, pre-partum monitoring may not be as appropriate (Lucchese *et al.,* 2015). Identification of chronic *C. burnetii* milk shedding cattle may be more effective in preventing environmental contamination, decreasing the risks of transmission among animals, and preventing the spread of the bacterium.

Only five of the seventeen articles selected included analysis of factors associated with *C. burnetii* infection. Herd size, cattle density and purchasing replacement animals from external sources were all linked with *C. burnetii* infection (Van Engelen *et al.,* 2014; Boroduske *et al.,* 2017). Additionally, the presence of ticks on cattle was associated with BTM PCR positivity (Van Engelen *et al.,* 2014).
For both cattle and small ruminants, a positive correlation between herd size and herd prevalence of *C. burnetii* has been reported (McCaughey *et al.,* 2010; Schimmer *et al.,* 2012). The association among herd size, density of animals, and enhanced risk of *C. burnetii* infection has been well demonstrated (Agger *et al.,* 2014; Nusinovici *et al.,* 2015a). Close contact between cows is an intrinsic characteristic of dairy herd management systems, and larger herds offer even greater chances for contact and

transmission. Densely populated farms are prone to a higher risk of transmission of the pathogen within the herd after *C. burnetii* is introduced into the farm. Additionally, high animal density leads to greater bacterial load and thus higher environmental contamination (Suman *et al.,* 2016), which may represent an increased risk of transmission to either cattle or people. This meta-analysis has shown that elevated prevalence of *C. burnetii* is associated with large-sized herds, where the odds of a BTM sample testing positive double with every unit increase in $\log_e$ herd size (odds ratio $CI_{95\%}$1.24-3.52). Accordingly, of the moderators analysed, average herd size had the largest effect, accounting for 33.0% of the observed level of heterogeneity among studies.

While Q fever has been studied in both European and non-European countries, these two contexts have not previously been contrasted. The overall prevalence of *C. burnetii* infection was remarkably similar in European and non-European studies (both 37%). The greater variability among non-European studies ($CI_{95\%}$ 18.0%–58.5%) than among European studies ($CI_{95\%}$ 22.8%–52.2%) could be accounted for by the differences in the numbers of studies and herds investigated.

The mandatory notification of a disease should be helpful not only for early identification of outbreaks but also for enabling the evaluation of the effectiveness of control strategies. For instance, legislation implemented by the Dutch government in the face of the largest Q fever outbreak ever recorded included compulsory notification of Q fever (Schimmer *et al.,* 2008). In the current meta-analysis, a remarkable similarity was noted between overall weighted prevalence of *C. burnetii* in BTM samples from countries with mandatory (37.0%, $CI_{95\%}$22.3–52.9%) and non-mandatory (36.9%, $CI_{95\%}$19.4–56.4%) notification legislation.

In our meta-analysis, the GNI per capita seems to have a minor effect as a moderator of the prevalence of *C. burnetii* in BTM samples. When the studies were stratified according to this indicator of economic development, high-income countries had twice the overall weighted prevalence of upper-middle income countries, albeit this difference was not statistically significant (P=0.24). All publications matching the inclusion criteria were conducted in high and upper-middle income countries. None of the studies conducted in low-middle and low-income countries that were identified in the initial search fulfilled the inclusion criteria and thus all were rejected from the meta-analysis. For instance, an ineligible study carried out in Egypt reported a 22% molecular prevalence of *C. burnetii* in individual milk samples (Amin *et al.,* 2009) and one carried out in Bangladesh reported 15.6% seroprevalence in herd milk specimens (Rahman *et al.,* 2016). These findings suggest that further field studies could prove rewarding. The overall prevalence in low-middle and low-income countries remains unknown. There is evidence of extensive ruminant infection with *C. burnetii* throughout African countries where the

threat of human exposure and significant economic impact are possibly underestimated (Vanderburg *et al.,* 2014).

Although the moderator analysis identified average herd size as one source, most of the heterogeneity remained unexplained (residual heterogeneity I^2=97.0%; P<0.01). It is quite possible that other factors, not currently addressed, influence the *C. burnetii* herd-level prevalence. Unsurprisingly, two of the moderators were highly correlated; studies in European and in high-income countries showed a significant and positive correlation (r=0.627, P<0.01). Awareness of the relationships between moderators that may potentially induce bias in the analysis should be considered when drawing conclusions (Lipsey *et al.,* 2003).

3.5 Conclusions

This meta-analysis reports a high overall global prevalence of *C. burnetii* in BTM samples of 37.0% ($CI_{95\%}$25.2-49.5%), showing widespread herd-level circulation of this agent in bovine dairy farms. These results should be of interest not only in European countries where *C. burnetii* is a well-known health threat, but also in countries where epidemiological investigations have been limited, its importance as a zoonosis may be underestimated and prevention strategies may need to be implemented. Information on local biosecurity practices and environmental conditions would be valuable for a full understanding of *C. burnetii* prevalence globally, but such descriptions were lacking in most of the publications considered in the meta-analysis. While this study has shown the global herd prevalence of *C. burnetii* in dairy cattle to be high, in many countries, including high-income countries such as Belgium, Italy, Portugal and UK, the disease is not currently notifiable, and control is not mandatory. To make it so might represent an additional burden on dairy farmers and would require justification on economic or public health grounds for which further study might be required. The high herd-level circulation of *C. burnetii* in bovine dairy farms in several countries showed by this study reinforces the need for further investigations into this globally important zoonosis.

Chapter 4: ***Occurrence and quantification of Coxiella burnetii DNA in pooled milk samples from commercial herds in Uruguay, cross-sectional study***

4.1 Introduction

The culture of *Coxiella burnetii* is associated with biohazard and is not a common practice in most veterinary laboratories. As an intracellular organism, *C. burnetii* can only be isolated by employing animal hosts, embryonated eggs, or cultures of mammalian cells. Recent investigations have led to the alternative of axenic growth of *C. burnetii* by utilising acidified citrate cysteine medium (ACCM) (Omsland *et al*., 2009; Singh *et al*., 2013; Omsland *et al*., 2013). Due to the very low dose of *C. burnetii* needed for infection, all the work involved in the culture of this bacterium must be done under biosafety level III (BSL-III) laboratory conditions. A BSL-III laboratory has essential features such as unidirectional airflow created by using negative pressure gradients, exhaust air cleaning by high-efficiency particulate air filters, which are capable of even filtering 0.3-micron air-borne particles, and adequate biomedical waste disposal and effluent decontamination (Richmond, 2001). Consequently, the isolation of *C. burnetii* is conducted by only a few specialised laboratories around the world.

The obligate intracellular nature of *C. burnetii* makes extremely difficult its diagnosis by culture standard tests; therefore, *C. burnetii* infection is frequently investigated by non-culture tests, most frequently by molecular tests (polymerase chain reaction (PCR)) or serology, or the combination of both approaches. However, neither of these two methods can establish the bacterial viability (Lockhart *et al*., 2012). Given the limited sensitivity and specificity of serological tests, they have restricted utility for diagnostic purposes (Natale *et al*., 2009). Indeed, molecular PCR tests represent a valuable *C. burnetii* DNA detection approach on clinical samples worldwide.

PCR is an enzyme-driven method for the *in vitro* amplification of short regions of DNA. This technique depends on the *a priori* knowledge of at least partial sequences of the target DNA. The amplification based on nucleic acid is founded on the sequence-specific hybridisation of two oligonucleotide primers to a template DNA. The target DNA (in this case DNA from *C. burnetii*) is used to design primers that are complementary to a specific region of this DNA target. An enzymatic reaction occurs driven by a DNA polymerase that has thermostable characteristics. This polymerase is responsible for copying the specific target region that is bounded by the pair of primers, with the fundamental presence of oligonucleotides that must be included in the reaction. The process is done in a thermocycler where several cycles of heating and cooling during strict periods produce rounds of nucleic acids denaturation, primer annealing/hybridisation, and primer extension, and finally, the DNA region of interest is amplified exponentially (Figure 4.1) (Yang & Rothman, 2004). In theory, after each amplification cycle,

the number of target DNA molecules is doubled, assuming 100% reaction efficiency (Yang & Rothman, 2004). The amplificated products generated in a conventional PCR (end-point PCR) are finally observed by agarose gel electrophoresis. The PCR method has been widely revised to improve its functionality and adaptability. The widespread use of conventional PCR was followed by the development of the real-time polymerase chain reaction (commonly called real-time PCR or quantitative polymerase chain reaction qPCR) test that allows the detection and the precise quantification of specific target nucleic acids. The qPCR uses the continuous collection of fluorescent signals from one or more polymerase chain reactions over a range of cycles (Dorak, 2007). The qPCR represents one substantial advance in this technology in which the steps of amplification and evaluation of the amplified product are combined in a single reaction tube. The qPCR has noticeable advantages since it eliminates the need for time-consuming post-amplification handling steps required to detect the amplicon, allowing the evaluation of the PCR products simultaneously with that of the DNA synthesis (Yang and Rothman, 2004).

One approach to the simultaneous monitorisation of amplicon production is to use a fluorescent-labelled internal DNA probe, which specifically anneals within the target amplification region (Yang & Rothman, 2004). This allows the monitoring of the fluorescent signal corresponding to the cycles during amplification. The probes are typically single-stranded, short and fluorophore-labelled DNA segments. The internal probe produces a fluorescent signal during each cycle of amplification only in the presence of the target sequences (Figure 4.2). Considering that the probe is an oligonucleotide sequence, its attachment to its complementary region can enhance the assay's specificity. The intensity of the signal increases in proportion to the quantity of amplified products generated. Thus, the number of starting templates drawn from a wide range of samples can be estimated by comparing the cycle number at which amplified products accumulate considerably over baseline using a pre-derived quantitative standard, which leads to its widespread use in different disciplines (Pääbo *et al*., 1989; Yang & Rothman, 2004). While conventional PCR allows the measurement of the amount of produced PCR product by the end of the thermocycling process using gel electrophoresis, qPCR enables the measurement of PCR product as the PCR reaction progresses, and dsDNA is generated (Gilliland *et al*., 1990; Diviacco *et al*., 1992).

The PCR technique was first described almost three decades ago in the 1990s, and since then, due to its remarkable capacity for amplifying sequences from a small amount of template target, it has become a widespread method used in a broad range of areas and applications (Higuchi *et al.*, 1993). The qPCR test permits the swift finding of microbial DNA with the independence of the culture procedures, which is especially relevant in the case of zoonotic pathogens. Bacterial nucleic acid from *C. burnetii* can be found rapidly by PCR in a sample; this does not immediately imply that this bacterium is of disease significance given carriage in healthy/asymptomatic animals. However, PCR does not provide evidence for an active infection as distinct from a recent trail of colonisation/infection. Additionally, a significant constraint of PCR is the chance of exposing bacterial DNA in the lack of viable cells.

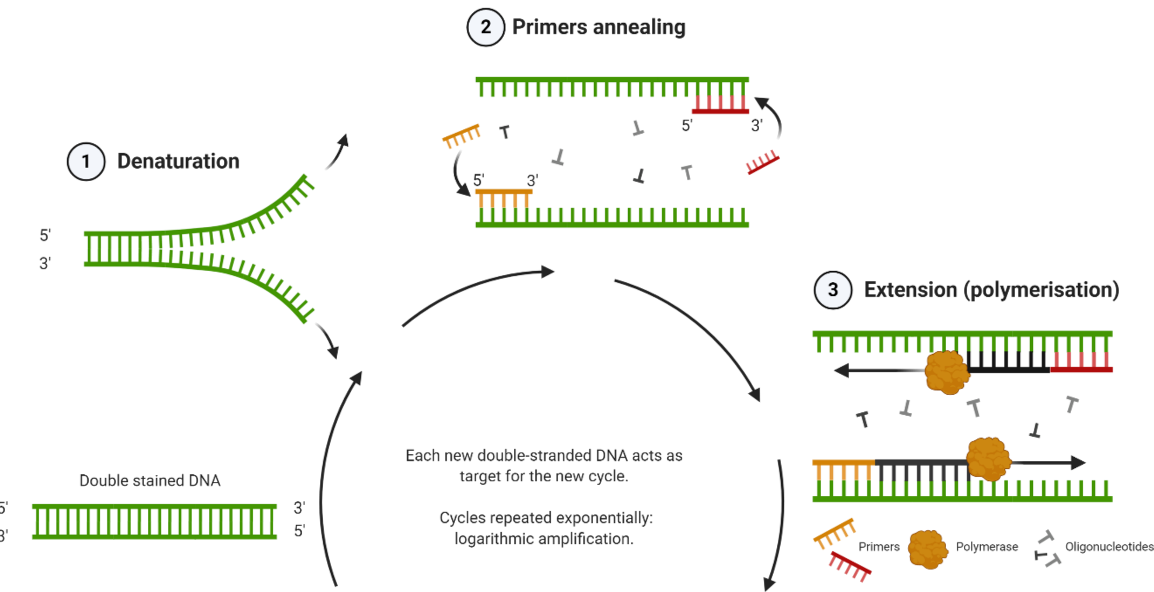


Figure 4.1: Schematic cycle of PCR. In each cycle, a DNA template is i) first denaturalised into two single-stranded DNA chains; ii) the primers anneal to the complementary target sequences; and finally, iii) a new copy of the target DNA is generated when DNA polymerase extends the primers. Each cycle is followed by a new cycle. Newly synthesised strands of target DNA act as a further template for subsequent cycles. (Schematic unmodified from Yang & Rothman, 2004).

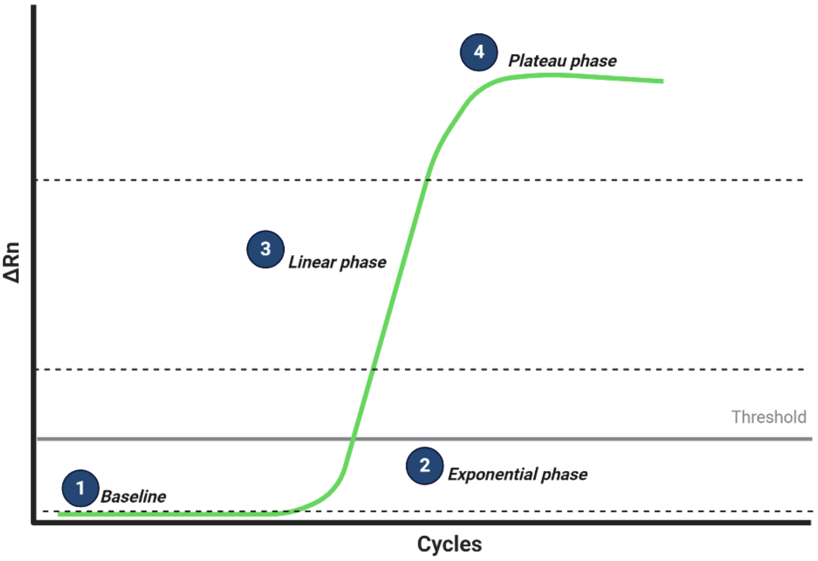


Figure 4.2: Amplification kinetics. Each amplification curve has four phases, a *baseline*, where the signal is being made but is not measurable by the device; *exponential*, detectable signal with maximal efficiency of PCR; *linear*, post-geometric with declining PCR efficiency; and finally a *plateau*, where no, or very few, new products are generated.

The baseline comprises the amplification that is below the level of detection of the equipment. Despite no signal is being detected, exponential amplification of the template is taking place during these cycles. The exponential phase is characterised by the first detectable signal from the reaction and this is where amplification progresses at its maximal rate. The length of the exponential phase depends on the template concentration and the assay quality. Ideally, there are two complete molecules synthesised from every template presented in the exponential phase. During the linear phase, the reaction's efficiency starts to decline, so that amplification is no longer two products from every one template molecule in each cycle. The efficiency continues to decrease further until the last phase known as a plateau, where amplification concludes during the remaining cycles (Dorak, 2007).

The sequence of probes is complementary to that of the target nucleic acid. The probes are single-stranded nucleic acid molecules with a fluorescent molecule attached to one end and a non-fluorescent quencher attached to the other end. The fluorescence resonance energy transfer (FRET) determines the quenching of the fluorescence signal when fluorophore and quencher are in close proximity. The FRET is a physical property in which the excitation of a donor molecule is transferred to an acceptor molecule when the fluorescence emission band and the excitation band of the latter overlap (Stryer, 1978). The fluorescent signal from the fluorophore is evidenced by ultraviolet light illumination when donor and receptor molecules are not in proximity (Tyagi & Kramer, 1996).

The TaqMan PCR protocols include a non-extendable probe and a pair of primers. The hydrolysis probe binds by complementary to the target in the region flanked by the primers, enhancing this way the assay's specificity. Commonly, the end named 5' of the probe is labelled by the fluorophore, considered as the "reporter" and the end, 3', has the quencher. The TaqMan probe hydrolysed to the sequence positioned downstream of one of the primers. This primer starts to be extended, and the probe is degraded by the 5'-3' exonuclease activity of Taq DNA polymerase. This exonuclease battery determines the elimination of any sequences in the enzyme path. This determines the probe dissociation; thus, the fluorophore and the quencher are no longer in proximity, interrupting the FRET and producing the dye release. Fluorescence is detected using a dye-specific wavelength after the application of illumination. The generated signal is proportional to the amount of the final PCR product (Nagy *et al.*, 2017). A third class of fluorescent molecule is typically used in qPCR and is identified as a reference dye, it is common in all reactions and does not interact with the assay components, being central to normalise the signal from well to well in the computational software (Dorak, 2007). A reference dye aims to check the fluorescence signal from every well and rectify any well to well discrepancies in detection efficiency within the device.

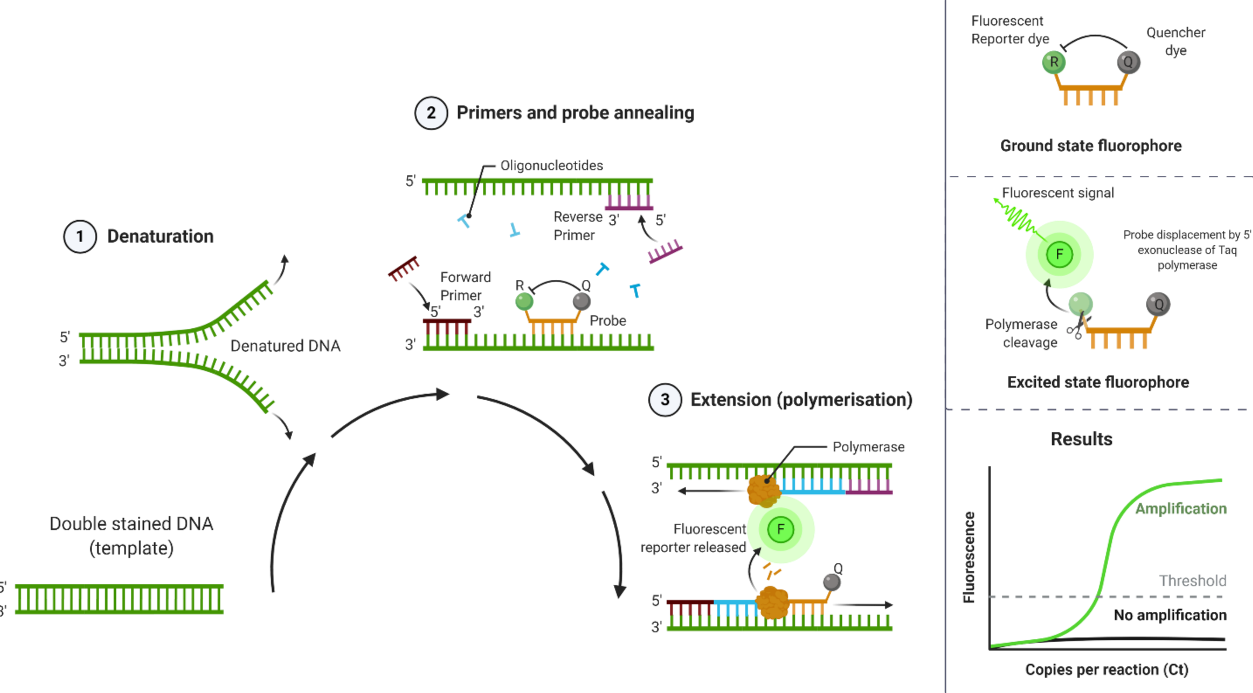


Figure 4.3: TaqMan Real-time PCR. TaqMan probe is a single-stranded oligonucleotide labelled with two different fluorescent dyes. The 5′-terminal has the reporter dye while the 3′-terminal contains the quenching dye. The sequence of the probe is homologous to an internal target sequence in the PCR amplified product. During the extension step of the PCR amplification, the probe is cleaved by the 5′ exonuclease activity of Taq polymerase, thus releasing the reporter from the quencher and generating a rise in the reporter emission intensity. As the amplification progresses, the amount of reporter dye signal detected is proportional to the amount of PCR product generated. When the probe is intact, the proximity of the two fluorescent dyes results in quenching of the reporter dye emission by the quencher (Yang & Rothman, 2004).

The reporter signal is frequently normalised to the reference dye by dividing the reporter's raw fluorescence by the fluorescence of the passive reference dye. This strategy to compensate for well to well variation (Dorak, 2007). This normalised reporter signal is known as the Rn. When the background value has been deducted from the Rn, then this is known as delta (Δ) Rn, the normalised background-adjusted fluorescence value.

The design of the primers is a point especially relevant to the workflow of PCR. The primer‘s design based on the target is followed by various assessments such as the computational evaluation of specificity, typically using BLAST, and the testing of dimmers formation. The optimal annealing temperature and best concentration of use are evaluated by gradient PCR reactions coupled with a concertation matrix. Additionally, a specificity panel that includes related DNA templates is employed

to fully assess primers' specificity. The measurement of a DNA target can be done either by absolute quantification or by relative (comparative) quantification. Absolute quantification consists in the estimation of the target copy number by using a standard curve as a reference. The standard curve is obtained by plotting the Ct values as the y values against log-transformed concentrations of serial ten-fold dilutions of the target nucleic acid (known-concentration standards) as the x values. The range of concentrations used for the standard curve construction should cover the expected unknown concentrations range of samples. This absolute approach quantifies unknown samples by interpolating their quantity from the constructed standard curve (Dorak, 2007). In the relative quantification, the relative change in target copy number is estimated in relation to a reference and is based on the concept that target and reference have similar amplification efficiency (Pfaffl, 2001). The standard curve (SC) is generated by serial dilutions (often more than 5 points). This SC is utilised to estimate operating parameters such as amplification efficiency, detection limits and quantification, dynamic and linearly range. A broad dynamic range facilitates the accurate quantification of even a target copy number differing by a significant amount (Bustin & Huggett, 2017; Forootan *et al.*, 2017). A line of best fit is determined for the standard curve using the least-squares method of linear regression as:

$$y = \mathrm{m}\,x + \mathrm{b}$$

where:

$y = C_{t,}$

m = slope,

$x = \log_{10}$ template amount,

b = y-intercept.

The efficiency of a PCR indicates how well the reaction has progressed and is calculated by the slope of the SC based on the linear regression of the Ct against the log of ten-fold dilutions of the target nucleic acid quantity of DNA. Thus, the efficiency of a PCR reaction is estimated from the standard curve slope (using the equation: Log E = 10(−1/slope)-1). Ten-fold dilution is typically used for SC construction, and under ideal conditions a perfect doubling arises in each amplification cycle, giving an efficiency of 100% with a Cq value increment of 3.3 at each ten-fold dilution point. A constant amplification efficiency in all contrasted samples is an essential criterion for consistent comparison between samples. The y-intercept gives insight into the sensitivity of the reaction and how precisely the template has been measured. The coefficient of determination known as r^2 describes the integrity of the data fit to the theoretical line (Dorak, 2007).

The first investigations of *C. burnetii* using polymerase chain reaction were registered in 1990 (Mallavia *et al.*, 1990; Frazier *et al.*, 1990). These first studies were based on the premise that *C. burnetii* contains DNA sequences sufficiently unique to permit its detection among other bacterial species. Since then, several investigations had been carried out on vertebrates (mammals: humans, wild, domestic, and

productive animals; birds) and invertebrates (ticks). Different molecular approaches had been used in a broad range of matrices, such as cell cultures and clinical specimens (milk, sera, several types of tissues, birth products). Additionally, *C. burnetii* was investigated in diverse environmental samples (dust, sewage water, soil), especially from places housing animals. The direct investigation of *C. burnetii* relied on cell culture is restricted to laboratories with stringent level three safety conditions. In those places where BSL-III is not available, the molecular approaches are explored as a promising alternative.

4.1.1 *Coxiella burnetii* excretion through milk

Coxiella burnetii can be shed into the environment through many routes. Birth products are the primary excreting source, but *C. burnetii* can also be shed in faeces, urine and milk (Guatteo *et al*., 2007). Milk appears as the most important route of *C. burnetii* shedding in cattle (Rodolakis *et al*., 2007), where active excretion lasts even throughout two consecutive lactation periods (Piñero *et al*., 2014ab).

Coxiella burnetii exhibits a broad range of cell type tropisms, including macrophages and monocyte, trophoblasts, epithelial cells, and adipocytes (Bechah *et al*., 2014; Boarbi *et al*., 2016; Sobotta *et al*., 2017). The predilection of *C. burnetii* for udder tissues has been investigated in *in vitro* conditions where the susceptibility of diverse epithelial cells for *C. burnetii* infection was evaluated. A recent study evidenced that bovine epithelial cells presented diverse propensity to *C. burnetii* invasion and facilitated in different degrees its replication. From the different epithelial cells lines inoculated with the bacterium; F3 (fetal placenta), BCEC (maternal placenta), BEL-26 (lung), PS (udder), FKD-R 971 (small intestine), the udder cells showed the highest laxity for the bacterium spread and as well as the highest replication rates (Sobotta *et al*., 2017). They developed large vacuoles containing *Coxiella*.

The localisation of this bacterium in the bovine udder is essential for its long-term secretion through milk; however, the mechanisms behind the persistence and the facilitated replication of *C. burnetii* within mammary glands remain unclear (Muskens *et al*., 2011). Udder epithelial cells may allow the bacterium's effective propagation mainly by supporting bacterial replication once invasion occurs (Sobotta *et al*., 2017). This known tropism of *C. burnetii* for mammary tissues in bovines appears to lay the foundation for the high-level bacterial shedding by milk that occurs in cows (Biberstein *et al*., 1974; Aitken *et al*., 1987; Rodolakis *et al*., 2007).

Additionally, the detection of both cell variants of *C. burnetii* (SCV and LCV) strongly suggests that this bacterium undergoes a complete life cycle within udder cells (Sobotta *et al*., 2017). *Coxiella burnetii* invade and replicate within bovine epithelial cells, particularly mammary epithelial cells, with no destruction of cell integrity or without a considerable stimulus of the host's immune

response; therefore, the bovine udder works as a niche for bacterial replication without warning the immune response of the animal (Sobotta *et al*., 2017).

The activation of immune cells is strain specific for Coxiella organisms, although this has not been observed in udder epithelial cells. Typically, avirulent strains stimulate greater pro-inflammatory cytokine production, in contrast with virulent strains (Graham *et al*., 2013), possibly due to the differences of the LPS phase during bacterial attaching to host cells (Dellacasagrande *et al*., 2000b). This phenomenon was not evidenced in udder cells, where a general failure to stimulate immune responses was exhibited independently of the phase-type (Sobotta *et al*., 2017).

As milk is a central route of *C. burnetii* excretion in cattle, the evaluation of milk samples by PCR has been proposed as a useful approach to assess bacterial shedding in infected animals. *Coxiella burnetii* was identified in cows' milk for up to 32-month postpartum (Marrie, 1990b). Indeed, cows may continue to shed the bacterium in milk during successive lactating periods (Biberstein *et al.*, 1974). However, there is no conclusive evidence about the mechanism behind the bacterium's persistence within the host. The possible reinfection from *C. burnetii* staying elsewhere in the organism for the duration of the inter-pregnant period, for example, in the mammary tissue, may be a potential explanation (van den Brom *et al*., 2013).

Two different sorts of *C. burnetii* milk shedding patterns have been identified within cows; a sort typical of milk heavy-shedder animals (cows with high and persistent shedding) had been postulated and another sort typical of cows with sporadic/intermittent bacterial shedding (Guatteo *et al.*, 2007; Rodolakis *et al*., 2007). Considering that milk shedding of *C. burnetii* can be intermittent in some animals, the monitoring of dairy farms using bulk-tank milk (BTM) samples appears to be an appropriate strategy. Moreover, BTM sample assessment allows the preliminary screening of herd status by implementing an easy and relatively inexpensive sampling method.

4.1.2 *Pooled milk samples utility*

Both BTM samples and individual milk samples had been broadly subjected to molecular assessments and antibody level determination in investigations of *C. burnetii* in diverse dairy herds (cattle, sheep, goats and camels) (Rahimi *et al.*, 2011). BTM analysis is a convenient large scale epidemiological tool for investigating the status of different diseases within herds (Ruiz-Fons *et al*., 2011). Surveillance evaluations frequently use BTM samples since they are easy to collect and most of the milking herd is represented in a single sample.

An extensive review has shown that *C. burnetii* shedding in milk had been described in many countries (Pexana *et al*., 2018), and a BTM sample is a promising target specimen for the investigation of this bacterium under field conditions (Kim *et al*., 2005; Rodolakis *et al*., 2007; Czaplicki *et al*., 2009). Among other approaches, qPCR targeting the IS*1111* has been broadly utilised to detect and quantify *C. burnetii* shedding in milk samples (Kim *et al*., 2005; Boarbi *et al*., 2014). BTM analysis is a useful initial approach to evaluate *C. burnetii* in a herd and, when possible, this first evaluation should be followed by a more complete study to confirm the existence of active Q fever in the herd. However, the mere finding of *C. burnetii* by PCR in a milk does not imply disease, because *C. burnetii* milk shedding has been identified in samples from aborted cows and healthy cattle as well (Anderson *et al*., 2013ab).

Whole milk is a complex matrix that poses difficulties for PCR assays due to the abundance of PCR inhibitors. Besides the protein and lipid components naturally found in milk samples, DNA from other bacteria and somatic animal cells can affect the detection of the target DNA sequences (Rossen *et al*., 1992; Ongol *et al*., 2009). Naturally, factors inhibiting the amplification of nucleic acids in PCR assays affects testing through several routes: by interfering with the cell lysis needed for the DNA extraction, by affecting the nucleic acid degradation or capture, and by constraining the activity of the polymerase during the amplification of target DNA (Wilson, 1997). Additionally, Ca^{2+} ions in milk were identified as an inhibitory component, interfering by blocking the DNA and shielding it from the polymerase's access (Wilson, 1997).

4.1.3 Objectives

This chapter's specific objectives have been to evaluate, validate, and optimise a qPCR test to investigate and quantify *C. burnetii* in compose milk samples. It aimed to investigate the occurrence and the *C. burnetii* burden in bovine bulk pooled milk samples from commercial Uruguayan dairy farms. Evaluate if the herd size has an association with the presence of the bacterium.

4.2 Materials and Methods

4.2.1 Study Design and Sampling Approach

The commercial farms were selected among the ones that regularly send milk samples to the two referral laboratories for the assessment of milk quality in Uruguay, the Milk Quality Laboratory of the "Instituto Nacional de Investigación Agropecuaria" (INIA) and the "Cooperativa Laboratorios Veterinarios de Colonia" Cooperative Veterinary Laboratory of Colonia (COLAVECO). They are located in the core of the principal milk-producing region of the country (Figure 4.4) and receive individual milk samples as part of a systematic monthly control program, popularly known as "control lechero". This evaluation estimates the quantity and quality (fat and protein) of the milk produced in each lactation of each milking cow. The data generated by this control enables the implementation of strategies such as the selection of cows based on production and the selection of heifers for animal replacement and food management. The laboratories made available an anonymised list of the farms that regularly send milk samples for analysis coupled with information about the farms' localisation (department) and the number of milking cows on each farm at the time of sampling.

Based on this information, farms were first divided/stratified into more homogenous groups based on the number of their milking cows in each herd. Accordingly, the five groups were stratified as follows: (tiny farms-group 1 [< 100 animals], small farms-group 2 [101 to 300 animals], medium-size farms-group 3 [301 to 500], large-sized farms-group 4 [501 to 1000], and very large-sized farms-group 5 [> 1000]). The selection of farms was made using stratified sampling as farms were randomly selected from within these groups (stratum). The sample size was calculated by considering an unknown *C. burnetii* infection status in the local dairy herds. The sample size was estimated assuming a herd level prevalence (which was actually unknown) expected to be around 50%, a confidence level of 95%, and a desired absolute precision of 10% (Thrusfield, 2007). The sample size was estimated using $n = (Z^2\ p(1-p))/\ \alpha^2$, where n= sample size, Z= 1.96 (the critical value at 95% of confidence level), p= expected herd prevalence, and α= type-1 error (0.10). According to this equation, the sample size was estimated to be 92 farms; therefore, 19 farms were randomly selected within each size stratum. This study was designed to be cross-sectional, proving the information of the population in a specific period, with stratified random sampling to ensure that a fully representative sample of all herd sizes was achieved.

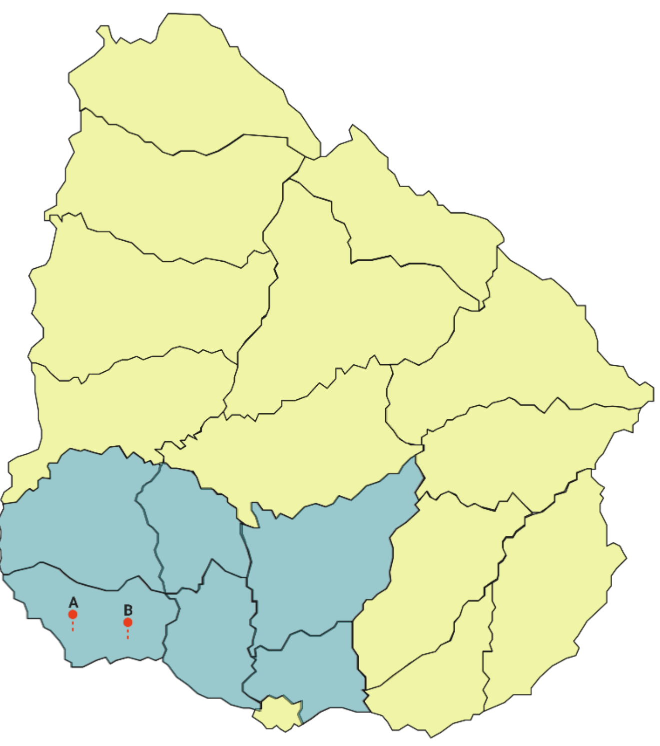


Figure 4.4: Location of the two referral laboratories used for milk quality analysis in Uruguay. A: Milk Quality Laboratory of the Instituto Nacional de Investigation Agropecuaria (INIA) and B: Cooperative Veterinary Laboratory of Colonia (COLAVECO). Area in blue represents the main milk producing region of the country.

4.2.2 Milk sampling and processing

As part of the laboratories' protocols, individual milk samples were routinely submitted in plastic tubes containing bronopol as a chemical preservative (2 brono-2-nitro-propane-1.3-diol). A composed sample from each farm was obtained by pooling individual milk samples and collecting them in sterile Falcon plastic tubes. These collective milk samples will be called throughout this chapter as pooled milk (PM) samples. This sampling approach was selected to approximate BTM sampling. Each tube of PM samples was labelled by an anonymised identification system which included an indication of the number of individual samples that gave rise to them.

The PM samples (50 ml) were concentrated before analysis (Renshaw *et al.*, 2000; Loftis *et al.*, 2010). Briefly, to obtain the cell fraction, the PM samples were first skimmed by low-speed centrifugation (1700 × g, 15 min at room temperature). The resulting pellet containing the concentrated cell fraction was resuspended in 40 mL of phosphate-buffered saline (pH 7.4) and again centrifugated. This step was repeated as needed to remove all residual cream. Finally, the resulting cell pellet containing somatic

cells, debris, and some milk solids, was resuspended in 500 µL of phosphate-buffered saline and utilised for DNA extraction.

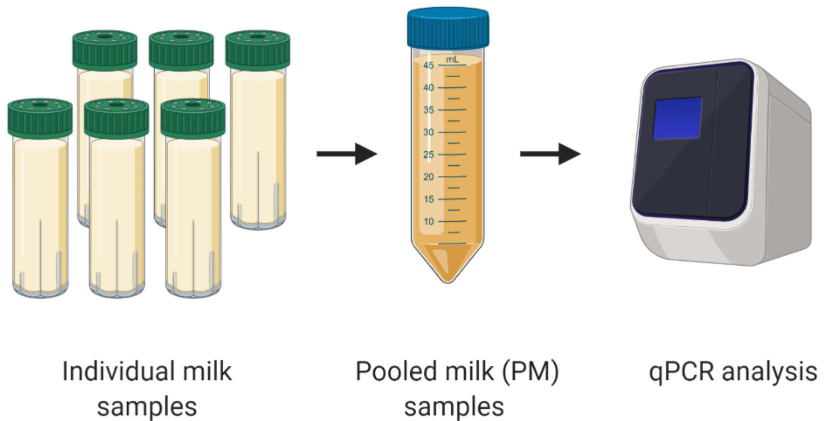


Figure 4.5: Workflow summary from individual raw milk samples to qPCR evaluation.

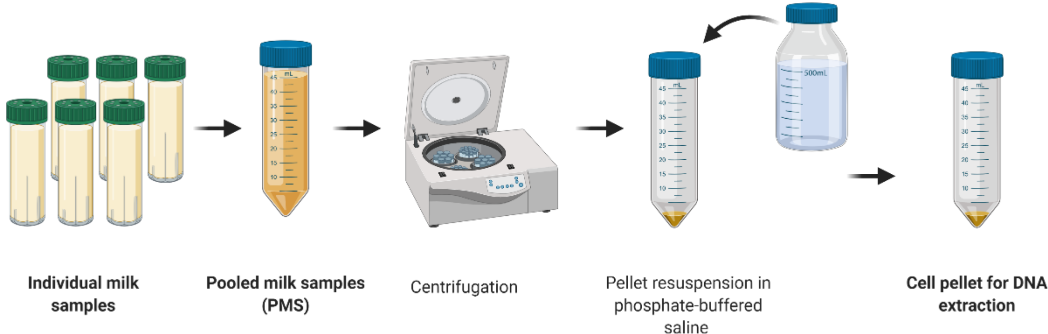


Figure 4.6: Milk sampling and general processing.

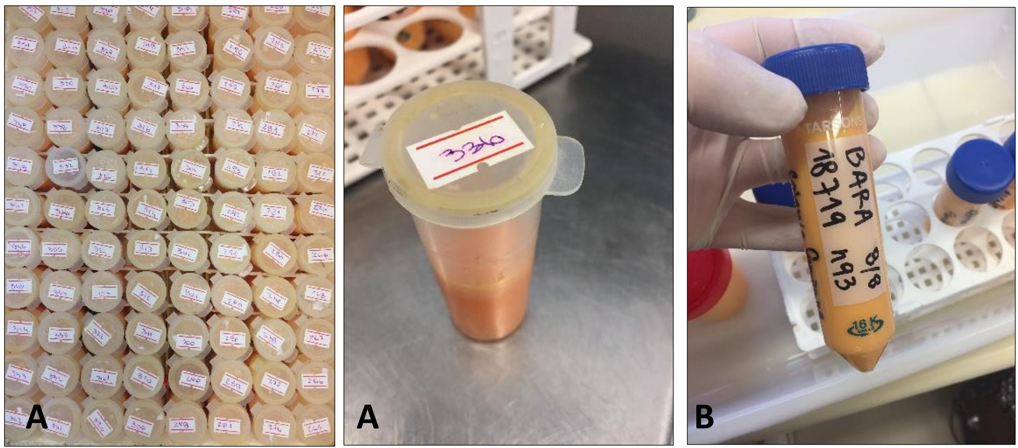


Figure 4.7: Milk sampling. A: individual milk samples. B: pooled milk (PM) samples. Anonymised samples labelled by the date of collection and number of individual samples that composed the sample pool.

4.2.3 Nucleic acid extraction

DNA was manually extracted from the milk pellets using a commercial magnetic particle DNA extraction kit. For this purpose, 300 µL of the sample were utilised, and the manufacturer's instructions were followed (MagMAX^{TM} Pathogen RNA/DNA Kit (Thermo Fisher Scientific, Waltham, MA, USA)). Briefly:

1. 300 µL of the homogenised PM sample were pipetted to a sterile 2 ml microtube.

2. 20µl of Proteinase K (600 mAU/ml solution) was added to the microtube.

3. 200µl buffer AL was added. Microtube was mixed thoroughly by vortexing.

4. Incubation of microtubes at 56°C for 10 min using a heat block was undertaken.

5. 200µl ethanol (96–100%) was pipetted into the microtube and mixed thoroughly by vortexing.

6. The product from step 5 was pipetted into a DNeasy Mini spin column, placed in a 2ml clean collection tube.

7. This was centrifuged at 6000 x g (8000 rpm) for 1 min. Flow-through and collection tube discarded.

8. The DNeasy Mini spin column was placed in a new 2 ml collection tube. 500µl buffer AW1 was added.

9. This was centrifuged for 1 min at 6000 x g (8000 rpm). Flow-through and collection tube discarded.

10. The DNeasy Mini spin column was placed in a new 2 ml collection tube. 500µl buffer AW2 were added.

11. This was centrifuged at 20,000g for 3 minutes. Flow-through and collection tube waste were collected.

12. The DNeasy Mini spin column was placed in a sterile 1.5ml microtube. The microtube tube was identified with a label.

13. 200 µl Buffer AE were pipetted directly onto the DNeasy membrane and incubated at room temperature for 1 min.

14. This was centrifuged for 1 min at 6000 x g (8000 rpm) to complete elution. DNeasy Mini spin column discarded.

All centrifugation steps have been carried out at room temperature (15–25°C). The extracted DNA was quantitatively and qualitatively analysed by spectrophotometry using a Nanodrop (ND1000, NanoDrop Technologies, Inc, USA) before downstream utilisation. After this, DNA samples were immediately stored at -20°C or -80°C depending on the expected moment of further utilisation.

4.2.4 *Coxiella burnetii* reference strain

A cultured strain (Nine Mile RSA-493) was generously provided by PhD T. Chisnall, Bristol Veterinary School, University of Bristol, Langford, United Kingdom.

4.2.5 Cloning into plasmid

The IS*1111* element was quantified by qPCR by the preparation of a plasmid harbouring this element as a template. A standard curve was constructed using averaged Ct values obtained from three technical replicates for each dilution point in each dilution series containing a fragment of the IS*1111* element of *C. burnetii*. First, the target sequence's 86-bp length fragment was amplified by conventional PCR utilising as a template a total genomic DNA extracted from *C. burnetii* Nine Mile RSA-493 strain, using the same primers as were used for the qPCR. Amplification was done by *Taq* polymerase to ensure 3′A-overhangs on the PCR product. The non-template-dependent terminal transferase activity of the *Taq* polymerase incorporates a single deoxyadenosine (A) to the 3´ ends of the products of PCR. This is essential for the correct ligation of the PCR inserted into the commercial vector because it has single, overhanging 3´ deoxythymidine (T) residues. The PCR product was analysed by agarose gel electrophoresis, in which a discrete band of the expected sized was visualised. This PCR product (amplicons) was cloned into a pCR®2.1-TOPO vector using the TOPO®TA cloning kit (Invitrogen, Carlsbad, CA, USA). After the ligation, the recombinant vector was transformed into chemically competent *Escherichia coli* TOP10 cells (Invitrogen). For competent cell production, *E. coli* TOP10 cells were grown Luria-Bertani (LB) broth medium at 37°C until an optical density of ~0.4 OD at 600 nm was reached. Then came the steps of successive centrifugations and resuspensions step of the pellet in 50 mM cold CaCl2, followed by a final 30 min on-ice incubation to make the cells competent. For the transformation, 2 µL of the TOPO® cloning reaction were added to 50 µL of competent *E. coli* cells. After a 20 min incubation on ice, cells were subjected to a heat-shock (45 s at 42°C) without shaking. These transformed *E. coli* (100 µL) were plated on LB-Agar containing 50–100 µg/mL ampicillin and 40 mg/mL X-gal, and at 37°C for 24 hours. The enzyme beta-galactosidase (LacZ) utilises the X-gal as a substrate, turning a deep blue colour when the enzyme utilises it. When the insert is successfully incorporated into the vector, the gen LacZ is disrupted, leading to the production of white colonies.

Thus, an efficient TOPO® cloning reaction should produce several white colonies; those colonies with a disrupted lacZα were selected. The recombinant plasmid DNA was recovered utilizing a DNA-spin plasmid DNA purification kit, and the final plasmid was sequenced (Applied Biosystems, Foster City, CA, USA). The resulting plasmid was quantified spectrophotometrically at 260 nm by Nanodrop ND-1000 (Thermo Scientific, Fremont, CA, USA) and the 260/280 ratio assessed its purity. The copy number of plasmid was assessed based on the plasmid quantity by using the following equation:

Plasmid copies/µl = [plasmid DNA concentration (ng/µl) × ($6.02{\times}10^{23}$)] / [plasmid length (bp) × 660]

where plasmid length (bp) denoted the vector length (3.9 kb for the pCR®2.1-TOPO) and the length of the PCR product (86 bp), 660 indicates the average molecular weight of one base pair (g mol^{-1} bp^{-1}), and $6.02{\times}10^{23}$ represents the Avogadro number. The number of molecules in preparation of plasmid DNA was established from the concentration of DNA, the molecular weight of the plasmid and Avogadro's number.

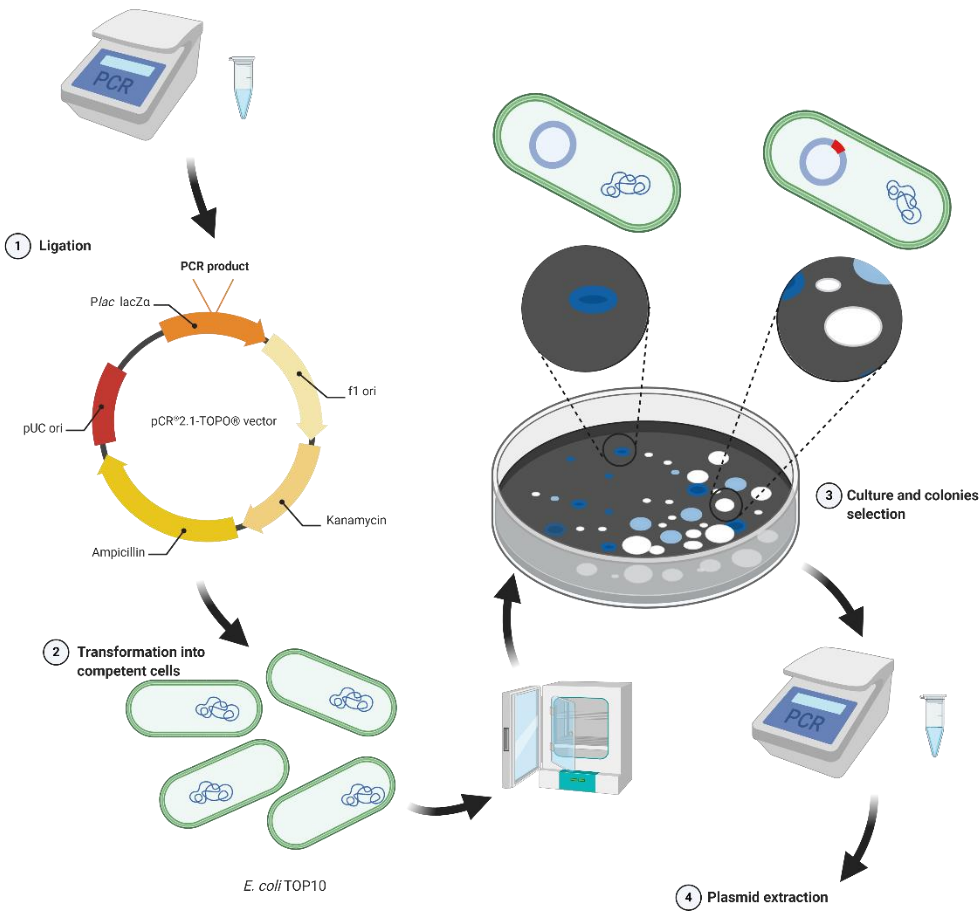


Figure 4.8: Plasmid cloning.

4.2.6 Construction of the qPCR standard curve

Ten-fold serial dilutions of the recombinant plasmid DNA were used to construct the quantitation assay's standard curves. Five independent serial dilutions ranging from 10^7 copies/µl to 10^2 copies/µl of the plasmid were utilized to create a standard curve from which each reaction's sensitivity could be determined and from which the amount of *C. burnetii* DNA in the original sample was determined. The dilution series were created to encompass all possible template quantities that might be encountered in the unknown samples. The dilutions were made in sterile dH_2O and were loaded in different PCR runs. Each dilution point was assessed in triplicate. Thus, five biological replicates of each dilution point and three technical replicates were obtained. Whereas technical replicates referred to the same samples triplicated in the qPCR plate set up to adjust for potential errors during pipetting, biological replicates involved temporally separated experimental replicates to correct for the experimental error. Standard curves were created by plotting the linear regression of the plasmid copy number against the quantified Ct values (also known as cycle quantification (Cq) or crossing point (Cp) for the 10-fold serial dilutions of the recombinant plasmid DNA employed as quantification standards. The Ct value shows the cycle number at which the fluorescence produced within a reaction crosses the threshold. It is inversely correlated to the logarithm of the initial copy number, and the Ct value from a specific well indicates the point during the reaction at which enough number of amplicons have accumulated (Dorak, 2007). For each dilution point, three technical replicated Ct values were obtained and averaged at each dilution series to construct the standard curve. The threshold is typically 10X the standard deviation of Rn for the early PCR cycles (baseline) set in the region of the PCR product's exponential growth (Dorak, 2007). The threshold is a numerical value given for each run to calculate the Ct value for each amplification. Despite this, considering that the lowest possible threshold is the best option, there is not a single optimal threshold value (Dorak, 2007). Some analysis using software corrects the threshold to make the standard curve present the highest r^2 value.

Serial dilution points were created to have an extensive dynamic range; this involves the range of initial template concentrations over which Ct values are acquired (Dorak, 2007). As long as the dynamic range is extensive, there is a greater capability to identify samples with high and low copy number in a single run. In absolute quantification, the interpolation within this range is precise, but the extrapolation beyond the dynamic range must be avoided as inaccurate estimations are obtained.

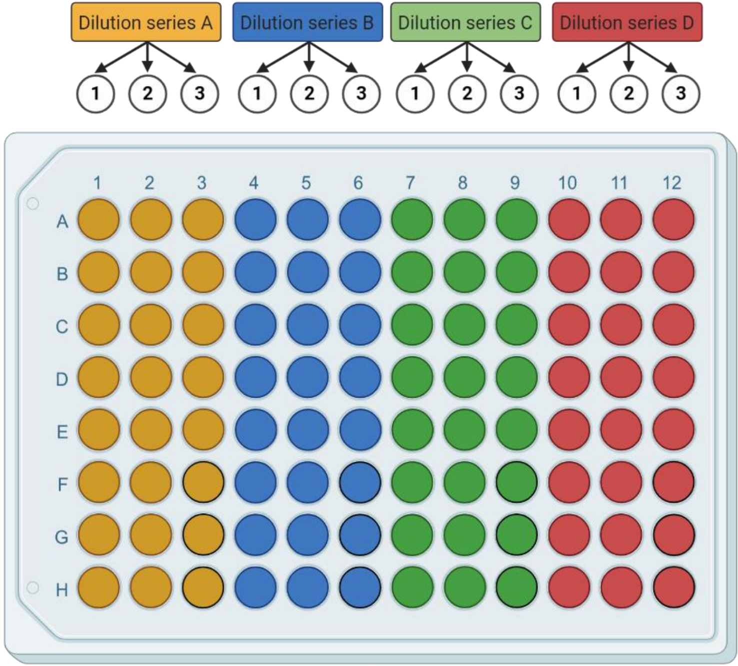


Figure 4.9: Biological and technical replicates shown in plates.

4.2.7 Thresholds and baseline settings

The threshold was set within the exponential phase of amplification and above any background amplification. Software auto-baseline function was used. The Ct values were generated when curves of amplification crossed the specified threshold.

4.2.7.1 qPCR efficiency and sensitivity

The qPCR amplification efficiency (E) was estimated using the equation stated in the introduction section as [$E = 10^{(-1/slope)} - 1$] or [$E(\%) = (10^{-1/slope} - 1) \times 100\%$] employing the resulting slope of the standard curve. The reaction's efficiency should be as close to 100% as possible, exhibiting a two-fold increase of amplicon at each cycle. The assay's precision and the variability between Ct values at each dilution among technical replicates were assessed. The mean Ct values, the standard deviation and CV (coefficient of variation) (%) from each dilution were determined. The detection limit of the assay was established using the highest dilution point with a Ct value.

4.2.8 Selection of primers and probes

The assessment of *C. burnetii* DNA was based on the multicopy IS*1111* insertion sequence amplification by adapting the method published by Panning *et al*. (2008) and then adjusted by Di Domenico *et al*. (2014). An internal control (IC) was also included to rule out DNA extractions failures, check for PCR inhibitors in the sample that affect the amplification, and control the sample loading. For this purpose, *β*-actin was chosen as the internal control gene (Wernike *et al*., 2011). The duplex qPCR targeting the IS*1111* element and *β*-actin genes generated two expected amplification fragments of 86-bp and 88-bp, respectively. The *C. burnetii* detection used the forward primer CburF, 5′- GAT AGC CCG ATA AGC ATC AAC -3′, reverse primer CburR, 5′- GCA TTC GTA TAT CCG GCA TC -3′ (Panning *et al*., 2008), and the probe FAM- TGC ATA ATT CAT CAA GGC ACC AAT GGT - TAMRA (Di Domenico *et al*., 2014). The *β*-actin assessment included the forward primer ACT2-1030-F 5′- AGC GCA AGT ACT CCG TGTG, reverse primer ACT-1135-R 5′- CGG ACT CAT CGT ACT CCT GCTT and probe ACT-1081-HEX 5′ HEX- TCG CTG TCC ACC TTC CAG CAG ATGT -BHQ1 (Wernike *et al*., 2011). All the primer sets, and probes were synthesised by Invitrogen (Beijing, China). First, each pair of primers was separately evaluated, and then the combination of the pairs of primers and the probes was tested in a duplex set-up. The amplification efficiency (E) was calculated for each assay, using the slope of the standard curve as $E (\%) = (10^{-slope} - 1) \times 100$. Negative control was also set up by replacing the DNA template with distilled water. Each run included a series of dilutions of the standard plasmid DNA along with the DNA templates.

4.2.9 Detection and absolute quantification of *C. burnetii* in pooled milk samples

The PCR assays for quantifying the initial copy numbers of *C. burnetii* in PM samples were performed in a QuantStudio® 5 Real-Time PCR System (Life Technologies Inc.) and optimised using a final reaction volume of 10 µl. Each reaction included: 5 µl of 2X SensiFAST™ probe No-ROX Kit (Bioline), 0.6 µl of each primer and 0.23 µl of the probe for *C. burnetii* (600nM and 220nM final concentration, respectively), 0.3 µl of each primer and 0.08 µl of the probe for the *β*-actin gene (300nM and 75nM final concentration, respectively), 1 µl of DNA template and 1.89 µl of free-nuclease water. The thermal profile used was as follows: an initial activation step at 95 °C for 5 min, 40 cycles of three-step denaturation at 95 °C for 10 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. Each sample was tested in triplicate using a 96-well plate. Each run included a non-template control (NTC), containing ultrapure DNAse/RNAse-free distilled water, as a template to check for primer-dimer and contamination. Only the PM showing a typical amplification curve with a cycle threshold (Ct) value

below 40 was considered positive. The concentration of unknown PM samples was extrapolated using linear regression based on the standard curve.

4.2.10 Data analysis

4.2.10.1 Variability of PCR

The coefficient of variation is calculated by the standard deviation divided by the arithmetic mean (Dorak, 2007). It is employed to assess the intra-assay reproducibility from well to well and to help calculate inter-assay variation from assay to assay. The coefficient of variation (CV) (expressed as a percentage) was determined to assess the variability presented between cycle quantification (Cq) values. The variability between Cq values produced at each dilution point between technical replicates was estimated as a percentage change as follows:

$$\text{Coefficient of variation (\%)} = (\text{standard deviation / mean}) \times 100$$

4.2.10.2 PCR standard curves

This sampling had a cross-sectional approach. Data were analysed in two steps, considering first the PM qPCR result as a binary variable (positive vs negative), and second, as a quantitative variable (estimated titres in *C. burnetii*/ml). Statistical analyses were done using a general linear model in RStudio.

The unknown amount of template in the samples can be calculated as:

$$\text{Log}_{10}\ \text{copy number} = C_t - y\text{-intercept / slope}$$

The copy number obtained using this formula refers to the copy number in each reaction. The number of copies in the original milk sample can be approximated by considering the factor of dilution, the volume of the extracted DNA, and the volume of the original milk sample utilised for DNA extraction (Rantala-Ylinen *et al*., 2011).

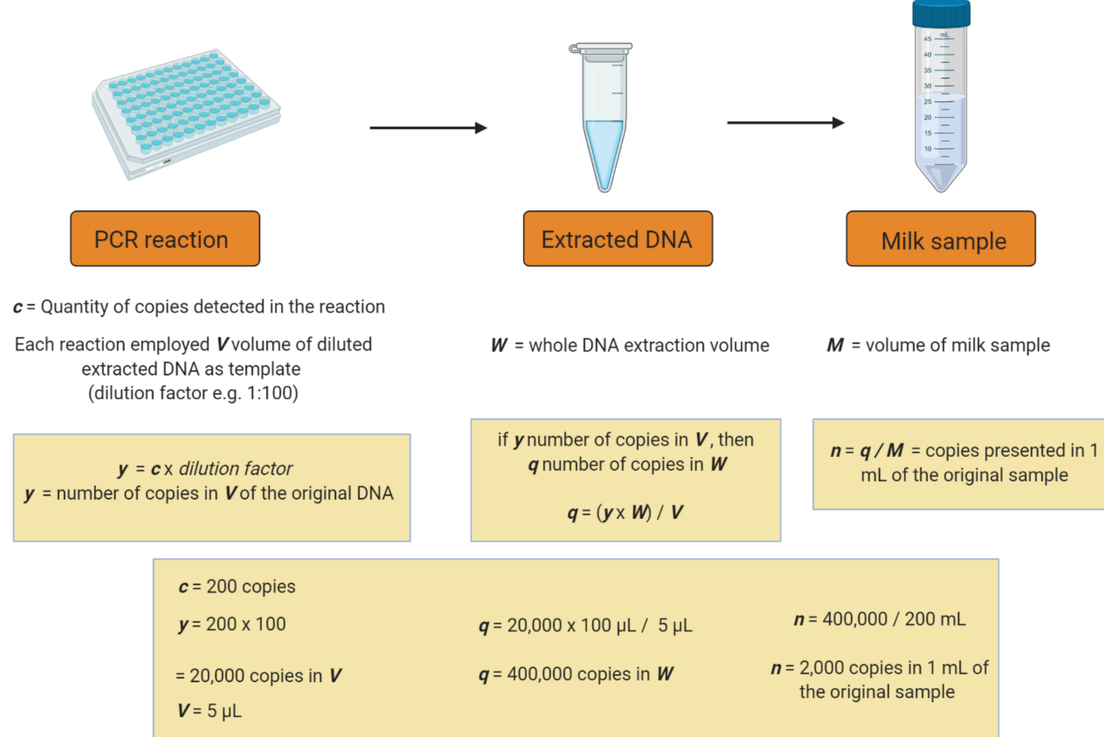


Figure 4.10: Estimating the number of copies in the original sample (adapted from Rantala-Ylinen *et al*., 2011).

For the statistical analyses, the qPCR values obtained were transformed to an ordinal scale to include negative test results: 0 (negatives, Cq $\geq$40), 1 (33 $\leq$ Cq <40), 2 (26 $\leq$ Cq <33), and 3 (Cq <26).

4.3 Results

4.3.1 Descriptive data of farms

A total of 115 PM samples were analysed from the 200 farms that regularly submit milk samples to the two leading milk quality laboratories in Uruguay. Each PM sample corresponded to one dairy farm. The current PM sampling indirectly evaluated 36,200 dairy cows, representing 11.75% of the national dairy herd, considering the total number of dairy cows in the country (308,000).

Regarding localisation of farms, the department of origin rather than the full address was made available to retain the farms' confidentiality. Seven of the eight departments located in the main dairy region of the country were represented in the sampling. Most of the farms analysed were located in Florida and Colonia (42 and 40 farms, respectively), followed by Canelones (19 farms), San José (6 farms), Río Negro (4 farms), Soriano (3 farms) and Durazno (1 farm) (Figure 4.11).

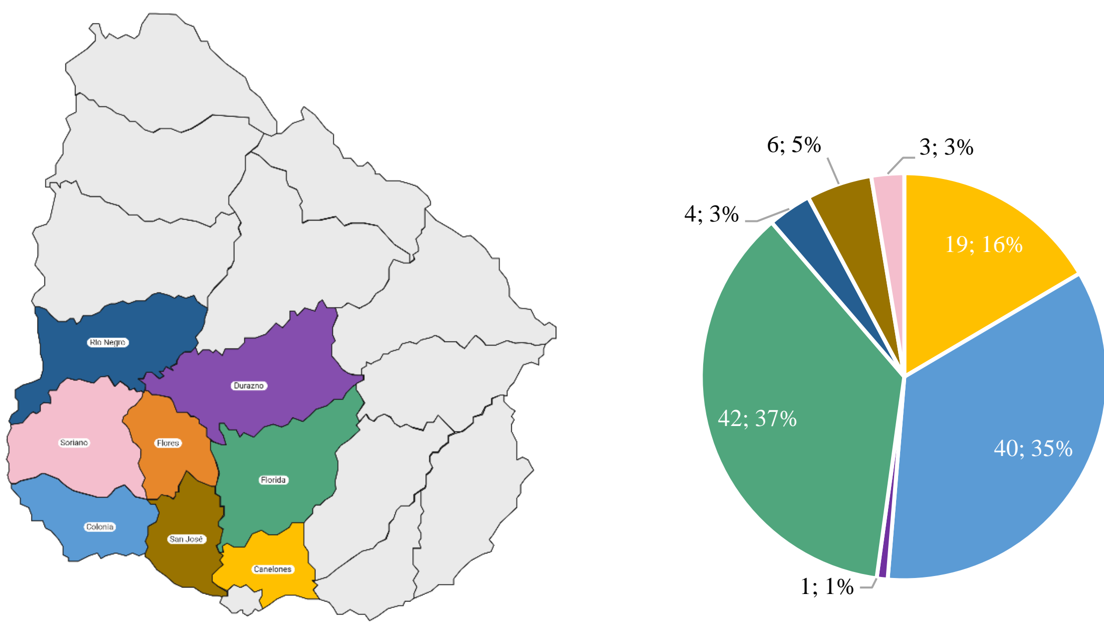


Figure 4.11: Descriptive data of farms.

4.3.2 Establishment of the standard curve and its sensitivity

The recombinant plasmid DNA concentration was 113.42 ng/µl, the A260/A280 ratio was 2.00, and the A260/A230 ratio was 2.35. The conversion into copy number of plasmid DNA was 2.64×10^{10} copies/µl.

The resulting plasmid was first diluted at 1:100. The standard curve was constructed employing the ten-fold serially diluted plasmid DNA from 10^8 to 10^1 copies/µl. The threshold was set within the exponential phase and above background amplification. The Ct values were plotted against the known copy number of each dilution point of the standard control. The slope to the calibration curve was -3.3778, and the Y-intercept was 38.34. The unknown samples were quantified by employing the formula Y=-3.3778X+38.34 (Y=threshold cycle, X=log starting quantity). For all standard curves, r^2 values were high ($r^2 > 0.99$).

Serial dilutions of the recombinant plasmid DNA ranging from 10^8 to 10^1 copies/µl were evaluated by qPCR to assess the sensitivity of the test. The detection limit was 10 copies per reaction. The assay's precision was investigated by measuring Ct values for the three replicates of each point of the serial dilution. Data from the three replicates from each concentration were used to calculate the mean Ct, the standard deviation, and the coefficient of variation (CV). The CVs values showed the variability among results, giving a sense of how reproducible the assay was.

The cloned plasmid was used to create standard curves for *C. burnetii* using the copy numbers and mean Ct values. Standard curves were linear when starting plasmid copy numbers ranged from 10^1 to 10^8 (Figure 4.12). Linear regression of Cq value versus $\log_{10}$ means concentration for corresponding ten-fold serial dilutions provided the equation later applied to Cq values obtained from PM samples to obtain copies/ul. The IC gave expected Cq values for all PM samples, suggesting effective DNA extraction and proving proof supporting no PCR inhibition. The genome copy number of *C. burnetii* from the PM was reliably quantified. Low variability, shown by reduced CV range, indicates efficient assay reproducibility. A slope of -3.3778 can be estimated from this graph, suggesting a high amplification efficiency of the PCR around 90%.

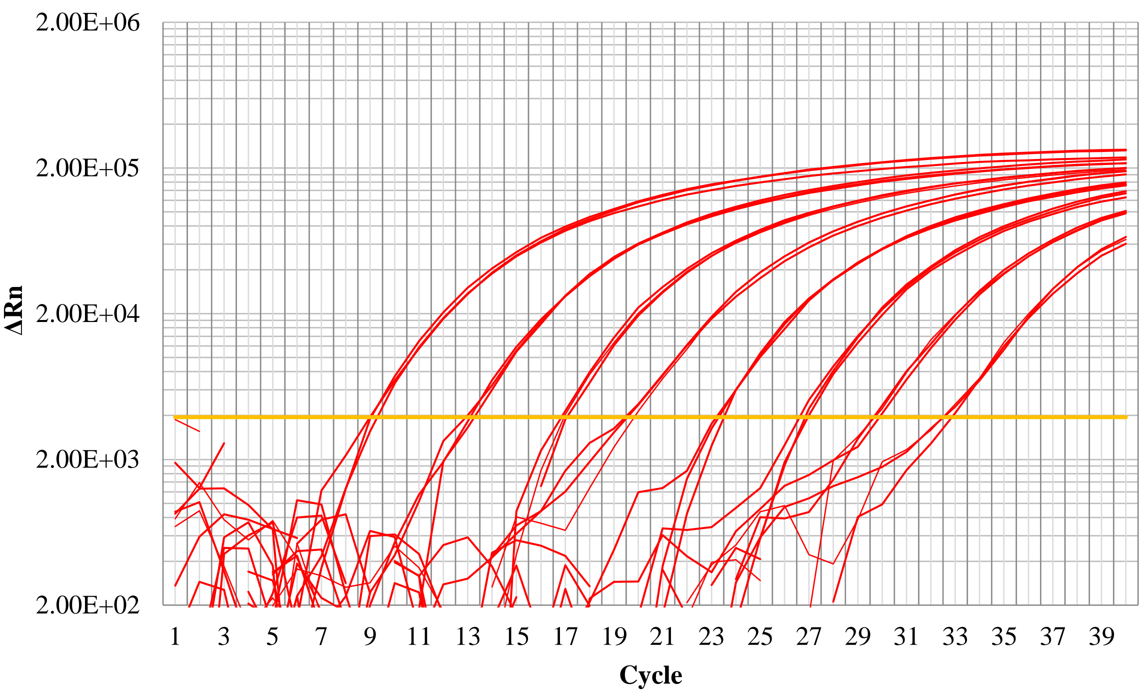


Figure 4.12: Amplification plot for *Coxiella burnetii*. Amplification plot for *Coxiella burnetii* over ten dilutions amplified using primer pair CburF and CburR (Panning *et al*., 2008), and FAM-TAMRA probe (Di Domenico *et al*., 2014). Delta Rn represents adjusted absorbance and is calculated by the formula (Rn+)-(Rn) (Dorak, 2007); the x-axis shows the cycle number. The horizontal line shows the threshold settled at 0.01.

Table 4.1: Inter-assay variability of *Coxiella burnetii* amplification using primer pair CburF and CburR (Panning *et al*., 2008), and FAM-TAMRA probe (Di Domenico *et al*., 2014) in pooled milk samples based on the repetitive insertion sequence *IS1111* element.

Nº of copies/µl	**Replicate 1**	**Replicate 2**	**Replicate 3**	**Mean Ct**	**SD[a]**	**CV[b] (%)**
2.6×10^8	10.068	10.142	9.780	10.064	0.06	0.657
2.6×10^7	14.672	13.068	13.302	13.681	0.70	5.732
2.6×10^6	18.615	16.742	17.391	17.582	0.776	4.416
2.6×10^5	21.559	20.175	20.962	20.898	0.567	2.712
2.6×10^4	23.844	22.795	23.636	23.425	0.454	1.937
2.6×10^3	27.628	26.637	27.549	27.241	0.433	1.589
2.6×10^2	31.506	29.201	30.267	30.325	0.942	3.107
2.6×10^1	34.476	33.388	33.536	33.800	0.482	1.425
2.6	UN	UN	UN	NA	NA	NA

[a] SD: Standard deviation
[b] CV (%): Coefficient of variation
UN: undetermined. No template detected.
NA: not applicable, no target nucleic acid detected for calculation of mean, standard error or coefficient of variation.
A threshold value of 0.001 was set for the determination of Ct values.

This table shows the amplification plot for *C. burnetii*. The Cq values increased along the concentration of the starting template is reduced. The absorbance dropped off in at plateau phase due to gradually diluted samples. The threshold was set at 0.01 above the background amplification signal and within the exponential phase.

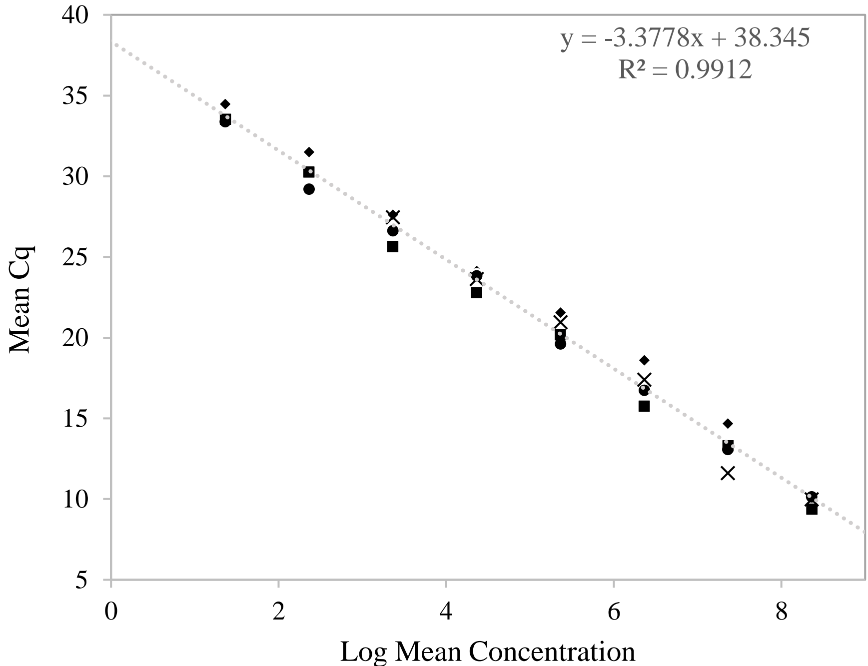


Figure 4.13: Amplification and standard curve construction.

The amplification curves were constructed using ten-fold dilutions of recombinant plasmid DNA ranging from 10^8 copies/µL to 10^1 copies/µL. The standard curve equation was Y=−3.3778X+38.345. The concentration means to the template copy number in each reaction. Cycle threshold (Ct) values are shown on the y-axis.

4.3.3 Descriptive data for the qPCR testing

Coxiella burnetii DNA was found in two of the 115 PM samples analysed (1.7%[1]*). A sample was deemed positive if it presented a standard amplification curve and a Ct value below 40. All the PM samples showed IC amplification suggesting that DNA extraction from the samples was appropriate and signalling no false-negative results produced by inhibition or failed loading of samples. The two positive PM samples found presented Ct values of 31.4 and 32.6, respectively. The amount of *C. burnetii* DNA, calculated based on the formula stated above on section 5.1.14.2 and using the generated standard curve, was 7.3×10^1 and 3.6×10^1 $\log_{10}$ copy numbers. *Coxiella burnetii* DNA copy number in the original PM sample was 1467 and 2163 copies/µl, respectively.

Using the serial dilutions of the recombinant plasmid containing the *C. burnetii* DNA, the assay's detection limit, in other words, the highest dilution point with a Ct value, was estimated to be approximately three copies per reaction.

[1] *It is worth to mention that the results presented here include the analyses completed so far. However, other 50 PM samples remain to be analysed (these are underway at the time of writing). The statistical analysis of the results will be conducted once all the samples are completely processed.

4.4 Discussion

One of this thesis's research goals was to evaluate the utility of a diagnostic strategy based on a qPCR assay using pooled milk samples to detect *C. burnetii*-infected dairy herds. This goal was seeded in the need for a tool for large-scale investigation of *C. burnetii* in dairy farms in Uruguay, aiming to estimate the bacterium's national prevalence among bovine dairies and potentially evaluate the dynamics of *C. burnetii* excretion in infected animals. A qPCR assay was adapted from previous publications (Panning *et al*., 2008; Di Domenico *et al*., 2014) and optimised with the incorporation of internal control of amplification for the detection and quantification of *C. burnetii* in pooled milk (PM) samples. This chapter's specific aim was to determine *C. burnetii* occurrence and quantify *C. burnetii* DNA's levels in PM samples from Uruguay. Unfortunately, many PM samples were not analysed due to disruption of access to laboratory facilities because of the Covid-19 pandemic and lockdown. The current discussion will focus on samples wholly analysed.

This study is the first investigation of the occurrence of *C. burnetii* in collective milk samples from commercial herds in Uruguay. Results obtained so far showed that raw milk from clinically healthy cows from two commercial farms that regularly sell milk to the industry presented milk contaminated with *C. burnetii* DNA. Numerous studies have reported healthy cows shedding in milk, even over long periods (Kim *et al*., 2005, Rodolakis *et al*., 2007, Guatteo *et al*., 2007). These findings reinforce the likelihood of bacterial shedding from asymptomatic animals. The *C. burnetii*-positive herds commercialise the milk produced for some processing units, ensuring adequate milk pasteurisation before it reaches consumers. However, farmers and their families' on-farm milk consumption cannot be dismissed, nor can on-farm artisanal cheese production.

The utilisation of collective milk samples in dairy ruminants, either BTM samples or pooled milk samples, has been proposed as a practical sampling approach to assess epidemiological aspects such as the occurrence and distribution of *C. burnetii* and infection dynamics at the herd level (Kim *et al*., 2005, Lockhart *et al*., 2011). Bulk-tank milk specimens an provide valuable information about a dairy herd's health status about several infectious agents with milk-borne transmission (Ruiz-Fons *et al*., 2011). Recent studies had conducted the molecular evaluation for *C. burnetii* in raw BTM samples in Chile and Colombia, possibly the only two investigations reported in South American countries (Contreras *et al*., 2015; Cornejo *et al*., 2020).

In Uruguay, raw milk trade was first regulated in 1984 (law number 15,640), and the commercialisation of raw milk for direct consumption by humans is currently banned; however, the use of raw milk in rural areas, especially the intra-farm consumption, is difficult to estimate, and therefore, to control. Of

the nearly 18,000 tons of cheese consumed yearly in Uruguay, ~50% represents artisanal cheese produced on ~1,000 dairy farms. Consumers' preferences for raw milk products is emerging as a growing global trend. Due to its indigenous microbiota, raw cheeses have specific organoleptic characteristics of gastronomic value, such as a strong flavor and a peculiar texture, much appreciated by consumers (Yoon *et al*., 2016; Barandika *et al*., 2019), and these characteristics are frequently attributed to the use of unpasteurised milk. Thus, most artisanal cheeses are manufactured using raw milk and either sold directly to consumers at the farms of origin or commercialised in unregulated local markets. This practice may embody a hazard to public health, considering the high stability of *C. burnetii* in final dairy products even with acidic pH or reduced water activity (Barandika *et al*., 2019).

The occurrence of *C. burnetii* from PM samples from across Uruguay assessed by qPCR was low (1.7%). However, as this was the first local evaluation of this bacterium in PM samples, there is no available data to contrast with. Despite the low incidence found, this bacterium's zoonotic potential makes the results disturbing, especially in Uruguay, where most of dairy products, predominantly cheeses, are produced using cattle milk. *Coxiella burnetii* is mainly transmitted aerogenously, although it can be persistently shed in bovine milk and survive in unpasteurised dairy products (Guatteo *et al*., 2007; Barandika *et al*., 2019). Transmission by the digestive route due to the ingestion of contaminated raw dairy products had been proposed, although the available body of evidence is contradictory. Infection by *C. burnetii* has been described after the consumption of raw cow milk (Signs *et al*., 2012) and cheese made from contaminated caprine milk (Hatchette *et al*., 2001; Maltezou *et al*., 2004), suggesting that the risk of *C. burnetii* exposure through the ingestion of dairy products should not be underestimated (Gale *et al*., 2015). Somewhat surprisingly, neither clinical evidence of Q fever infection nor antibodies were detected in people after the deliberate consumption of unpasteurised milk contaminated by *C. burnetii* (Krumbiegel & Wisniewski, 1970).

A clear correlation between the results obtained by collective milk tests and those from tests on individual cattle in a herd is a requirement for utilising pooled milk samples as the target specimen in epidemiological assessments. Surprisingly, there has been limited investigation of the correlation between PCR test results on, for example, BTM samples and individual samples, despite BTM samples being a frequent evaluation subject (Guatteo *et al.*, 2007). An evaluation comprising thirty-seven BTM samples and 1522 individual samples from cows contributing to those collective samples, revealed that herds with positive BTM presented a significantly higher within-herd prevalence of milk-shedder cows. Additionally, the prevalence of cows shedding *C. burnetii* through milk in a herd, as well as the proportion of heavy *C. burnetii* shedder animals, increased considerably with the title of *C. burnetii* DNA found in the BTM samples (Guatteo *et al.*, 2007).

The amount of *C. burnetii* load transmitted by the routes varies among ruminants species (Rodolakis *et al*., 2007). Birth products are the main excretion route in small ruminants and relevant in cows; however,

milk is the main shedding route of *C. burnetii* in dairy cattle. Even asymptomatic animals (Guatteo *et al*., 2007) or seronegative cattle (Barberio *et al*., 2014) have been identified as *C. burnetii* milk shedders. *Coxiella burnetii* can be excreted in milk for up to 13 months (Roest *et al*., 2011a; Kargar *et al*., 2013), although this may be intermittent (Rodolakis *et al*., 2007). Two shedding patterns have been seen in cattle. They can be persistent heavy shedders or sporadic shedders (Guatteo *et al*., 2007). These heterogeneous shedding patterns, make composite samples of particular importance for massive scale investigations. A positive result provides robust evidence for identifying infected herds; for instance, BTM testing is the preferred diagnostic approach for disease notification in many countries (van der Hoek *et al*., 2010). It has epidemiological value for the monitoring of infection status over time in follow-up evaluations (Muskens *et al*., 2011) during monitoring campaigns or after medical interventions (Taurel *et al*., 2014) or vaccination (Astobiza *et al*., 2013; Boarbi *et al*., 2014). It must be borne in mind that the failure of detection of *C. burnetii* DNA in PM samples is not enough evidence to consider a farm as free from the bacterium because a PM sample is a restricted/partial picture of the sanitary herd's status as just milking cows, but no unhealthy animals and dry cows, are included in the sample. The likelihood of detection may have been conditioned by the prevalence of shedder cows within the herd and the shedding intensity.

Numerous PCR techniques, such as conventional PCR, qPCR, multiplex PCR and nested PCR, have been developed and are frequently utilised to investigate the presence of *C. burnetii* and to quantify its DNA in individual and composed milk samples. Contrary to the serologic assays that detect antibodies developed in milk only after a while, the PCR approaches can identify *C. burnetii* immediately after milk contamination. Incorporating a housekeeping gene encoding *β*-actin as an IC of amplification has helped increase results' reliability, especially bearing in mind how milk sampling is challenging, and this approach saves time and cost. As the IC was incorporated in the reaction tube, if all the steps of the qPCR were appropriately followed, the IC would produce a positive signal even though the occurrence or absence of the bacterial target region, giving additional information about the quality of the sample by revealing the existence of inhibitors of the reaction.

The two available reports on *C. burnetii* molecular investigation in BTM samples in South America yielded dissimilar results. For instance, the study conducted in Colombia constituted a random sampling of BTM specimens from 11 dairy herds in the region of Montería. Five dairy herds showed positive amplification on an *IS1111*-based PCR, constituting a prevalence of 45.5% from the total samples analysed (Contreras *et al*., 2015). The Chilean evaluation was a convenient sampling focused on Chile's southern region, where a human Q fever outbreak affected dairy farmworkers and their families in 2017 (Cornejo *et al*., 2020). This evaluation found 2.1% *C. burnetii*-positivity among the samples.

Coxiella burnetii shows tropism by alveolar macrophages and monocytes (Fernandes *et al*., 2016); however, the bacterium can infect diverse types of cells (Sobotta *et al*., 2017). While keeping cell

viability, udder cells permitted the greatest replication rates to form large cell-filling *C. burnetii* containing vacuoles (Sobotta *et al*., 2017). This may be one explanation behind the central role of milk in *C. burnetii* shedding in cattle, strengthening the need for the evaluation of milk as the target sample when attempting to detect infected cows. Numerous worldwide publications reported a widespread shedding of *C. burnetii* through milk in cattle (Kim *et al*., 2005; Muskens *et al*., 2011; Astobiza *et al*., 2012). The prolonged excretion of the bacterium in cattle through milk that can continue even for several months, contrasted with the briefer excretion described for small ruminants, could explain the elevated *C. burnetii* DNA prevalence reported by these studies (Rodolakis *et al*., 2007; Astobiza *et al*., 2010).

As the DNA detected can be derived from either viable or dead bacteria (Gyawali *et al*. 2016), the mere identification of *C. burnetii*-DNA in PM samples does not imply the identification of infectious *C. burnetii*. Conversely, the investigation of RNA should be used to detect living organisms because RNA degradation occurs quickly after bacteria death, becoming RNA a more reliable indicator of possible infectivity (Centurion-Lara *et al.*, 1997).

4.4.1 Is Q fever a relevant problem in Uruguay?

The epidemiology of coxiellosis in ruminants in Uruguay is broadly unknown. So too is the epidemiology of Q fever in the Uruguayan population. C*oxiella burnetii* is an occupational hazard for slaughterhouse and dairy farm workers in Uruguay. Since 1956 at least 18 human outbreaks involving abattoir and meat-processing workers have been identified in this country by complement fixation, capillary agglutination test and layer microagglutination, and traced directly to cattle exposure (Salveraglio *et al*., 1956; Somma-Moreira *et al*., 1987; Ortiz-Molina *et al*., 1987). The first Uruguayan cases of Q fever epidemiologically linked to a dairy farm were diagnosed in 1988, using the indirect fluorescent antibody test (IFAT) for anti-*Coxiella* IgM (Braselli *et al*., 1989). The five cases, all adults, presented IgM titres of 1/20, without the antigenic phase specificity being determined. The infection was assumed to result from the inhalation of contaminated dust; none of the five patients had ingested raw milk. A local Q fever outbreak linked to wildlife occurred during 2003-2004 (Hernández *et al*., 2007). Workers from an experimental wildlife breeding station became infected apparently through the inhalation of contaminated particles during grass mowing. Epidemiological investigation identified the pampas deer (*Ozotoceros bezoarticus*) as the presumed source of infection.

The current findings enlarge the body of evidence as they confirmed *C. burnetii* DNA's presence in collective milk coming from commercial herds. Even though most people infection arises through the inhalation of aerosols contaminated by *C. burnetii*; the oral route of infection by consuming unpasteurised milk and dairy products containing the virulent bacterium had also been postulated

(Rodolaskis, 2006; Berri *et al*., 2000). None of the local Q fever outbreaks which have occurred so far had been epidemiologically linked to *C. burnetii*-contaminated milk consumption. However, it should be considered that human acute Q fever is clinically characterised by a broad range of non-specific symptoms and signs, thus often leading to misdiagnosis and underreporting of the disease events (Anderson *et al*., 2013a).

4.4.2 Limitations

The utilisation of a primer set specific to a single-copy gene is generally recommended for accurate quantification using qPCR. The use of a gene that is presented in one copy per *C. burnetii* genome, such as *com1,* is translated as one genome equal to one *C. burnetii* organism (Klee *et al*., 2006; Kersh *et al*., 2010). In the current study, the absolute quantification of *C. burnetii* was done using a qPCR based on the repetitive transposon-like sequence IS*1111* element, which could be seen as a limitation. This approach, albeit at the expense of some quantification accuracy, was chosen to enable the detection of the bacterium with enhanced sensitivity. The IS*1111* is highly conserved among different bacterium strains and is presented at several (10–30) copies per bacterium, becoming a suitable target for detection/diagnostic purposes (Willems *et al*., 1994; Lorenz *et al*., 1998; Klee *et al*., 2006). The potential *C. burnetii* strains circulating in Uruguay are still unknown, as is the number of copies of the target IS*1111* within their genomes. However, it is not unreasonable to think a low genetic diversity among Coxiella strains infecting the Uruguayan dairy cattle population. Thus, the quantitative values shown in this chapter assumed that the copy number of IS*1111* was equal in *C. burnetii* strains in all the herds. Some degree of accuracy in quantification was knowingly lost when using this high sensitivity qPCR. Having a high sensitivity was very important, especially as pooled milk samples, instead of individual samples were subjected to analysis. The utilisation of a duplex PCR targeting a single copy gene (for example, *com1*) and the repetitive transposon-like sequence IS*1111* element, would have been an alternative approach to overcome this limitation by combining adequate sensitivity and accuracy.

As the molecular test was performed on PM samples, only cows that were actively milking, assuming these animals to be healthy adult cows, were actually evaluated. Thus, special consideration needs to be paid to the dry cows, heifers, and treated cows with milk withdrawal, which are not included in the samples, thus not evaluated (Frössling *et al*., 2006). These animals may, potentially, later excrete massive amounts of *C. burnetii* during parturition and subsequent lactation. Therefore, as PM samples exclude non-lactating cows, repeated analyses over time should be conducted to evaluate the entire herd. Additionally, as the current study employed a single PM sample from each corresponding herd, it is possible to estimate that the infection went unnoticed in those herds with few infected cows. Milk

from non-shedding cows would have, to some extent, diluted the concentration of *C. burnetii,* which could lead to restricted detection.

The utilisation of convenience sampling could be seen as a limitation because some selection bias could have been introduced; thus, likely the sample was not representative of the whole population of dairy farms in the country. Possible other types of sampling methods, such as random sampling, in which all the farms present the same probability of been chosen, could have improved the sample's representativeness. Despite being aware of this risk of bias, we have selected this convenience sampling approach as the only accessible option in terms of costs and resources, at the time of research.

Because of the samples' confidentiality, the farms' exact location was not available. Thus, unfortunately, a within-herd evaluation of the animals following this at herd-level assessment will not be plausible.

4.4.3 Further research

This investigation uncovers evidence about the presence of *C. burnetii* in collective milk samples from local dairy bovine herds and sets a precedent for an extensive scale assessment. The current sampling evaluated 115 dairy herds and indirectly assessed 36,200 dairy cows, representing 11.75% of the national dairy herd population. The current evaluation represents the first attempt to investigate *C. burnetii* in Uruguay using milk as the target sample. The findings confirm that testing milk by qPCR may aid in the detection of *C. burnetii* infected herds.

Identifying infected herds would have been the first step to an in-depth evaluation within herds by evaluating Q fever's clinical signs, such as abortions or infertility. This study is the foundation for future studies assessing the within-herd prevalence of *C. burnetii* which is crucial considering that the identification of *C. burnetii* shedder animals is a critical step to limit the bacterium spread among cows, as well as to reduce zoonotic risk (Guatteo *et al*., 2006; Guatteo *et al*., 2007). Identifying chronic *C. burnetii* milk shedding cattle may be important in order to avoid or minimise contamination of the environment, decrease the chances of spread among animals, and prevent the transmission of *C. burnetii*. These results support implementing a national surveillance plan, using bulk-tank milk, to explore the prevalence of *C. burnetii* on dairy farms in the whole country. A large-scale investigation is required to gain more exact knowledge of the *C. burnetii* epidemiological situation in Uruguay. The national surveillance proposed could evaluate paired BTM samples collected from each farm to evaluate on a larger scale the status of herds in terms of *C. burnetii* excretion, and possibly to evaluate the efficacy of measures of control after their implementation.

As mentioned above, as no previous systematic investigation about *C. burnetii* had been conducted in Uruguay, no data were available regarding the occurrence of this bacterium in commercial farms. The multicopy element IS*1111* was chosen, aiming to have a qPCR with high sensitivity. However, some arguments against this decision could point out the loss of accuracy in quantification (Klee *et al*., 2006). Further evaluations using the same PM sample already stored can be conducted targeting a single copy gene in order to achieve much more precise quantification.

The detection of *C. burnetii* in dairy herds may also imply potential food security concerns. Recent findings revealed that *C. burnetii* could remain viable in raw cheeses up to 8 months after maturing (Rozental *et al*., 2020). Genotyping investigations have suggested that dairy products' predominant genotypes are the same as those identified in bovine dairy herds (Tilburg *et al*., 2012). This alerts us to the possibility of dairy products, especially those made using raw milk such as artisanal cheeses, being a potential source of the bacterium facilitating human infection. There are ~1000 dairy farms that produce artisanal cheese in Uruguay, predominantly located in San José and Colonia. These producers sell around 9.000.000 kilos of cheese per year, representing 50% of the local consumption. Based on this, the evaluation of the occurrence and viability of *C. burnetii* in dairy products ready to be commercialised would be critical. For this purpose, while the current qPCR can be employed for quantification, the bacterium's viability should be analysed by other techniques. As *C. burnetii* isolation or inoculation on experimental animals is not an available option in the local contexts. No BSL-III laboratories are currently operative. Other procedures should therefore be explored. RT-qPCR can approximate *C. burnetii* viability based on mRNA from gene *ARNr16S.*

The qPCR assay should be accompanied by serology tests on BTM samples, not at the individual level, as the combination of the two approaches provides complete information on a herd's infection level.

4.5 Conclusions

Hitherto, the lack of diagnostic tools has limited epidemiological investigations in Uruguay, where no information about the prevalence of *C. burnetii* among ruminants is currently available. This study is the first attempt to detect *C. burnetii* in collective milk samples from Uruguayan dairy herds. The findings showed that *C. burnetii* is shed by clinically healthy cows from commercial dairy herds in Uruguay, reaffirming the bacterium's shedding in milk in asymptomatic animals. These animals were presumed as asymptomatic as they were being milked at the moment of sampling. The evidence obtained supports further surveillance investigations by bulk tank milk testing. Considering the survival of *C. burnetii* as a milk-borne pathogen in unpasteurised milk and raw dairy products, this report raises awareness of Q fever as potential food safety and public health concern.

A low incidence (1.7%) of *C. burnetii* was revealed among the analysed samples; however, due to its zoonotic threat, as infection is frequently asymptomatic but can lead to serious illness under certain circumstances, people from at-risk exposure groups should be advised about Q fever and take the steps necessary to avoid infection. Environmental factors had been identified as primary aspects in the transmission of *C. burnetii* between cattle, though cattle trade and transport are also relevant factors (Nusinovici *et al*., 2015a; Pandit *et al*., 2016). The movement of animals from one herd to another should be done using control measures, such as testing and quarantine, to avoid infection spread.

Both *C. burnetii*-positive herds identified so far were large herd size farm; however, the effect of herd-size on the frequency of *C. burnetii* DNA detection in collective milk samples has not yet been evaluated because of the samples still needing to be analysed. Complete analysis of all samples is needed to find a reliable link.

*Chapter 5: **Investigation of*** **Coxiella burnetii** ***and*** **Chlamydia** ***spp. in aborted bovine placentas, a case-series sampling***

The work presented in this chapter had been already partially published on the journal *Brazilian Journal of Microbiology*. This publication can be founded in the Appendix A.

Rabaza, A., Macías-Rioseco, M., Fraga, M., Uzal, F.A., Eisler, M.C., Riet-Correa F. & Giannitti, F. *Coxiella burnetii* abortion in a dairy farm selling artisanal cheese directly to consumers and review of Q fever as a bovine abortifacient in South America and a human milk-borne disease. Brazilian Journal of Microbiology, (2021). https://doi.org/10.1007/s42770-021-00593-1

5.1 Introduction

Coxiella burnetii, Chlamydia abortus and Chlamydia pecorum are obligate intracellular Gram-negative bacteria. They induce reproductive disturbances in domestic ruminants (cattle, sheep and goats), including abortion, leading to significant economic losses. These pathogens have been detected not only in domestic livestock but also in wild animals, and they pose a zoonotic threat to humans. These animal-borne bacteria are comparable in terms of epidemiology and how humans are infected. They have a low infective dose, posing a severe concern, especially to those people handling livestock and people indirectly exposed to animals by working with clinical samples. Human infection with *C. abortus* occasionally arises, while *C. burnetii* infection, known as Q fever or coxiellosis, frequently occurs as outbreaks in which many individuals may be affected.

Coxiella burnetii and *C. abortus* infection in animals often present an asymptomatic course; however, these infections have been associated with different alterations. *Coxiella burnetii* has been demonstrated to be mostly implicated in bovine reproductive disturbances, such as abortion, weak offspring, and stillbirth (Hopper, 2015). Chlamydial infections can also produce different sorts of problems, comprising polyarthritis, conjunctivitis, mastitis, other urogenital tract infections, and encephalomyelitis (Barkallah *et al*., 2014). For instance, *C. pecorum* primarily produces inapparent enteric infections (Berri *et al*., 2009), but is also known to trigger pneumonia, arthritis and conjunctivitis (Aitken & Longbottom, 2007). When the infection leads to abortion, it takes place during the last trimester of gestation in ruminants. These pathogens are massively shed in birth products, even from normal births or abortions, such as placenta, amniotic fluid, and fetuses.

Both bacteria pose a danger for humans, and the inhalation of aerosols or contaminated dust with the bacteria seems to be the primary source of human infection. There is currently a discussion ongoing about the importance of raw dairy products as a source of *C. burnetii* for human infection (Cerf &

Condron, 2006; Gale *et al*., 2015). Transmission of *C. abortus* to people has been repeatedly associated with enzootic abortion in sheep (Sachse & Grossmann, 2002). Similarly, most of the Q fever cases reported in humans had been originated from small ruminants.

The *C. abortus* zoonotic risk induces various clinical forms ranging from asymptomatic infection to mild influenza-like illness or, rarely, pneumonia cases, sporadically with severe complications (Rodolakis & Mohamad, 2010; Sillis & Longbottom, 2011). Abortions in pregnant women after *C. abortus* infection have also occasionally been described (Pospischil *et al*., 2002c; Meijer *et al*., 2004; Aitken & Longbottom 2007; Psarrakos *et al*. 2011). It has also been linked to other human health disturbances at pregnancy (PID and sepsis) (Rodolakis & Mohamad, 2010; Walder *et al*., 2005; Wheelhouse & Longbottom 2012). Most of the human Q fever cases are asymptomatic. Occasionally, infection in people can produce an acute presentation characterised by self-limiting influenza-like symptoms, headache, pneumonia, hepatitis; or a chronic presentation frequently involving endocarditis and granulomatous hepatitis. *Coxiella burnetii* infection has also been associated with premature delivery or abortion in pregnant women (Maurin & Raoult, 1999).

Chlamydiosis caused by *C. abortus,* and Q fever could be underestimated, and possibly underdiagnosed, diseases both in people and animals, fundamentally because of the unspecific clinical outcome of their infections. As these bacteria can infect ruminants even without clear clinical signs revealing infection, these infections may often course inapparently. Precisely this lack of clear clinical signs of infection reinforces the necessity of introducing routine monitoring of these pathogens.

Coxiella burnetii and *Chlamydia* spp. belong to phylogenetically unconnected species (Woese, 1987); however, these bacteria demonstrate some similarities regarding the way they interact with the host and characteristics of the pathogenesis of the infection (Lukacova, 1996). Additionally, similar clinical characteristics appear in the abortions generated by these bacteria.

5.1.1 *Coxiella burnetii* and *Chlamydia* spp. mixed infection

Articles reporting *C. burnetii* and *C. abortus* mixed infections in abortions in goats and sheep (Schöpf *et al*., 1991; Parisi *et al*., 2006), and cattle (Sting *et al*., 2000) are available. The concomitant infection of these bacteria was reported in an outbreak of enzootic abortion in small ruminant flocks in Italy investigated by PCR on fetal and placenta samples (Masala *et al*., 2007).

5.1.2 *Placenta*

The placenta in cows, sheep and goats is classified based on gross anatomical features as cotyledonary and based on its histological characteristics it is classified as synepitheliochorial. The placenta is the

only route for the transferral of nutrients to the conceptus, and its requirements rise exponentially to term, simultaneously with a broad variety of changes (Wooding & Flint, 1994). In terms of gross anatomical characteristics, ruminants have a cotyledonary placenta with discrete areas of attachment called placentomes constitute the interaction of the chorioallantois with the maternal endometrium, where the fetal portion is known as a cotyledon, and the maternal contact sites are the caruncles (Igwebuike, 2006). During the blastocyst stage of the fertilised oocytes, the embryo acquires the blastocoel, which is a fluid-filled central cavity surrounded by the trophectoderm that is a single layer of cells. The trophectoderm and the somatic (parietal) mesoderm represent the chorion. The allantois, a structure that resembles a sac, finally expands to fuse with the chorion, constituting the chorioallantois (Schlafer *et al*., 2000). The vascularised embryonic chorioallantois is lined externally by cells of the trophectodermal epithelium. The cells constituting the trophectodermal epithelium undertake specific functions and are named as trophoblast cells.

The placenta from ruminants has two main regions, one called the interplacentomal region engaged in histotrophic exchange, and the placentomal region that functions in the haemotrophic transfer of metabolites and nutrients between the mother and the fetus (Schlafer *et al*., 2000). Within the interplacentomal areas, there is simple apposition between the fetal membranes and the epithelium of the uterus. In the placentomes, however, there are deep caruncular crypts among the endometrial surface, infiltrated by extended, abundantly branched cotyledonary villi of the chorioallantois (Davis *et al*., 2000). This placentomal structure permits a considerably increased surface area between fetal and maternal compartments. In contrast to the placenta presented in humans or rodents, the cotyledonary synepitheliochorial placenta found in ruminants (Wooding, 1982) has reduced trophoblast invasion of maternal tissue limited to the placentomes (Gogolin-Ewens *et al*., 1989).

The trophoblast cells constitute a continuous epithelial layer of the chorion, over the whole surface of the chorioallantois (Schlafer *et al*., 2000). Within these, there are mononucleate and binucleate trophoblast cells, which are distinct cell types in terms of morphology and functionality (Wimsatt, 1951; Greenstein *et al*., 1958; Igwebuike, 2006). While the mononucleate cells are mostly engaged in the exchange of nutrients and cover the majority of the interface; the binucleate cells have a synthetic function and produce hormones (Duello *et al*., 1986; Myers & Reimers, 1988).

The mononucleate trophoblasts are cells located on a basal lamina having cuboidal to columnar shape and show typical features of epithelial cells (Wooding *et al*., 1994). The mononucleate comprise about four-fifths of the trophoblast population (Boshier & Holloway, 1977). The apical surface membranes of these cells form microvillar structures that interdigitate with analogous processes occurring from the uterine epithelial cells, establishing the fetus-maternal connection (Bjorkman, 1969; Dent, 1973). The binucleate cells are large and have a different structure from the surrounding mononucleate epithelial cells. The binucleate trophoblast cells embody around 20% of the trophectodermal cells (Wooding & Wathes, 1980; Wooding *et al*., 1982). There is a migration of the binucleate cells across the interface

between fetus and mother, latter fusion with columnar cells of the uterine epithelium which give rise to the trinucleate hybrid cells (Wooding, 1984; Wooding, 1982; Wango *et al*., 1990). The continue migration of the binucleate cells and fusion with trinucleate cells, finally lead to the enlargement of the trinucleate cells forming multinuclear syncytial plaques (Wooding, 1982, 1984). This particular capacity showed by binucleate cells to migrate and fuse with epithelial cells from the uterus result in the categorisation mentioned above of the ruminant placenta as synepitheliochorial (Wooding, 1982).

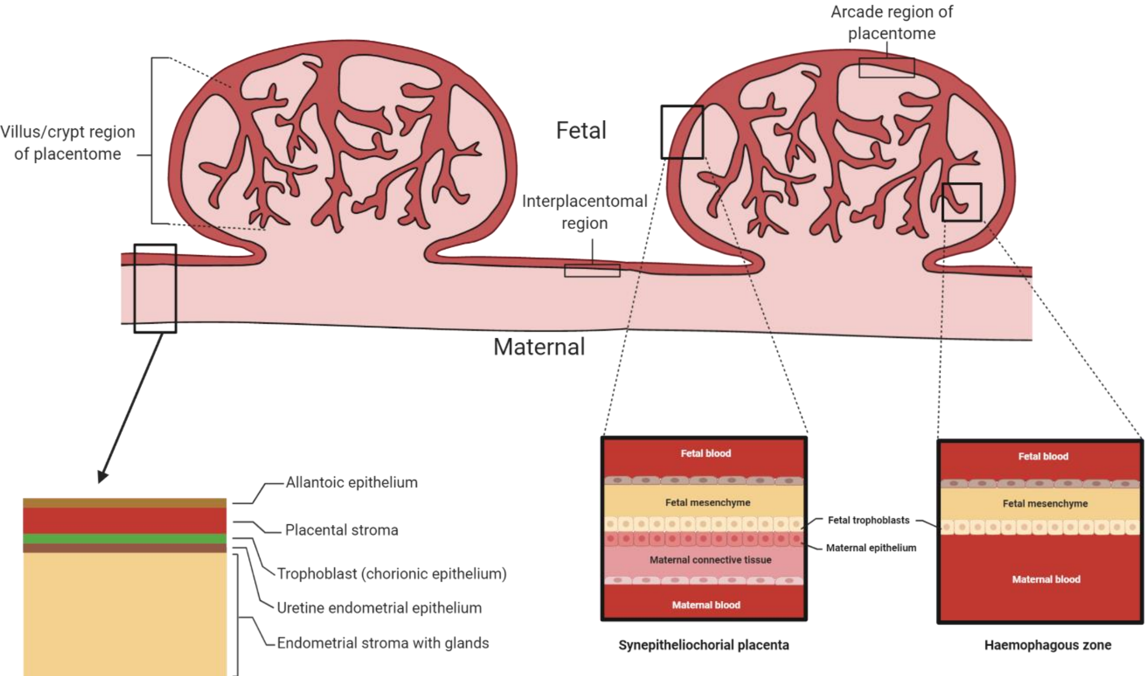


Figure 5.1: The bovine placenta. Adapted from Schlafer *et al*., 2000.

5.1.2.1 The common predilection for trophoblast cells

After infection, *C. burnetii* and *C. abortus* colonise trophoblasts in the allantochorion, these cells being the primary target for their multiplication (Navarro *et al*., 2004; Sanchez *et al*., 2006; Roest *et al*., 2012).

Abortion caused by *C. abortus* appears to be initiated by the destruction of the layer of trophoblasts (chorionic epithelium) which are the primary site of bacterial multiplication in the placenta. These cells also play a relevant role in the beginning and spread of placental inflammation throughout chlamydial infection (Wheelhouse *et al*., 2009). The infection becomes established within the trophoblast and the *C. abortus* intracellular biphasic developmental cycle occurs.

From these cells, the bacterium spreads into the adjacent chorion, leading to the damage of the placenta, affecting nutrient procurement and hormonal adjustments, which may produce premature fetal expulsion (Longbottom & Coulter, 2003).

The experimental inoculation of *C. burnetii* in pregnant goats by the intranasal route showed marked tropism of the bacteria towards the placenta at two to four weeks after the administration when bacteria become detectable in trophoblasts of the allantochorion which shows signs of inflammation (Roest *et al*., 2012). The marked tropism of *C. burnetii* toward the trophoblasts could indicate that only pregnant dams are plausible hosts of *C. burnetii* infection (Roest *et al*., 2012). However, the hypothesis of undetectable numbers of the bacterium being hidden in the body and emerging to infect trophoblasts when they become available cannot be entirely ruled out (Alsaleh *et al*., 2011).

As mentioned above, evidence obtained in goats reinforced the point suggesting that *C. burnetii* replication appears to take place fundamentally in the trophoblasts of the placenta and not in other organs of the infected dam or in the fetus (Roest *et al*., 2012). Several aspects regarding this strong tropism showed by *C. burnetii* to the placenta, and reproductive organs have not yet been elucidated; similarly, the precise mechanism behind the bacterial infection in trophoblasts remains unclear. The gradual increase of *C. burnetii* DNA detected in the placenta until parturition, followed by a radically marked drop in detection after birth makes sense and could be explained by the disappearing of trophoblasts, which removes the bacterium replication niche (Sanchez *et al*. 2006; Roest *et al*., 2012).

Despite the central importance of the trophoblast in these infections, the histologic detection of large numbers of coccoid bacteria within these cells is not specific to *C. burnetii* or *C. abortus*. Although this is a typical finding in infections due to these bacteria, as trophoblasts are phagocytic cells, and other microorganisms such as *Brucella abortus* are likely to proliferate within them easily leading to confusion (Anderson *et al*., 1986; Hazlett *et al*., 2013).

Available evidence supports the idea that *C. abortus* infection of the placenta starts from the maternal side. For instance, the *C. abortus* antigen was first detected in the endometrial stroma within mononuclear cells, and focal necrosis with bacterial colonisation of maternal epithelial cells was revealed during an experimental infection done in pregnant sheep (Navarro *et al*., 2004). Once the colonisation of the fetal placenta occurs, antigen from *C. abortus* appears to be limited to trophoblast cells in the placentome and periplacentome areas of the chorioallantoic membrane, with no evidence of antigen occurrence in the intercotyledonary trophoblast until normal birth or abortion (Buxton *et al*., 1990). In contrast to the controlled infection that seems to occur in the maternal placenta, the extended infection evidenced in the fetal placenta suggests that trophoblast cells are one of the central niches for *C. abortus* multiplication and dissemination (Navarro *et al*., 2004). The central role of trophoblast in *C.*

abortus infection is likely built on two aspects, the lack of MHC I antigen expression and the lack of induction of an enzyme responsible for tryptophan degradation. As trophoblast cells fail to express the MHC I antigen, these cells are hardy to CD8 T cell-dependent cytolytic processes. Chlamydia strains have a tryptophan synthase that enables the bacteria to produce tryptophan from indole, which is obtained from the genital tract microbiome (Caldwell *et al*., 2003). This mechanism is essential because host responses to infection activate the production of interferon-γ (IFN-γ), which facilitates the induction of the enzyme indoleamine 2,3-dioxygenase (IDO) that degrades tryptophan as a defence strategy by starving the bacteria of tryptophan (Beatty *et al*., 1994ab, Leonhardt *et al*., 2007). The trophoblasts fail to stimulate the expression of IDO (Entrican *et al*., 2002) so these cells offer an ideal environment with plenty of tryptophan, a vital amino acid for *C. abortus* growth (Brown *et al*., 2001).

The two pathogens share the characteristic of having a sort of latent subclinical phase in non-pregnant dams, that is activated during pregnancy. Animals become oronasally infected with *C. abortus* by contact with pasture or water contaminated with abortive material or infected offspring that survive (Longbottom & Coulter, 2003). Studies conducted in sheep showed that primary infection of non-pregnant ewes is likely to produce first a mild or even no symptomatic latent infection, that is conducive to the trophoblast cells colonization in the following pregnancy, affecting the chorionic epithelium and causing abortion that often occurs in late gestation (Buxton *et al*., 2002; Longbottom & Coulter, 2003; Navarro *et al*., 2004). At present, the mechanism behind the interruption of the latency stage is not well understood. Whenever the infection occurs, most of the time abortion takes place during the last weeks of gestation (Entrican *et al*., 2001).

In the case of *C. burnetii*, after initial infection, the bacterium primarily replicates in lymph nodes, leading to a stage of bacteremia followed by migration to predilected organs with a strong tropism toward the placenta (Maurin & Raoult, 1999; McQuiston *et al*., 2002). *Coxiella burnetii* infection in animals is most persistent. In chronically infected animals, it is not well explained how and where Coxiella remains during the non-pregnant stage and what the process is which lies behind the activation of bacteria multiplication in placental tissues.

Analogous latent persistence followed by recrudescence has been documented in human cases in which researchers similarly disagree about the organ site where latent infection persists. Nevertheless, in a fatigue syndrome, the bone marrow was found to be the likely focus of *Coxiella* infection from which other sites such as the placenta and endocardium could be seeded for recrudescence (Harris *et al*., 2000, Marmion *et al*., 2005).

The *C. burnetii* infection in cattle is characterised by a wide variety of outcomes, from inapparent infection to abortion (Bildfell *et al*., 2000; Rodolakis *et al*., 2007). Once in the uterus, *C. burnetii* triggers an infection that could remain latent and restricted to the placenta or might occasionally spread to the fetus (Agerholm *et al*., 2013). An active infection which is haematogenous or amniotic-oral

spread to the fetus is more likely to compromise the fetus and cause abortion (Agerholm *et al*., 2013). Aborted fetuses are predominantly fresh with scarce or even no lesions; the strong placental tropism of *C. burnetii* makes this tissue be heavily infected (Bildfell *et al*., 2000; Roest *et al*., 2012; Ammerdorffer *et al*., 2014). This is the key explanation why the placenta should be the preferent specimen to be analysed when investigating active *C. burnetii* infections (Arricau-Bouvery *et al*., 2003; Macías-Rioseco *et al*., 2019). Additionally, as the placenta is heavily *C. burnetii* loaded, its expulsion during parturition results in a vast bacterial shedding into the environment enhancing contamination and the risk of disease spread and zoonotic exposure (Roest *et al*., 2012). Therefore, infected birth materials need careful handling.

Due to some characteristics of these bacteria, such as airborne transmission, low doses causing infection, stability and resistance to environmental factors, and culture being time-consuming and hazardous, isolation of these agents can solely be done under BSL-III facility conditions (Berri *et al*., 2009; Heddema *et al*., 2015). In this context, the use of culture-independent technologies appears essential, considering that BSL-III cabinets are not available in all laboratories. This is the case for Uruguay, where these biosafety conditions in veterinary laboratories are not currently operative. In conditions where culturing of the agents cannot be performed, molecular tests based on the investigation of nucleic acids appears a useful alternative.

5.1.2.2 Main placental lesions

Tissular changes linked to *C. burnetii* abortion often include mild to very severe placentitis, especially in the intercotyledonary areas (Palmer *et al*., 1983). The trophoblasts are typically filled with basophilic intracytoplasmic bacteria. Placental vasculitis involving mixed inflammatory cell infiltrations (mononuclear cells, eosinophils or neutrophils) had been observed on maternal and fetal chorioallantois of aborted fetuses from sheep and cows (van Moll *et al*., 1993). Bovine abortion associated with *C. burnetii* infection is linked with necrotising inflammation of the cotyledons (Rády *et al*., 1985; van Moll *et al*., 1993; Bildfell *et al*., 2000). By contrast, histological examination of cotyledons from *C. burnetii* infected dairy cattle with normal calving showed a variety of generally mild alterations; the bacterial infection was only infrequently related to inflammation (Hansen *et al*., 2011). The detection of placental inflammation is a relevant indicator of the impact of the infection. Placental inflammation may result in dysfunction of the organ, with consequent adverse effects on the offspring (Carpopino *et al*., 2007).

A relationship has been proposed between the species, the lesion severity found in the aborted placenta, and the severity of infection. While coxiellosis in sheep has been linked to abundant bacterial antigen accumulation and serious necrotising and purulent placentitis, *C. burnetii* infection in cows has presented mild or even absent lesion, with a smaller number of bacteria demonstrated

immunocytochemically (van Moll *et al*., 1993). This aspect can be connected to some epidemiological features of coxiellosis in cattle, such as why bovine Q fever is frequently clinically inapparent.

Regarding chlamydial infection, placentitis is deemed as the most consistent pathological feature among the chlamydial experimentally induced abortions in cattle (Idtse, 1984; Perez-Martinez & Storz, 1985). Severe placental vasculitis has also been reported as a typical lesion in fetuses aborted due to Chlamydial infection (Palmer *et al*., 1983; Zeman *et al*., 1989).

As *C. burnetii* and *C. abortus* typically colonise trophoblasts (Navarro *et al*., 2004), it is not surprising that frequently a larger number of these organisms has been found in cytoplasmic vacuoles in placenta samples than in fetal lungs or stomach content samples.

5.1.3 *Confirmatory diagnostic tests*

The gold standard for definitive diagnosis of *C. burnetii* from aborted samples is culture and bacterial isolation from either fetus or placenta (Ozkaraca *et al*., 2017). Similarly, the *C. abortus* isolation from aborted samples is the technique of preference for definitive diagnosis (Li *et al*., 2015). However, because both agents present zoonotic risks, culture could not be the method of choice in many laboratories which lack high biosafety facilities. As mentioned in previous chapters, in recent years, different PCR approaches have been developed and widely implemented with successful results. However, in abortion assessments, PCRs enable pathogen detection but give no indication of their implication in causing the abortion. Therefore, when *C. burnetii and C. abortus* are investigated in cases of abortion, the diagnosis should not rely merely on positive PCR outcomes. Other ancillary tests, coupled with the evaluation of histological changes, are needed to arrive at a diagnosis.

5.1.3.1 Fluorescence in-situ hybridisation

Among the tests to demonstrate bacteria within the tissue samples, there is the fluorescent *in situ* hybridisation (FISH), whose usefulness has been broadly demonstrated worldwide. FISH is a molecular diagnostic tool that complements the investigation of infectious diseases caused by an intracellular and fastidious microorganism (Prudent & Raoult, 2019). The utilisation of FISH using rRNA-targeted oligonucleotide probes has developed into a diagnostic alternative for the investigation of difficult-to-cultivate bacteria. This approach is founded on a reaction of hybridisation between specific fluorescent labelled probes and the complementary sequences of RNA or DNA targets while maintaining cell integrity. Four steps are usually involved, sample fixation, sample permeabilisation, probes hybridisation, and finally, detection and analysis by microscopic visualisation (Prudent & Raoult, 2019). The nucleotide sequences can be studied with no alteration of the cell morphology (Moter & Göbel,

2000). FISH has the advantage of permitting the direct visualisation of the bacterium, and it might be feasible to roughly estimate the number of bacteria associated with the tissular lesion.

There are no published FISH-based studies for the investigations of *Chlamydia* spp. in clinical samples collected from aborted ruminants. However, the applicability of this tool for C*hlamydia* spp. diagnostic research, as well as for the assessment of probe sets for the differentiation of *Chlamydia* species, has been investigated supporting its suitability for clinical diagnosis (Poppert *et al.* 2002).

By contrast, FISH has been frequently used for *C. burnetii* investigation in human and veterinary medicine. The first FISH attempts to identify *C. burnetii* in clinical samples were made on animal samples. The first report of *C. burnetii* detection in placentas of ruminants using FISH was done in 2007 (Jensen *et al.*, 2007). In this study, formalin-fixed paraffin-embedded aborted placentas samples were assessed for the detection of *C. burnetii* by a FISH hybridisation using rRNA-targeted oligonucleotide probes. From a total of 90 ruminant abortions submitted for routine laboratory assessment, only one case was positive (Jensen *et al*., 2007). The same investigation provided evidence supporting FISH as a feasible technique for the identification of *C. burnetii* in tissue samples, providing results completely similar to those obtained by the IHC technique (Jensen *et al.,* 2007).

Reports of *C. burnetii* investigation by FISH of both bovine and human heart valves sections are available (Kumpf *et al.*, 2016; Agerholm *et al.*, 2016; Aistleitner *et al.*, 2018). Additionally, patients with B-cell non-Hodgkin lymphoma had been examined for the detection of *C. burnetii* using this method (Melenotte *et al*., 2016; van Roeden *et al*., 2018). Lymphoproliferative alterations have been found in patients with Q fever (Eldin *et al.,* 2017), and evidence supporting *C. burnetii* as a possible lymphoma cofactor is available (Melenotte *et al.,* 2016). For example, a case report showed positive-*C. burnetii* FISH evidence from a retroperitoneal lymphoma tissue sample collected from a 58-year-old male patient with vascular infection and lymphoma (van Roeden *et al*., 2018).

The main strength of FISH is the direct visualisation of the rRNA target at the cell level. Both PCR tests and FISH allow the molecular detection of *C. burnetii*; however, the latter allows the visualisation of the agent within the histological context, showing morphology, abundance and spatial distribution.

Additionally, PCR is unable to differentiate viable bacteria and non-viable cells or free nucleic acid. In 16S rRNA-target FISH testing, the fluorescence signal generated provides information about bacteria replicative activity. For example, a positive result obtained in FISH furnishes evidence that bacterial forms found are viable microorganisms (Atieh *et al.*, 2013). Considering that each replicating and metabolically active cell has a high copy number of 16S rRNA, this gene provides enough targets to detect even single bacterial cells (Prudent *et al.*, 2018). Despite the fact that *C. burnetii* is a cultivable microorganism, pursuing bacterial culture is challenging as it requires BSL-III conditions that are not available in many veterinary laboratories. In those laboratories with conditions not suitable for *C.*

burnetii culture, a cultivation-independent method like FISH appears as a great alternative for agent detection.

RNA-FISH employs a fluorophore-conjugated nucleotide probe to identify target RNAs within the fixed cells. The fluorophore acts as a marker to visualise the hybridisation between the synthesised nucleotide sequence and the target sequence. The use of different fluorescent dyes permits the simultaneous detection of diverse bacterial species. The inclusion of the 16S rRNA-targeted bacterial probe (EUB338), deemed as a general hybridisation probe, the entire bacterial population in the samples is revealed (Amann *et al*., 1990ab). This probe permits the recognition of a wide range of bacteria and is commonly included as a positive control in all analysis using FISH. Typically, the non-specific binding is excluded by the use of the negative control probe NON-EUB338, the complementary sequence of EUB338 (Amann *et al*., 1990ab).

Molecular diagnosis is built on the study and detection of nucleic acids, either DNA or RNA. FISH is founded on the hybridisation reaction between a particular fluorescent labelled probe and complementary target sequences (RNA or DNA). This tool is utilised not only for the detection and localisation of nucleotide sequences but also for gene expression studies. FISH procedures include four fundamental stages. First, the sample being analysed (microorganisms, smear, or tissue specimen) is fixed to the glass slide. Then the sample is subject to a pre-treatment and permeabilisation; this process gives the probes access to the nucleic acids. The following step is the hybridisation of the labelled probes to the target (DNA or RNA). The final step is washing to remove unbound probes, mounting and imagining by either flow cytometry or microscopy (Moter & Göbel, 2000; Prudent & Raoult, 2019).

The fluorescent oligonucleotide probes are constituted by two elements, a sequence of nucleotides that is complementary to the target sequence that is under study, and a fluorescent marker that reveals hybridisation. The interaction between the probe and the target sequence is founded on the molecular hybridisation reaction that occurs between two complementary sequences (Prudent & Raoult, 2019). The oligonucleotide probes can be made by DNA or RNA sequences to obtain RNA/RNA, DNA/DNA, or RNA/DNA targets. Generally, the oligonucleotide probes are 15–30 bases long, single-strand and attached to a single dye (Prudent & Raoult, 2019). Ribosomal RNA (rRNA)-targeted probes have become more and more common. Each prokaryotic ribosome contains one 16S rRNA of ~1600 nucleotides in the small subunit. As each cell has several ribosomes, these targets are naturally massive, amplified, and raise the intensity of the fluorescence signal (Amann & Fuchs, 2008). Therefore, the 16S rRNA gene is an accurate and useful choice for indicating the majority of microorganisms. The visualisation, even of a single bacterial cell, is facilitated by enough target available due to the elevated number of 16S rRNA in each single metabolically active cell able to replicate. The RNA as a single-stranded molecule can be easily hybridised to the probe without requiring an initial denaturation step.

Post-hybridisation washes prevent non-specific binding of FISH probes and decrease the level of background noise (Barakat & Gribnau, 2014).

Using the FISH technique enables the location of the pathogen within the cell as this method preserves cell integrity (Moter & Göbel, 2000; Prudent *et al.*, 2018). A noteworthy advantage of the FISH approach is the possibility of detecting more than one type of bacteria in the samples. The combined use of the Bacteria-specific probe (EUB338) with a species-or-genus-specific probe permits the visualisation of co-infections. The potential co-existence of different species in the presence of *C. burnetii* or *Chlamydia* spp. would open an exciting field of investigation. The interactions between bacteria during co-infection involving microorganisms with similar interactions with host and pathogenic mechanisms are worth further analysis.

As mentioned above, due to the high copy number of ribosomal RNA molecules, the sensitivity of the *in-situ* detection of rRNA seems not to be a cause of concern. However, some regions of the ribosomal RNA have constrained access due to strong secondary structures or because of the presence of complex structures with ribosomal proteins. This embodies a limitation because specific probes generate sparse hybridisation signals (Bridger & Volpi, 2010). As rRNA-targeted FISH is limited to identifying bacteria involved in active growth with active protein synthetization, a negative result can be obtained at low infection levels or when metabolically inactive bacteria predominate in the sample (Moter & Göbel, 2000; Kumpf *et al*., 2016). As cellular rRNA content of the targeted microorganisms influences the results obtained by rRNA-targeted FISH, low rRNA would be the most frequently recurrent limitation of the technique. Reduced rRNA content generates dim and weak fluorescence signal intensity, which is frequently not detectable against background fluorescence, leading to unsuccessful detection. Some authors claim that FISH needs a minimum of target molecules per cell (400 ribosomal), only identifying intact and viable bacteria at tissue fixation (Hoshino *et al*., 2008; Aistleitner *et al*., 2018). High intensity of the signal brightness indicates a high level of rRNA, evidencing physiological cellular activity (Kemp *et al*., 1993; Wallner *et al.,* 1993). In this scenario, the metabolic cellular rate would be relevant in order to detect the pathogen. Another weakness of FISH relates to its poor standardisation, where by the interpretation of results will be significantly affected by the technical experience of the reader (Prudent *et al*., 2018).

Contrasted with other molecular approaches, the use of primary material omits the need for DNA extraction steps, thus reducing the time of analysis (Wellinghausen *et al*., 2006). FISH represents a complementary technique providing ancillary information fundamentally about pathogen localisation in a relatively short time (Prudent *et al*., 2018).

As mentioned above the mere presence of nucleic acids material of either, *C. burnetii* or *Chlamydia* spp., in aborted samples is not enough to confirm the disease or to deem these agents as responsible for

the abortion. One substantial limitation of the PCR technique is the lack of distinction between non-viable and viable microorganism. The utilisation of FISH overcomes this limitation, as it is based on rRNA detection. FISH yields positive results only when active bacterial cells are present.

Curiously, and even though *C. burnetii* has a strong tropism to the placenta; coxiellosis rarely produces inflammation in placentas from cows (Hansen *et al.*, 2011). This may explain why coxiellosis is only considered a sporadic agent of bovine abortion (Bildfell *et al*., 2000). *Coxiella burnetii* may be located in the placenta after infection but remain quiescent, producing a condition which is not clinically apparent. It must be noted that positive results obtained by these molecular techniques do not strictly indicate that *C burnetii* infection was the cause of the abortion, so results need to be interpreted with caution. Positive result obtained by PCR coupled with a negative FISH result may indicate inactive forms of *C. burnetii*, which may explain the absence of inflammation in these placentas. The visualisation of bacteria-loaded macrophages indicates intracellular multiplication. The presence of inactive bacteria, thus a negative FISH result, may explain the absence of inflammation reported in the case of *C. burnetii* abortion in cattle. This finding would, to some extent, explain why Q fever in people is rarely linked with cattle. The potential discrepancy between FISH and IHC findings could suggest the detection of inactive intracellular bacteria by FISH, which again, is in line with the lack of inflammation. *Coxiella burnetii* rarely causes abortion in cattle (van Moll *et al*., 1993; Bildfell *et al*., 2000; Jensen *et al*., 2007). If *C. burnetii* is generally inactive in parturient bovine placentae, then this may be an essential part of the reason why outbreaks of Q fever in humans are infrequently associated with aborted cattle, and most cases are linked to small ruminants.

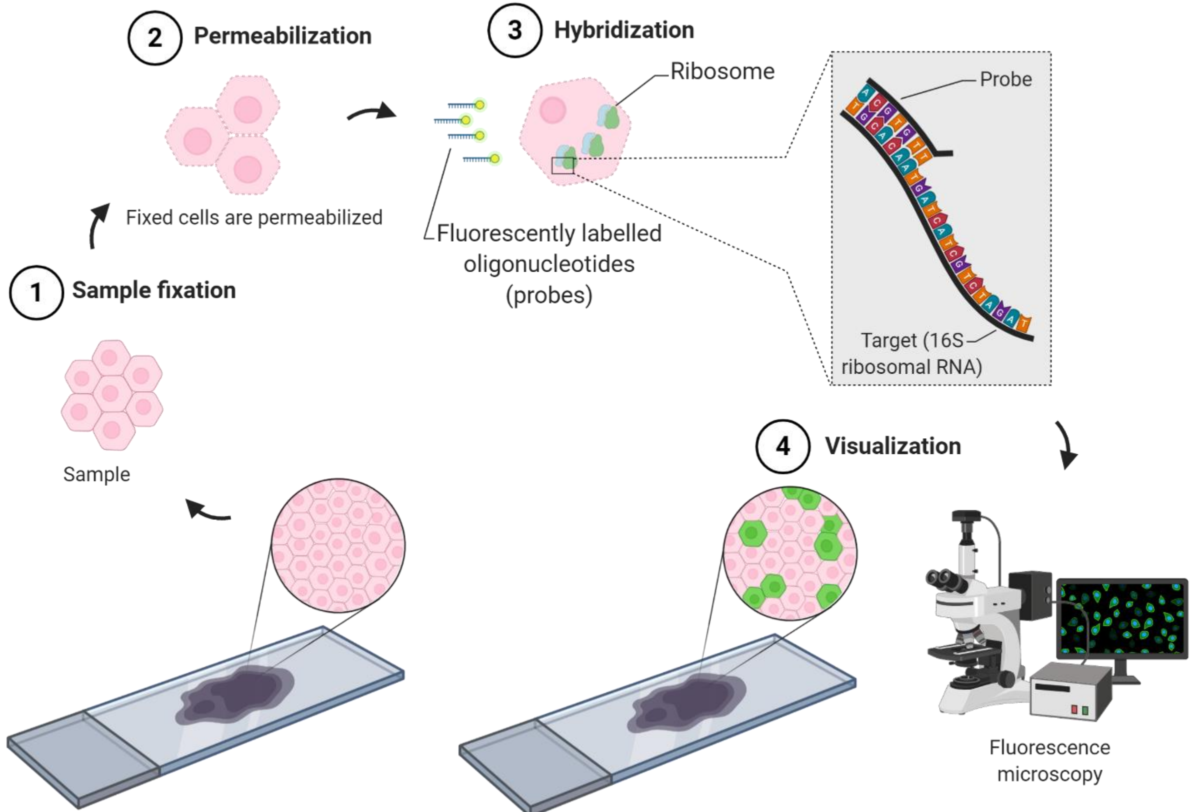


Figure 5.2: Essential steps of fluorescence *in situ* hybridisation (FISH). Biological samples are first fixed to stabilise the cells and permeabilise the cell membranes. Then the labelled oligonucleotide probe is included and permitted to hybridise to its intracellular targets. Then the sample is ready for analysis, commonly by epifluorescence microscopy (Adapted from Amann & Fuchs, 2008).

5.1.3.2 Immunohistochemistry

Immunohistochemistry (IHC) is used to visualise the localisation of cellular components by exploiting the natural ability of antibody proteins to bind to specific molecular structures (epitopes, antigens), usually a short sequence of amino acids from a peptide or protein (Gibbins, 2012; Coons, 2017). Antibodies labelled with a fluorescent tag retain specificity for their antigen (Coons *et al*., 1941). This phenomenon is the foundation of this technique, as tissue samples incubated with fluorescently labelled antibodies and examined for signal emission under fluorescence microscopy, reveal the localisation of antibodies. Hence, the tags permit the direct detection and location of antigens within the tissue samples. The antibodies, also known as immunoglobulins, are polypeptide structures that are implicated in humoral immune reaction. Antibodies detect and bind to the antigen with high specificity. Usually, the antigen is a cluster of protein consisting of five or six amino acid residues, known as the epitope. Antigens can contain several identical epitopes (homopolymeric) or various distinct epitopes (heteropolymeric) (Lipman *et al*., 2005). The shape of antibodies resembles the letter “Y” with polypeptide heterotetramers comprised of two “heavy” chains and two “light” chains. Disulfide

linkages maintain this quaternary structure. They can be further structurally divided into the constant (fixed) region and the antigen-binding domain. The latter contains a variable region and a hypervariable region. The hypervariable region of the antigen-binding domain of the antibody defines the epitope‘s specificity (Janeway, 2001).

Both mono- and polyclonal antibodies are commercially available. While monoclonal antibodies work against a single epitope of a single antigen, polyclonal antibodies are targeted against multiple epitopes of one antigen (Coons, 2017). The ability of a molecule to be recognised by its antigen (antigenicity) is of central importance. Antigens must remain structurally intact to enable antibodies to recognise them. If any of the antigen properties (shape, size, amino acid sequence or charge) is altered, despite the antigen being present, the staining could fail, causing false-negative results (Khoury *et al*., 2009).

When IHC is used on preserved tissue samples, (mostly fixed tissue samples), a diminished antigenicity of epitopes could occur. This phenomenon is likely to occur due to modifications of its structure within the tissue during the fixation process. Antibodies identify their target molecules based upon unique characteristics of the epitope (shape, size, and charge profile) (Janeway *et al*., 2001). If the alteration compromises the structure of the intracellular molecular target, then the immunoglobulin will no longer be capable of detecting it and subsequently binding to it (Coons, 2017). In this context, the implementation of antigen retrieval methods appears essential to moderate antigenicity loss. Down the years, the direct detection of the presence of antigens in tissue samples has benefited from improvements. Facing relatively low levels of detectable staining when using tags attached directly to antibodies, researchers developed the technique of amplifying the intensity of staining by the addition of a step in the process. This consists of the incorporation of an unlabelled primary antibody, as a substitute for hybridising a labelled antibody to the target antigen. Then, a labelled secondary antibody, specific against the primary unlabelled antibody, is incorporated into the sample (Coons, 2017). Amplification of the signal is achieved by adding this second layer of staining because multiple secondary antibodies can now attach to every single primary antibody.

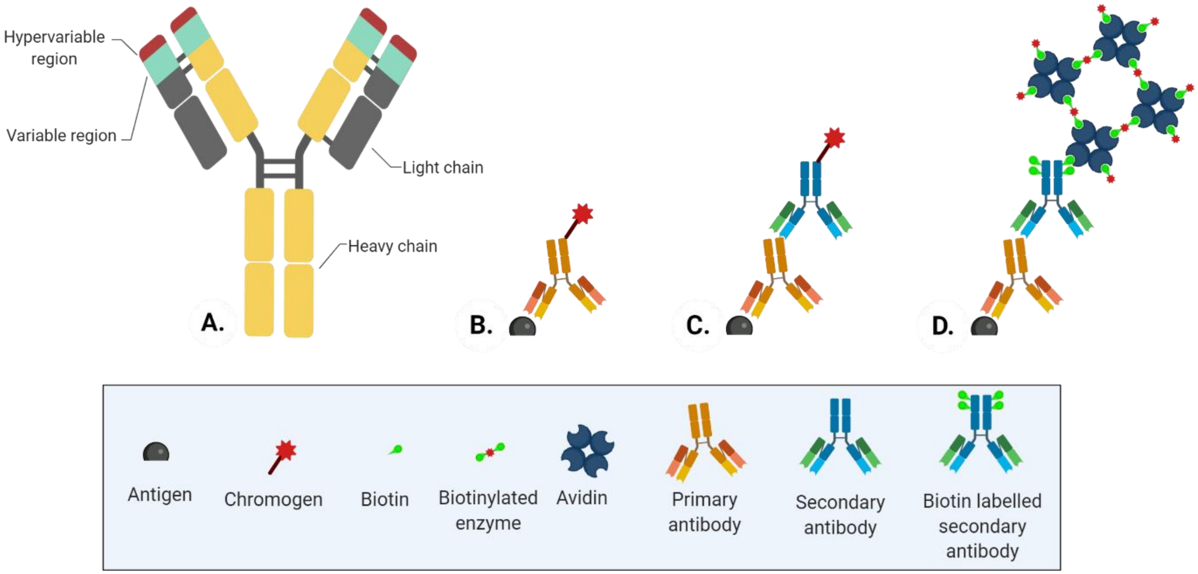


Figure 5.3: General antibody structure. Direct, indirect, and avidin-biotin-peroxidase complex immunohistochemistry staining. A. General antibody structure. An antibody protein is a heterotetramer, comprising two heavy chains and two light chains. Disulfide bonds link the four polypeptides. The variable and hypervariable regions provide the specificity against a specific antigen. Among the various detection methods in immunohistochemical staining: B. Direct method: the label is attached to the immunoglobulin that targets the epitope being studied; C. Indirect (sandwich) method: permits the utilisation of the same secondary immunoglobulin with numerous different primary immunoglobulins. (Adapted from Coons, 2017). D. Avidin-biotin-peroxidase complex immunohistochemistry staining: the biotin is attached to the primary antibody and avidin is linked to peroxidase and presents strong biotin affinity. Enhanced sensitivity is seen due to the numerous biotin molecules linked to the primary antibody.

Immunohistochemistry staining is an approach used frequently for the investigation of *C. burnetii* and *Chlamydia* spp. An avidin-biotin-peroxidase complex staining technique was the first method created for *C. burnetii* investigation in the formalin-fixed placenta; thus, no need for fresh samples for diagnosis (Dilbeck & McElwain, 1994). The first identification of *C. burnetii* as an agent causing abortion on a dairy goat farm occurred in 2005 and diagnosis was done by the IHC exposure of *C. burnetii* in tissue sections (Wouda & Dercksen, 2007). For *C. burnetii* infections, IHC has been utilised more on maternal samples such placentas than on fetal tissues (Bildfell *et al*. 2000; Sanchez *et al*. 2006; Jensen *et al*. 2007; Muskens *et al*. 2012). Muskens *et al*. (2012) has supported the concept that IHC has lower sensitivity than PCR testing, which seems especially relevant when there is some degree of autolysis as frequently occurs in abortion material. Despite the high sensitivity shown by PCR, its sole use is not enough for a

confirmatory diagnosis, and this tool should be combined with another method seeking confirmation and accurate diagnosis of active infection with *C. burnetii*.

Immunohistochemistry can also be utilised to identify chlamydial antigens (for example LPS or MOMP) in tissue sections. A direct immunoperoxidase method was first established for investigating *C. abortus* in formalin-fixed tissues (Finlayson *et al*., 1985). *Chlamydial* LPS was detected within placental lesions, smears and lymph nodes in ewes and goats after abortion by using formalin-fixed, paraffin-embedded tissues (Buxton *et al*., 1996; Buxton *et al*., 2002). Lately, *C. pecorum* has been studied by IHC in placenta and intestine samples from caprine abortions (Giannitti *et al*., 2016), and in joint samples from ovine carcases with clinical arthritis (Lloyd *et al*., 2017).

5.1.3.3 Detection of bacteria is not a synonym of disease

The occurrence of both *C. abortus* and *C. burnetii* DNA in genital organs of healthy non-aborting small ruminants has been verified (Berri *et al*., 2001; Livingstone *et al*., 2009; Jones *et al*., 2010). Identifying these bacteria as responsible for abortions is difficult, especially considering that these organisms may be part of the normal resident microflora or may be present without leading to disease. A simple positive result obtained by PCR can lead to over interpretation and be misleading when an evaluation is done using this tool alone, leading to misdiagnosis. The presence of nucleic acids of the agent, indirectly interpreted as the presence of the bacterium does not reliably mean occurrence of the disease. Positive PCR results need to be interpreted in light of other assessments.

The submission of the placenta to evaluate possible active infection by these bacteria seems to be fundamental. Although farmers are encouraged to send placenta samples to diagnostic laboratories, frequently placentas are lost to scavengers. In such cases, fetal samples, like stomach content and lungs, can be utilised as an alternative (Hazlett *et al*., 2013).

Despite being phylogenetically unrelated species (Woese, 1987), *C. burnetii* and *Chlamydia* spp. present some similarities when interacting with a host and also have similar pathogenesis (Lukacova, 1996). Likewise, clinical characteristics of the abortions produced by these bacteria are often similar. Local availability of a single-assay that simultaneously detects and distinguishes these bacteria by the utilisation of a single sample would be advantageous. The evaluation of a clinical sample by using a single reaction may reduce not only the time it takes to obtain several results as in other methods but also the cost of biochemical reagents, materials and labour.

5.1.4 *Objectives*

The objectives of this chapter were to evaluate and optimise a m-PCR test for the simultaneous study of *C. burnetii*, *C. abortus* and *C. pecorum* from aborted bovine placentas. This study is the first attempt to locally provide insight into the presence of co-infections of these bacteria in abortions. Additionally, this chapter aims to evaluate the utilisation of fluorescence *in-situ* hybridisation as an ancillary molecular approach to investigate active *C. burnetii* infection within placenta samples.

5.2 Materials and Methods

5.2.1 Study Design and Sampling Approach

Placentas samples from aborted cattle submitted over three years (2017-2019) to the Plataforma de Salud Animal from the Instituto Nacional de Investigacíón Agropecuaria (INIA), Uruguay, were examined. All samples came from commercial dairy farms. This investigation constituted a case series study with no follow-up evaluation or interventions. The aborted materials were gathered by a convenience sampling method where samples have been non-randomly selected. The submission of aborted material to the laboratory was voluntary. Thus, the participation in this project depended on the farmers' willingness to take part. With this being the case, several efforts were made to spread the word among veterinary practitioners and farmers about the work that was being done, and to encourage them to be part of the project and explain the importance of their involvement. The study was publicised at field days, technical talks and meetings, and also information on how to participate was made available in each of the regional veterinary centres where veterinarians gather weekly or fortnightly.

Aborted bovine material was delivered to the laboratory by commercial delivery companies or directly by the farmers or vets. The biological material was frequently, but not always, accompanied by epidemiological information such as the age of the pregnancy, age of cow, utilisation or not of reproductive vaccines, and type of breeding (bull, artificial insemination (IA), IA and bull).

Samples were first macroscopically assessed for gross alterations. A placenta sample was stored in a sterile microtube at -20°C until DNA extraction. For the purpose of histological analysis, another placenta sample was formalin-fixed in 10% buffered formalin (pH=7.2) by immersion over 48 h before tissue block preparation.

Figure 5.4: Fetus and placenta from a cattle abortion.

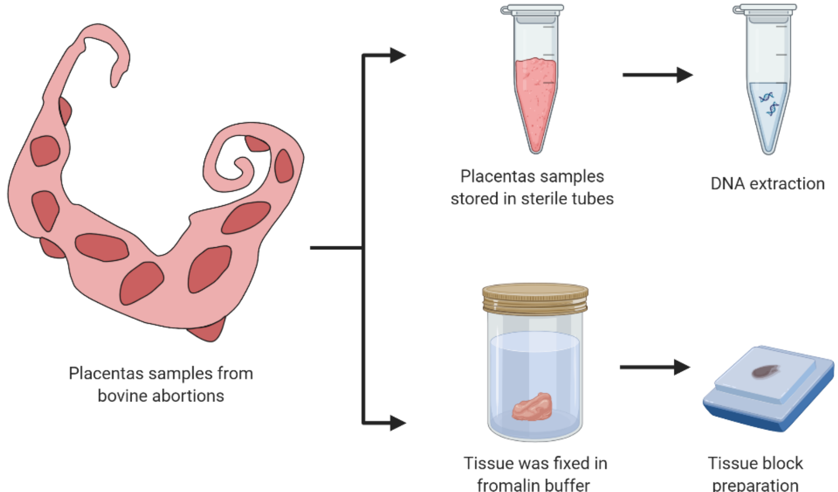


Figure 5.5: Workflow of placenta samples from bovine abortions.

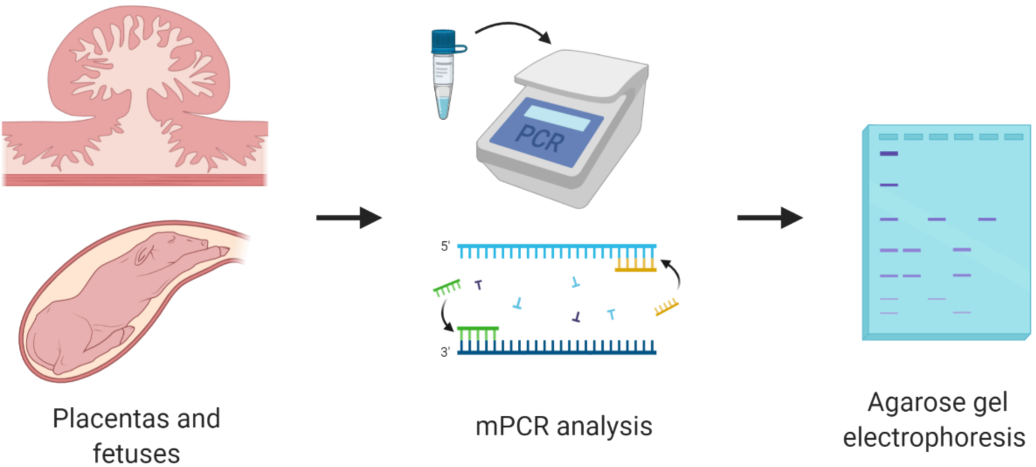


Figure 5.6: Bovine placentas analysis workflow.

5.2.2 DNA extraction from placenta samples

High-quality DNA was manually extracted from placenta samples using the QIAgen DNeasy Blood & Tissue DNA extraction kit (GmbH, Hilden, Germany), following the manufacturer's guidance. This kit utilised the column centrifugation system of DNA extraction and comprised of the following steps:

1. 25 mg of tissue sample were cup up into small pieces and placed in a 1.5 ml microtube.

2. 180µl of ATL were added to the microtube.

3. 20µl of Proteinase K (at 600 mAU/ml solution) was pipetted into the microtube.

4. This was incubated at 56 °C until the tissue was completely lysed. Samples were occasionally vortexed to disperse the sample.

5. 200 µl Buffer AL was included to the sample. Samples were mixed thoroughly by vortexing.

6. 200 µl ethanol (96–100%) were added to the sample and mixed again thoroughly by vortexing.

7. The mixture described in the previous step was pipetted into the DNeasy Mini spin column placed in a 2 ml collection tube.

8. Centrifuged at 6000 x g (8000 rpm) for 1 min. Flow-through and collection tube discarded.

9. The DNeasy Mini spin column was placed in a new 2 ml collection tube. 500µl buffer AW1 was added.

10. Centrifuged for 1 min at 6000 x g (8000 rpm). Flow-through and collection tube discarded.

11. The DNeasy Mini spin column was placed in a new 2 ml collection tube. 500µl buffer AW2 was added.

12. Centrifuged at 20,000g for 3 minutes. Flow-through and collection tube discarded.

13. The DNeasy Mini spin column was placed in a sterile 1.5ml microtube. The microtube tube was identified with a label.

13. 200 µl Buffer AE were pipetted directly onto the DNeasy membrane and incubated at room temperature for 1 min.

14 Centrifuged for 1 min at 6000 x g (8000 rpm) to complete elution. DNeasy Mini spin column discarded.

Centrifugation are done at room temperature (15–25°C). The extracted DNA was quantitatively and qualitatively analysed by spectrophotometry using a Nanodrop (ND1000, NanoDrop Technologies, Inc, USA) before downstream utilisation. After this, DNA samples were immediately stored at -20°C or -80°C, depending on the expected moment of further utilisation.

5.2.3 Formalin fixation and paraffin embedding

Placenta samples were routinely processed for histopathological examination by formalin fixation and paraffin embedding (FFPE). For this, a standard protocol was followed (Fischer *et al*., 2008).

1. Samples were fixed in buffer 10% formalin for 48 h

2. Tissue dehydration was done in ascending grades of alcohol

3. 50% Ethanol for 3-4 hours at room temperature.

3. 70% Ethanol for 3-4 hours at room temperature.

6. 80% Ethanol for ½ hour at room temperature.

7. 95% Ethanol for ½ hour at room temperature.

8. 100% Ethanol for 1 hour at room temperature.

10. Then samples were cleared by xylene for 1-2 hours at room temperature.

12. Samples were paraffin-embedded. Paraffin blocks were made by filling a tissue mold using melted paraffin (65°C).

13. Blocks completely solidified as they cool at room temperature.

Blocks were preserved to be sectioned later using a standard microtome.

5.2.4 End-point multiplex PCR

Coxiella burnetii, *C. abortus* and *C. pecorum* were investigated in a multiplex PCR for their simultaneous differential identification using clinical samples, following a protocol proposed by Berri *et al*., 2009 with minor adaptations. This assay used the following target genes, *IS1111a*, pmp 90/91 and CPC, for *C. burnetii* and *C. abortus, C. pecorum,* respectively. The assay targeted three specific 687-bp, 526-bp, and 821-bp long fragments. The PCR was done in 25 µL final volume reactions, with a concentration of 0.8 µM of each primer (Trans-1: 5'-TATGTATCCACCGTAGCCAGT-3', Trans-2: 5'-CCCAACAACACCTCCTTATTC-3'; pmpF: 5'-CTCACCATTGTCTCAGGTGGA-3', pmpR821: 5'-ACCGTAATGGGTAGGAGGGGT-3'; Cpc-F: 5'-TTCGACTTCGCTTCTTACGC-3', Cpc-R: 5'-TGAAGACCGAGCAAACCACC-3'), 1.5 U of Taq polymerase (New England Biolabs®, Ispwich, MA), 1× PCR buffer (New England Biolabs®, Ispwich, MA), 3 mM of $MgCl_2$, 0.2 mM of dNTPs, and 2 µL of the template. Amplification comprised a denaturation for 10 min at 94°C, followed by 35 cycles of denaturation for 30 s at 94°C, annealing for 1 min at 61°C, and extension for 1 min at 72°C, with a

final extension step for 10 min at 72°C. The multiplex PCR was run in a ProFlex™ PCR System (Applied Biosystems, Foster City, CA, USA). The *C. burnetii* Nine Mile phase II (RSA 493) strain, *C. abortus* reference strain S26/3, and *C. pecorum* reference strain P787 were used as positive controls. A non-template control (NTC) was included in each run containing ultrapure distilled water to rule out contamination. Visualisation of the multiplex PCR products was done by gel electrophoresis in 1.2% agarose gel stained with Good View® dye, for 40 min to 80 V and visualised by ultraviolet transillumination using a Bio-Rad GelDoc EZ imager (Bio-Rad Laboratories GmbH-Munich, Germany). Samples were classified as positive when a band with the expected size was obtained.

5.2.5 Quantitative PCR (qPCR)

As secondary analysis, those samples that revealed positive results in end-point multiplex PCR were subjected to bacterial burden quantification. For this purpose, probe-based quantitative PCRs were carried out for the absolute quantification of the investigated bacteria.

5.2.5.1 Coxiella burnetii

The protocol used was adapted from the method first published by Panning *et al.* (2008) and modified by Di Domenico *et al.* (2014). This qPCR included primers targeting the *IS1111* transposon of *C. burnetii* and generated an amplification fragment of 86-bp. The primer-pairs and probe sequences used were as follows: forward primer CburF, 5′- GAT AGC CCG ATA AGC ATC AAC -3′, reverse primer CburR, 5′- GCA TTC GTA TAT CCG GCA TC -3′ (Panning *et al*., 2008), and probe FAM-TGC ATA ATT CAT CAA GGC ACC AAT GGT -TAMRA (Di Domenico *et al*., 2014). The assay was performed using the QuantStudio 5 Real-Time PCR System (Life Technologies Inc.) in a final reaction volume of 10 µl. Each reaction included: 5 µl of 2X SensiFAST™ probe No-ROX Kit (Bioline), 0.6 µl of forward primer, 0.6 µl of reverse primer and 0.23 µl of the probe for *C. burnetii* (600nM and 220nM final concentration, respectively), 1 µl of DNA template and 2.57 µl of free-nuclease water. The thermal profile used was as follows: PCR activation at 95 °C-5 min, 40 cycles comprising denaturation at 95 °C-10 s, annealing at 60 °C-30 s, and extension at 72 °C-30 s. The testing for each placenta was run in triplicate using a 96-well plate. A standard curve was constructed by ten-fold serial dilutions of a plasmid with *C. burnetii IS1111* as a template. Quantification was achieved by the calculation of Ct values against the standard curve. More details about the clonation protocol and standard curve generation have been described in **Chapter Four**. Each run included a no-template sample (NTC) that contained ultrapure DNAse/RNAse-free distilled water as a template. Samples exhibiting an exponential amplification curve up to cycle 39 were classified as positive.

5.2.5.2 *Family Chlamydiaceae*

Placenta samples that showed positive results for *Chlamydia* species in the end-point multiplex PCR were quantified on a QuantStudio 5 Real-Time PCR System (Life Technologies Inc.) instrument utilising a 23S-rRNA gene-based *Chlamydiaceae* family-specific qPCR which has been previously published (Ehricht *et al*., 2006). For this purpose, the following primers and probe were employed: forward primer Ch23S-F, 5′-CTGAAACCAGTAGCTTATAAGCGGT-3′, reverse primer Ch23S-R, 5′- ACCTCGCCGTTTAACTTAACTCC-3′, probe Ch23S-p, FAM-CTCATCATGCAAAAGGCACGCCG -TAMRA. These specific primers were designed to amplify a 111-bp product, particularly for family members of *Chlamydiaceae*.

The 25 µl-reactions contained 12.5 µl of 2X SensiFAST^{TM} probe No-ROX Kit (Bioline), 0.8 µl of primers and probe (500nM final concentration), 2 µl DNA template, and 8.1 µl of free-nuclease water. The cycling conditions comprised an initial denaturation for 10 min at 95°C, followed by 50 cycles of 15 s at 95°C and 30 s at 60°C. The total cycle lasted one h and 37 min. Each sample was run in triplicate. A standard curve was generated by serial dilutions of a plasmid with *OmpA C. abortus* as a template. Serial ten-fold dilutions of the plasmid were used in triplicate as the template in qPCR reactions to produce a standard curve. Absolute quantification was performed using the standard curve. Each PCR run included an NTC that contained ultrapure distilled water as a template.

5.2.6 Fluorescent *in situ* hybridisation (FISH) for *Coxiella burnetii* detection

This protocol was planned to be used for FISH evaluation on aborted placentas at the diagnostics laboratory of the Bristol Veterinary School at Langford. Unfortunately, due to the COVID-19 lockdown, this assessment was not conducted. However, as this was a relevant activity in the project, even the placenta samples initially processed in Uruguay had been transported to the UK. The laboratory protocol for FISH analysis of placentas will be described.

Three-micron-thick sections were obtained from each block containing placenta tissue using a standard microtome. These sections were placed and mounted on silane-coated slides. The silane is used to increase tissue adherence to the slide, preventing tearing and adverse effects throughout the steps of the process. First, the samples were deparaffinised in xylene, dehydrated in a graded series with decreasing concentrations of ethanol (100%, 90% and 70%), rinsed with distilled water and air-dried. The methodology published by Jensen *et al*., 2007 was followed with some modifications. The samples were first evaluated in the absence of probes to ruled out the tissue background and autofluorescence signals. The probes were selected from validated protocols published elsewhere, consisting of commercially synthesised oligonucleotide probes 5' end-labelled. The approach involved the

simultaneous application of species-specific probes and general bacterial probes. Two *C. burnetii*-specific probes labelled with the fluorochrome Cy3 (indocarbocyanine) were used (CB-0443, 5′-CTTGAGAAT TTCTTCCCC-3′ and CB-189, 5′- CCGAAGATCCCCCGCTTTGC-3′). The general bacterial probe EUB338, 5'GCTGCCTCCCGTAGGAGT-3' labelled with fluorescein isothiocyanate (FITC), which is complementary to a portion of the 16S rRNA gene conserved in the domain *Bacteria,* was also utilised (Amann *et al*., 1990ab). The samples were parallel tested for non-specific binding using the NON-EUB338, 5′-CGACGGAGGGCATCCTCA-3′, to exclude nonsense hybridisation and detect false positive results (Wallner *et al.,* 1993; Kaittanis *et al*., 2012). Samples were hybridised for 16 h with 40 ml a hybridisation buffer containing a mixture of the probes, each probe at a concentration of 200 ng (0.9 M NaCl, 0.1% sodium dodecyl sulphate (SDS), 100 mM Tris [pH 7.2]). No permeabilisation steps were used. Permeabilisation is intended to permit probe uptake into the sample. However, permeabilisation approaches are generally not required in case of short-fragment probes targeting rRNA in Gram-negative bacteria (Frickmann *et al*., 2017). Additionally, some chemicals, for instance, xylene or formaldehyde, typically employed during dehydration or fixation processes, have a weak detergent role and could lead to some degree of tissue permeabilisation (Frickmann *et al*., 2017). Incubation should be done in a dark, humid chamber at 45 ºC. Washing was done with 100 ml of prewarmed hybridisation buffer (at 45 ºC) for 15 min, followed by an additional washing step during 15 min with 100 ml of prewarmed washing solution (100 mM Tris [pH 7.2], 0.9 M NaCl). Samples were rinsed with distilled water and dried in air, always under dark conditions. Finally, samples were mounted using the mounting medium Vectashield for qualitative digital imaging on epifluorescence microscopy. Hybridised sections were read by an epifluorescence microscope, where the FITC and Cy3 signals were visualised at x 1000 magnification.

5.2.7 Immunohistochemistry IHC for *C. burnetii*, *C. abortus* and *C. pecorum*

Facing the difficulty of the COVID-19 lockdown, and because the FISH assessment was eventually not conducted the researcher decided that doing IHC would be conducted as a way of investigating the presence of the pathogens within the sample. After the PCR‘s evaluations, selected positive samples for various of the agents were subjected to IHC. Samples were sent for IHC evaluation abroad, as the required antibodies were not locally available. Following appears a brief explanation of the protocols.

Three-micron-thick sections cut from the formalin-fixed paraffin block placental samples were mounted on glass slides. Samples were deparaffinised in a xylene series and rehydrated over a declining ethanol series. Serial sections of placenta samples were analysed by IHC for the investigation of *Chlamydia* spp. and *C. burnetii* antigens, as previously described (Dilbeck & McElwain, 1994; Giannitti *et al*., 2016). The retrieval of antigens was done in a decloaking chamber. After the endogenous peroxidase

activity had been quenched by treating the samples with 3% hydrogen peroxide (H_2O_2) during a 5 min incubation, the primary antibody was added. Mouse anti-*Coxiella* and anti-*Chlamydia* monoclonal antibodies were used for IHC as primary antibodies (AB-COX-MAB; US Department of Defense, Critical Reagents Program; anti-Chlamydia lipopolysaccharide antibody; Virostat, Westbrook, ME) (Dilbeck & McElwain, 1994; Giannitti *et al*., 2016). Antigen retrieval was undertaken by incubation in acidulated pepsin for 15 min. Bound antibody was visualised after sequential incubations with anti-mouse polymer labelled with horseradish peroxidase (Biocare, Pacheco, CA), followed by an incubation with 3-amino-9-ethylcarbazol (AEC) (Thermo Scientific, Fremont, CA) chromogen substrate. For the exposure of *C. burnetii* antigen by IHC, the unmasking of antigens was not required. Placenta samples from goats naturally infected by *Chlamydia* spp. and *C. burnetii* were utilised as positive controls. Sections of bovine placentas in which the primary antiserum was substituted by non-immune serum, were utilized as a negative control.

5.2.8 Data analysis

Since this study followed the epidemiological design of a case series sampling, data analysis included descriptive statistics instead of including analytical epidemiology. The frequency of DNA detection of the agents under study is reported in terms of their number and percentage in relation to the total number of cases assessed.

5.3 Results

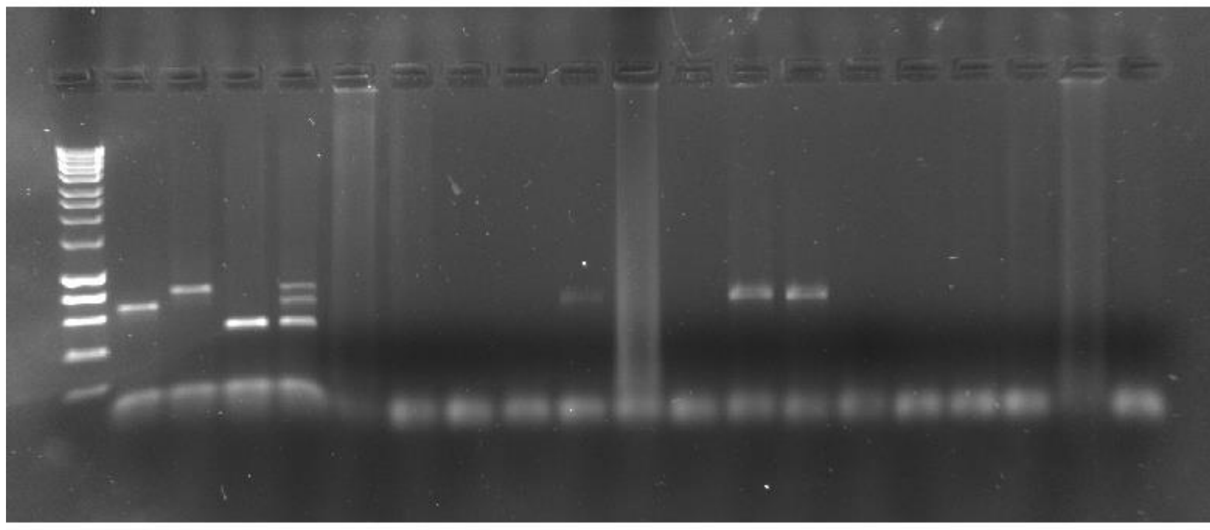
5.3.1 General description of the case series

In the three years (2017 to 2019), sixty-two placenta samples from aborted cattle were submitted to the local veterinary diagnostic laboratory Plataforma de Salud Animal of the Instituto Nacional de Investigación Agropecuaria (INIA), for pathologic examination and diagnostic work-up. All samples were taken from dairy animals raised on commercial farms in Uruguay. Samples were first investigated for the presence of nucleic acid material from *C. burnetii*, *C. abortus* and *C. pecorum* in the Molecular Biology Laboratory of the aforementioned Institution.

After the assessment stage, five placentas were classified as positive-*C. burnetii* samples because specific primers amplified bacterial DNA. No evidence of *C. abortus* or *C. pecorum* infection was found.

Table 5.1: General characteristics of cases with positive *C. burnetii* PCR in placenta.

	Biotype	Number of lactations	Stage of gestation	Main commercial activity from the dairy farm
Case 1	American Holstein	2	Not possible to be determined	Sell to a dairy plant
Case 2	American Holstein	2	Last third gestation	Sell to a dairy plant
Case 3	American Holstein	3	Last third gestation	Sell to a dairy plant
Case 4	New Zealand Holstein	4	Last third gestation	Sell to a dairy plant
Case 5	American Holstein	Aborted cow not identified	Not possible to be determined	Artisanal cheese manufacturing



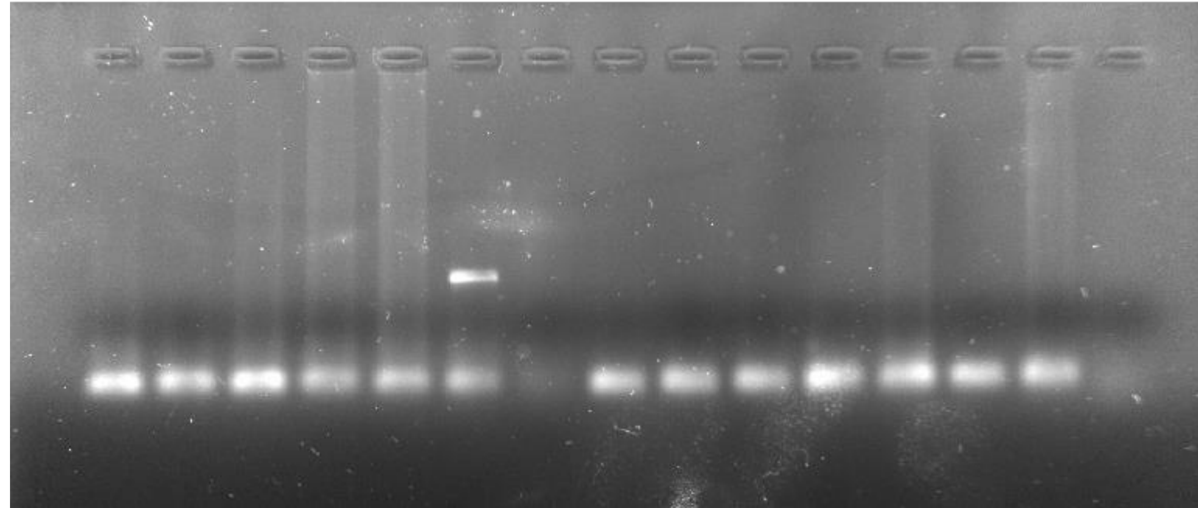


Figure 5.7: Gel electrophoresis multiplex PRC for *Coxiella burnetii*, *Chlamydia abortus* and *Chlamydia pecorum*.

5.3.2 Absolute quantification of *C. burnetii* burden in aborted placenta samples

The recombinant plasmid DNA presented a concentration of 113.42 ng/µl, with an A260/A280 ratio of 2.00, and an A260/A230 ratio of 2.35. The copy number of plasmid DNA was estimated based on the quantity of DNA and the length of DNA in base-pairs. The conversion into copy number of plasmid DNA was 2.64×10^{10} copies/µl. The resulting plasmid was subjected to a serial 10-fold dilution series and was utilised to construct the standard curve ($1 \times 10^8 - 1 \times 10^1$ copies/µl). The threshold was placed within the exponential phase and above noise of background amplification. The Ct values were plotted against the known copy number of each dilution point of the standard control. The slope to the calibration curve was -3.377, and the Y-intercept was 38.34. Finally, the unknown placenta samples

were quantified by employing the formula Y=-3.3778X+38.34 (Y=threshold cycle, X=log starting quantity). For all standard curves, r^2 values were high ($r^2 > 0.99$).

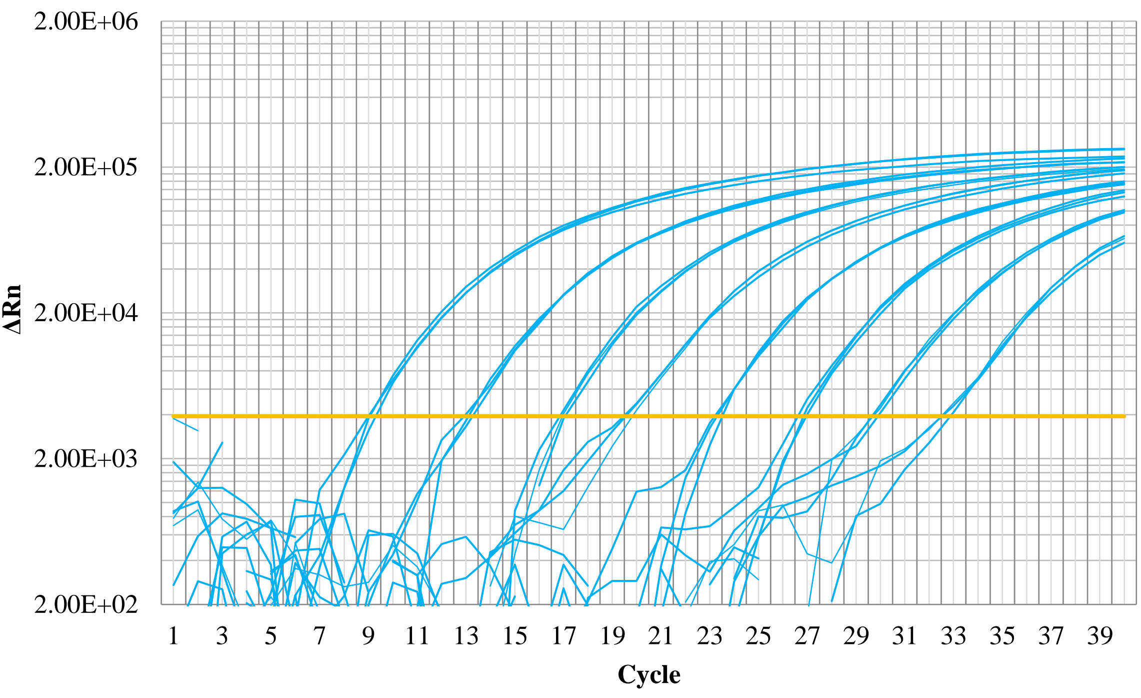


Figure 5.8: Amplification plot for *Coxiella burnetii*.

Amplification plot for *Coxiella burnetii* over ten dilutions amplified using primer pair CburF and CburR (Panning *et al*., 2008), and FAM-TAMRA probe (Di Domenico *et al*., 2014). Delta Rn represents adjusted absorbance and is calculated by the formula (Rn+)-(Rn) (Dorak, 2007); the x-axis shows the cycle number. The horizontal line shows the threshold settled at 0.01.

Table 5.2: Inter-assay variability of *Coxiella burnetii* amplification using primer pair CburF and CburR (Panning *et al*., 2008), and FAM-TAMRA probe (Di Domenico *et al*., 2014).

N° of copies/µl	Replicate 1	Replicate 2	Replicate 3	Mean Ct	SD[a]	CV[b] (%)
2.6×10^8	10.068	10.142	9.780	10.064	0.06	0.657
2.6×10^7	14.672	13.068	13.302	13.681	0.70	5.732
2.6×10^6	18.615	16.742	17.391	17.582	0.776	4.416
2.6×10^5	21.559	20.175	20.962	20.898	0.567	2.712
2.6×10^4	23.844	22.795	23.636	23.425	0.454	1.937
2.6×10^3	27.628	26.637	27.549	27.241	0.433	1.589

2.6×10^2	31.506	29.201	30.267	30.325	0.942	3.107
2.6×10^1	34.476	33.388	33.536	33.800	0.482	1.425
2.6	UN	UN	UN	NA	NA	NA

[a] SD: Standard deviation
[b] CV (%): Coefficient of variation
UN: undetermined. No template detected.
NA: not applicable, no target nucleic acid detected for calculation of mean, standard error or coefficient of variation.
A threshold value of 0.001 was set for the determination of Ct values.

This table shows the amplification plot for *C. burnetii*. The Cq values increased as the concentration of the starting template is reduced. A noticeable decrease in absorbance was observed durign the plateau phase. The threshold was set at 0.01 above the noise of background amplification signal and during the exponential stage.

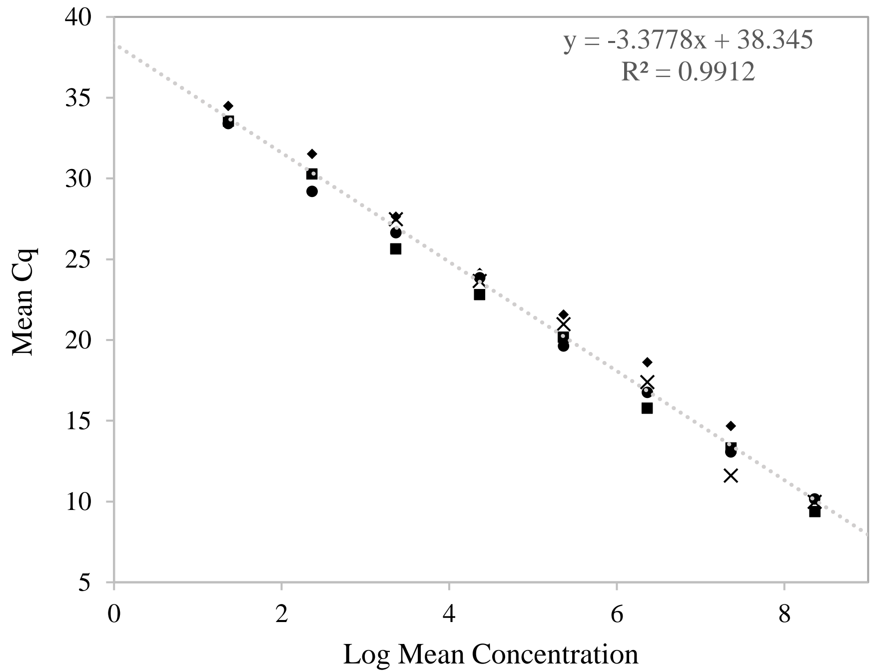


Figure 5.9: Amplification curve and standard curve. The amplification curves were constructed by using ten-fold dilutions of recombinant plasmid DNA ranging from 10^8 copies/μL to 10^1 copies/μL. The standard curve equation was Y=−3.3778X+38.345.

5.3.3 Fluorescent *in situ* hybridisation (FISH) for *Coxiella burnetii* detection in placentas

As stated previously in the Material and Methods section, the FISH assessment was not conducted due to the COVID-19 lockdown.

5.3.4 Immunohistochemistry of placenta samples

The IHC for *C. burnetii* showed strong positive immunoreaction, revealing the presence of abundant intralesional antigen, both in the cytoplasm of trophoblasts and macrophages, and extracellularly, in the allantois and chorion. *Chlamydia* spp. IHC was negative in all the samples. Although the chorion showed positive immunoreactivity by IHC, the signal was even stronger in the allantois.

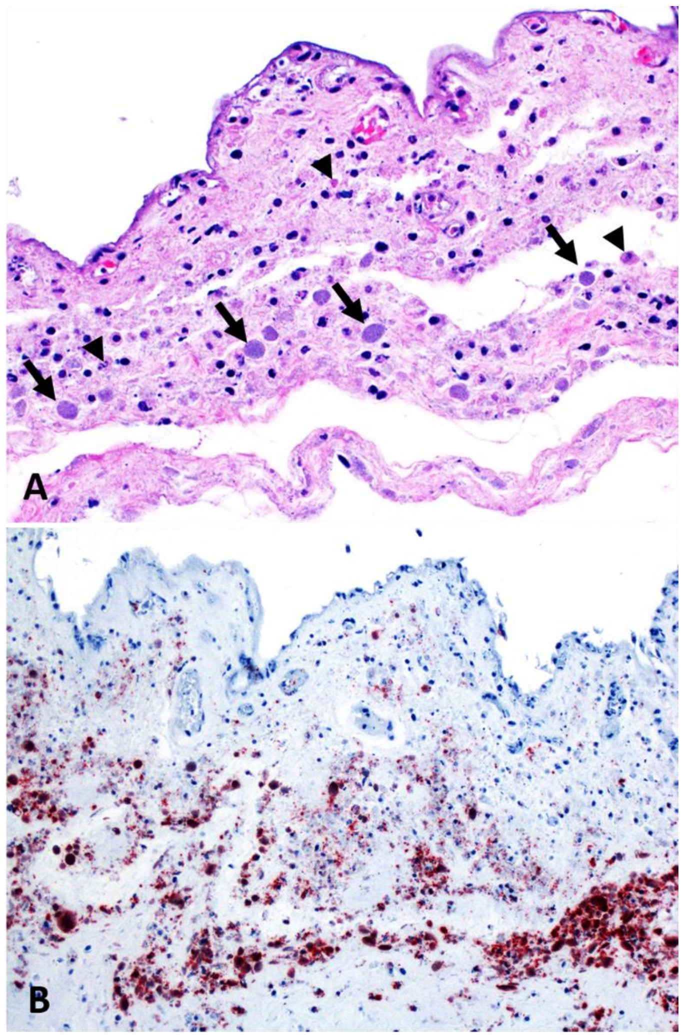


Figure 5.10: Microscopic lesions in the placenta of the Holstein cow with an abortion caused by *Coxiella burnetii*. **A**. Intercotyledonary region of the chorion. The chorionic stroma is infiltrated by neutrophils and macrophages that contain myriads of intracytoplasmic basophilic coccobacilli (arrows). Pyknotic and karyorrhectic hypereosinophilic cellular debris (arrowheads) are indicative of necrosis.

Hematoxylin and eosin stain. **B**. Serial section of **A**. The bacteria are strongly immunoreactive with *C. burnetii* antiserum, which is depicted as intracytoplasmic and extracellular granular brown chromogen deposition. Immunohistochemistry for *C. burnetii*, hematoxylin counterstain.

5.4 Discussion

The presence of nucleic acid material from *C. burnetii* was found in five bovine placentas of aborted animals using PCRs. In one of these samples, the definitive diagnosis of abortion due to coxiellosis was confirmed by the microscopic assessment of the placenta and IHC strong positive immunoreaction for *C. burnetii*.

Obtaining placenta from aborted ruminants under field conditions for laboratory investigation is frequently challenging. Often the presence of predators, mainly scavenger animals that eat scattered tissues, as well as the interval from parturition to placenta expulsion, may lead to failure to comply with placenta collection requirements. Additionally, once expulsed placenta may be easily contaminated. Despite these difficulties, for successful investigation of active infection it is critical that the placenta is submitted to the laboratory. For example, *C. burnetii* do not usually cause lesions in the fetal tissues, although pneumonia has been described in aborted bovine fetuses (Bildfell *et al*., 2000).

Similarly, even though striking, grossly visible intercotyledonary and cotyledonary placentitis may be observed (Bildfell *et al*., 2000; Macías-Rioseco *et al*., 2019), in some cases the infection can induce only subtle macroscopic placental alterations, while in others the placenta may appear wholly unremarkable (Bildfell *et al*., 2000). Despite the lack of noticeable characteristic gross placental lesions, as this is the primary *C. burnetii* target tissue with high tropism towards trophoblasts, the primary histological examination coupled with further molecular assessments are probably the most informative tests. Bacterial colonisation frequently induces a neutrophilic inflammatory reaction and necrotising placentitis, which along with the visualisation of the bacteria within trophoblasts, may guide evaluators towards the diagnosis of Q fever.

The collection of the placenta under field settings and it submission to laboratory is crucial for diagnostic purposes. Also any remain piece of placenta and birth products should be destructed by any method (incineration, burial) because the bacterial burned of ruminant placentas is the main cause of environmental contamination and may produce both human and animal infection.

When investigating either *C. burnetii* or *Chlamydial* abortion, the use of single laboratory tests may be misleading. The mere detection of the bacterial DNA in a sample does not necessarily imply disease causality, as subclinical infections are common (Agerholm, 2013). Similarly, in the case of *C. burnetii* infection, serologic approaches at the individual level are not informative enough, as seroconversion can occur without detectable lesions or bacterial shedding, animals can remain seropositive long after they have overcome infection, shed *C. burnetii* before the development of detectable antibodies, and even shed the agent without ever seroconverting (McQuiston *et al*., 2002). PCR, FISH and IHC are valuable tools, mostly, for *C. burnetii* detection in diagnostic settings. PCR based assays are sensitive

and quick screening methods used in a wide variety of samples, FISH and IHC enable the localisation of the bacterial rRNA or antigen within lesioned tissues, which is a powerful indicator of causality (Bildfell *et al*., 2000).

The use of PCR is suggested only as a part of an overall diagnosis protocol when investigating Q fever and chlamydiosis, and this should be combined with other tests and evaluations. For instance, the identification of a microorganism in the case of abortion does not certainly indicate that microorganism was responsible. For a better picture of the likely cause of the abortion, a more comprehensive interpretation of different approaches is needed.
As *C. burnetii* and Chlamydia spp are biothreat agents posing a great threat to public health, and researchers face a lack of proper facilities, culture of these agents is often not routinely conducted; other methods of assessments should be explored. Among those alternative approaches, PCR is a detection method with high sensitivity and specificity popularly employed in various matrices such as blood, milk, human and tissue samples (Willems *et al*., 1994; Lorenz *et al*., 1998; Klee *et al*., 2006; Guatteo *et al*., 2007; Agerholm, 2013; Kim *et al*., 2005; Saglam & Sahin, 2016). However, the major constraint of PCR is its inability to distinguish live and dead bacteria. FISH is able to do this and is thus a complementary molecular approach. A FISH method developed for *C. burnetii* detection founded on specific probes targeting the 16S ribosomal RNA of the bacterium detects live an active bacteria (Jensen *et al*., 2007). Therefore, the sensitivity of this method depends greatly on the bacterial metabolic rate, and therefore the detection of dormant or otherwise inactive *C. burnetii* could be complicated by this method (Jensen *et al*., 2010). By contrast, the IHC based on polyclonal antibodies against the whole bacterium identifies both active and inactive bacteria (Hansen *et al*., 2011).

Frequently, differences between results from PCR and IHC can be explained to some degree by the former's greater sensitivity (Duncan *et al*., 2013). When FISH and IHC methods were contrasted for *C. burnetii* detection in tissue samples, their results were completely equivalent (Jensen *et al*., 2007). While IHC typically detects intracellular antigens, FISH detects RNA, thus revealing positive results in only active infections. Therefore, discrepancies between FISH and IHC results can indicate the presence of inactive intracellular bacteria, and this could be correlated with histologic findings. FISH and IHC are both robust with differing strengths and weaknesses, their combined utilisation is more potent than the use of either test in isolation.

Q fever is a globally reported zoonosis, deemed as re-emerging or emerging in several countries. The inhalation of contaminated dust and aerosols, following the normal parturition or abortion of domestic ruminants, is the main path of *C. burnetii* infection in people. However, oral transmission after the ingestion of unpasteurised milk or raw dairy products containing the bacteria also appears to be a feasible route of infection. The significance of the digestive route for *C. burnetii* transmission under

spontaneous conditions is a subject of debate, and research groups are still working to define its risk, which has implications for food safety and public health. After the initial infection in cattle, the bacterium remains latent in lymph nodes and mammary glands, and bacterial shedding can occur in subsequent calving seasons and lactations, with milk shedding being a significant and persistent excretion route of *C. burnetii* (Guatteo *et al*., 2007).

Serological evaluations conducted in France linked the consumption of contaminated unpasteurised milk with seroconversion in people (Fishbein & Raoult, 1992). A serologic survey of a cohort of goat farmers, workers, and their contacts, involved in an outbreak of Q fever in the Canadian province of Newfoundland, identified the consumption of cheese made with pasteurised goat milk as well as contact with goat placenta as important independent risk factors for infection (Hatchette *et al*., 2001). Likewise, a two-year epidemiological evaluation conducted on 1,200 children hospitalised with different clinical manifestations in Greece, found that eating raw cheese from rural areas enhanced the risk of Q fever (Maltezou *et al*., 2004). Recently, a random sampling and molecular investigation performed on the most traditional and oldest type of raw-milk cheese in Brazil, known as Minas artisanal cheese and manufactured with bovine milk, revealed a high prevalence of *C. burnetii* in this ready-to-eat product. The study estimated that 1.62 tons of cheese produced daily are contaminated with this bacterium (Rozental *et al*., 2020).

Numerous investigations have revealed *C. burnetii* DNA in milk and derived products, such as cheese, cream, butter, and yoghurt from cows, sheep, and goats (Eldin *et al*., 2013; Pearson *et al*., 2014; Barandika *et al*., 2019; Rozental *et al*., 2020). However, only a few studies took a step further in the investigation of its viability and hazards. Viable *C. burnetii* was proven in raw cheese by culture in Vero cells and inoculation in mice (Barandika *et al*., 2019. The potential inactivating effect of cheese ripening was dismissed as viable *C. burnetii* was detected in samples from unpasteurised hard cheeses after eight months of maturing (Barandika *et al*., 2019). Experimental studies, recently conducted in mice, reinforced the digestive route as a feasible *C. burnetii* transmission mode (Miller *et al*., 2020).

Coxiella burnetii in milk is successfully inactivated by pasteurisation, which is fundamental, not only for the prevention of infection by *C. burnetii* and other serious milk-borne infectious illnesses such as tuberculosis and brucellosis. Despite the lack of a complete consensus about *C. burnetii* transmission by the digestive route, and the discrepancies that still exist about its role as a foodborne pathogen, this route of infection should not be neglected and farmers, particularly those producing cheese onsite instead of selling milk to the dairy industry, ought to be made aware of the relevance of pasteurisation.

In Uruguay, the raw milk trade had been regulated since 1984, however, the control of raw milk consumption in rural areas is challenging. Additionally, consumers' preferences for raw milk products is emerging as a growing global trend. Due to their indigenous microbiota, raw cheeses have particular organoleptic attributes, such as a strong flavour and a peculiar texture, much appreciated by consumers,

and these characteristics are frequently attributed to the use of unpasteurised milk. Thus, some cheeses are manufactured using raw milk and eithers are sold directly to consumers at the farm or commercialised at local markets. This practice may embody a hazard to public health, considering the high stability of *C. burnetii* in final dairy products even with acidic pH or reduced water activity (Barandika *et al*., 2019).

As no epidemiological information was available from the *C. burnetii*-positive placenta coming from the artisanal cheese manufacturing farm, it was not known if *C. burnetii* was recently introduced into the farm or was already endemic in the herd at the time of abortion occurrence. Q fever has a complex epidemiology; finding the source of the infection can be challenging and was outside the scope of this study. Farm-level risk factors for *C. burnetii* infection include the introduction of replacement cattle from other farms, tick infestation, and the cohabitation of cattle with goats or sheep. As the affected farm was in an area officially classified as free of cattle ticks, tick infestation was unlikely in the herd; however, none of these risk factors was investigated on this farm. Similarly, at the time of the aborted placenta submission, there were no laboratories running PCR tests for the identification of *C. burnetii* in milk in Uruguay. Additionally, no bulk-tank milk samples of the affected herd were available, and this is why its presence in a contemporary milk sample could not be assessed.

Q fever and Chlamydiosis are two zoonotic diseases distributed in many countries around the world. Their significance is not only related to animal production losses and associated economic impact but also to the serious risks posed to people (Rodolakis *et al*., 1998; Maurin & Raoult, 1999). Since *C. burnetii* is a serious zoonosis, occasionally with fatal consequences in humans with pre-existing medical conditions, this bacterium merits more attention from both veterinary and human health services. On the other hand, despite the intrinsic zoonotic threat of *C. burnetii*, this bacterium has also been associated with an improved risk of lymphoma because the occurrence of the bacterium in the tumour microenvironment appears to favour lymphomagenesis (Melenotte *et al*., 2016).

Abortions due to *C. burnetii* are more commonly reported in small ruminants than in cattle. A relationship among the species, the severity of lesion found, and the weight of infection has been proposed. This finding was based on the evidence of mild lesions, and a smaller accumulation of *C. burnetii* antigen immunocytochemically revealed in aborted placentas in cows compared with histologic findings in ewes (van Moll *et al*., 1993). *Coxiella burnetii* has been linked to sporadic abortion in cows (Bildfell *et al*., 2000), exhibiting infection rates that resemble those of opportunistic bacteria (Agerholm, 2013). In South America, as globally, scientific publications reporting evidence about bovine abortions caused by *C. burnetii* are scarce. A retrospective survey conducted in Brazil, where a pool of organs, gastric content, and brains from aborted bovine fetuses and stillborn calves were analysed by PCR for the identification of *C. burnetii* DNA, found an infection rate of 10.3% (3/28). However, none of these cases was assessed histologically for the presence of typical lesions of Q fever

(de Souza Ribeiro Mioni, 2018). A recent study from Uruguay reported the occurrence of a cluster of four cases of abortion due to *C. burnetii* in Holstein cows on one farm, based on gross and microscopic examination of the placentas, coupled with the identification of the agent by IHC and PCR (Macías-Rioseco *et al*., 2019). The lack of scientific reports on bovine abortions caused by *C. burnetii* in other South American countries suggests that the disease may have gone undetected or underdiagnosed. The significance of *C. burnetii* as an abortifacient agent in bovines should not be underestimated.

Chlamydiaceae species do not seem to play a central role in abortions in cows in Uruguay, as neither *C. abortus* nor *C. pecorum* were detected in the aborted placentas. As occurs in other countries, in Uruguay their role as abortifacient agents in small domestic ruminants would be more relevant than in cattle (Rodolakis & Souriau, 1989; Tlatli *et al*., 1999; Borel *et al*., 2006). Nonetheless, their zoonotic potential should be considered when handling excreted materials from aborting cattle. *Chlamydia abortus* is deemed as an emerging zoonosis posing significant risks for pregnant women. This bacterium has been related with PID in women (Walder *et al*., 2003) and reports of abortions in several countries such as The Netherlands (Kampinga *et al*., 2000), Switzerland (Pospischil *et al*., 2002a), the UK (Hadley *et al*., 1992) and USA (Hyde & Bernirschke, 1997). The evaluation of concomitant infection with *C. burnetii*, *C. abortus*, and *C. pecorum* did not reveal co-infection in the placentas from aborted cattle analysed.

5.4.1 Limitations

It is relevant pointing out that placenta samples analysed in this study came from a biased population as samples were cases that arrived at a local laboratory of veterinary diagnosis and were not gathered as part of a structured epidemiological investigation. The impossibility of carrying out FISH evaluation was also identified as a substantial limitation, as active infections due to *C. burnetii* were not possible assessed.

5.4.2 Further research

The farms of origin of the positive-*C. burnetii* placentas could be further investigated to establish links between the bacterium and other risk factors and conditions. For example, it would be interesting to detect *C. burnetii* in BTM samples and also investigate whether *C. burnetii* strains from milk corresponds to those causing abortion. Additionally, we could try to detect *C. burnetii* DNA in cheese samples and further investigate their bacterial viability, mostly considering that those dairy products are sold directly to consumers at local food markets. As a qPCR is now available, it would be worth to correlate the bacterial burden in placentas with the degree of histologic lesions.

5.5 Conclusions

Hitherto the lack of diagnostic tools, and the impossibility of culture because of the lack of adequate biosafety laboratories, has limited the epidemiological investigations of these agents in the local context. The current evaluation represents the first attempt to simultaneously detect *C. burnetii, C. abortus, and C. pecorum* in Uruguay. *Coxiella burnetii*-DNA was detected and quantified in placentas from aborted dairy cattle. Taken together these findings expand the evidence supporting *C. burnetii* as an agent presented in dairy farms in Uruguay, and for the first time, this bacterium was identified as the bovine abortifacient pathogen on an artisanal cheese-producing farm. No co-infections of these pathogens were found.

Besides the economic failures in animal production caused by these bacteria, a zoonotic threat to public health is also created. People with direct contact to ruminants such as those working in rural areas (farmers, vets), or those working in the industry (slaughterhouses/abattoirs), or even those with indirect exposure to animals (such as laboratory technicians), should be made aware of the presence of *C. burnetii* in cows in Uruguay.

Despite some discrepancies in the existing literature, there is evidence about unpasteurised milk and derived dairy products representing potential sources of Q fever transmission to humans. The detection of *C. burnetii* in an artisanal manufacturing cheese farm highlighted that the public health risk posed by *C. burnetii* should not be neglected, and the need for on-farm milk pasteurisation by artisanal cheesemakers should be highlighted.

*Chapter 6: **Quantification of*** **Chlamydia abortus** ***in vulvo-vaginal swabs from aborted cattle: a case-control evaluation***

6.1 Introduction

Recently the family *Chlamydiaceae* has been reclassified, and there is now one genus, the *Chlamydia*. As stated in previous chapters, the members of the genus *Chlamydia* are obligately intracellular Gram-negative bacteria that have a unique developmental biphasic cycle that includes two forms of the organism, an obligate intracellular (the replicative phase) and an extracellular (the infectious phase). *Chlamydia abortus* and *C. pecorum* are two among 13 species in this genus (Sachse *et al*., 2014).

Ruminants, especially goats, sheep, and cattle, are the primary hosts for *C. abortus* and *C. pecorum*. Whereas *C. abortus* may trigger subfertility and abortion, *C. pecorum* has often been detected in faeces of healthy animals and occasionally been shown to lead to clinical disease, although abortion due to *C. pecorum* has also been described (Rodolakis *et al*., 1998; Giannitti *et al*., 2016). Additionally, *C. pecorum* has been isolated from the gastrointestinal tract of cattle clinically ill by mastitis, conjunctivitis, fertility disturbances, and inflammation in the pulmonary system (Ruhl *et al*., 2009).

Chlamydia abortus is one of major microorganisms causing failure in reproductive performance in goats and sheep, particularly in flocks managed under intensive conditions. Specifically, *C. abortus* is the aetiological pathogen of the enzootic abortion of sheep (also identified as ovine enzootic abortion), which produces lamb loss in many sheep-rearing countries. Goats are affected by this illness, as well as other species but to a lesser degree (cattle, horses, pigs, and deer) although the incidence of these infections is quite unclear because of a lack of epidemiological information (Longbottom & Coulter, 2003). In cattle, chlamydial infection has been related to reproductive failure, including the occurrence of abortion, repeat breeding, endometritis, vaginitis and seminal vesiculitis, perinatal mortality and the birth of weak calves (Reinhold *et al*., 2011). In bovines, *Chlamydia* infection, including *C. pecorum, C. abortus*, and *C. psittaci*, has also been observed to be related to respiratory tract infection (Sachse *et al*., 2009).

Chlamydia abortus and *C. pecorum* are the main species accounting for genital disorders in cows (Biesenkamp-Uhe *et al*., 2007). Although *C. abortus* is a recognised agent of abortion and hypofertility in cattle, the much less frequent occurrence of this agent in cattle than in sheep and goats may indicate species-specific variations (Borel *et al*. 2006; Doull *et al*., 2015). When abortion due to chlamydiosis arises in cattle, it often occurs during the last trimester of pregnancy, and it is particularly prevalent during the first gestation of heifers (Barkallah *et al*., 2018). In bulls, the *C. abortus* infection alters the quality of semen by producing epididymitis, testicular atrophy and seminal vesiculitis; also the

organism can be shed by semen which can produce local infections leading to inflammatory reactions in the uterus, and afterwards can cause low fertility in heifers (Livingstone & Longbottom, 2006). The vaginal mucosa, as well as uterine mucosa in cows and sheep, are vulnerable to infection (Wittenbrink *et al*., 1993; Papp & Shewen, 1996), and it has been demonstrated that experimentally infected semen can also transmit chlamydial agents (Bowen *et al*., 1978; Appleyard *et al*., 1985).

Evidence supporting the point that the bacterium remains in a latent state in non-pregnant dams, possibly in lymphoid tissue, till a pregnancy occurs had been reported in naïve ewes (Nietfeld, 2001; Entrican, 2002; Da Silva *et al*., 2006). Even though the animals contained the bacterium, diagnosis of the infection could not be achieved either serologically or by direct exposure of the bacterium up to the time of the abortion, the time when the bacterium is excreted, and titres of maternal *C. abortus* antibody quickly rise (Gokce *et al*., 2007). Aborting dams may transform into inapparent carriers that shed *C. abortus* for years or even for the rest of their productive lives (Papp *et al*., 1994; Koehler *et al*., 1997; Rodolakis *et al*., 1998; Entrican *et al*., 2001). The clinical diagnosis of chlamydiosis in cattle is frequently complicated since neither the pathological lesions nor the clinical signs are specific or characteristic of *C. abortus* infection and may also be detected in abortions due to other causal agents.

In vitro studies have also provided information helping to explain the mechanisms behind the establishment of latent infections. It has been shown that *C. abortus* can develop an inapparent and persistent infection in cell culture under a variety of conditions (Rodolakis *et al*., 1989; Beatty *et al*., 1994ab; Entrican *et al*., 1998). However, strong evidence about latent infection in heifers followed by reactivation before the first pregnancy has not yet been provided but has not been dismissed either.

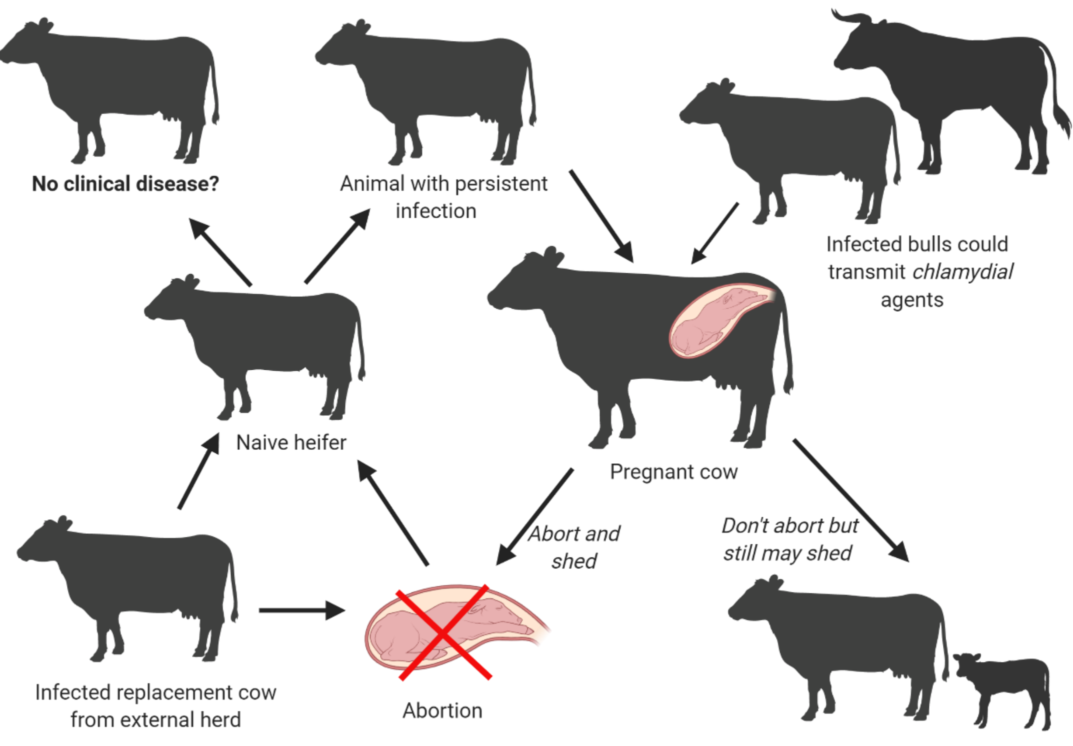


Figure 6.1: The transmission cycle of *Chlamydia abortus* in cattle.

Chlamydia abortus can be eliminated in nasal, ocular, uterine or vulvar discharges, placenta, urine, semen and faeces (Perez-Martinez & Storz, 1985; Rodalkis *et al*., 1998; Longbottom & Coulter, 2003). Healthy animals acquire the disease by ingestion, or by the inhalation of air contaminated with infected material, particularly discharges and fetal membranes, and bacteria can also be venereally transmitted (Schachter *et al*., 1975; Gerbermann, 1991; Papp & Shewen, 1996; Rodalkis *et al*., 1998; Longbottom & Coulter, 2003). Although *C. abortus* is most often oronasally transmitted, the venereal route should not be underestimated (Papp & Shewen, 1996; Rodolakis *et al*., 1998). After an abortion, *Chlamydiae* can be transmitted by contact with infected fetuses, membranes, vaginal and uterine discharges and even milk (Rodolakis, 2006b). The oronasal route is the primary transmission path, and this route is facilitated when animals are managed in proximity and in frequent contact (DeGraves *et al*., 2003). The products of abortions were identified as the primary sources of infection for transmission to naïve animals, an aspect that is central from the perspective of flock or herd management (Livingstone *et al*., 2009). Furthermore, wild animals, such as wild boar (*Sus scrofa L.*), act as potential reservoirs for *C. abortus* and may play an essential role in contamination of the environment and in the subsequent transmission of the bacterium (Hotzel *et al*., 2004).

Evidence of the vertical transmission of the infection was reported from sheep to offspring, although the significance of this route of spread of the disease is still under discussion (Rodolakis & Bernard,

1977). During vertical transmission, lambs may acquire infection from infected mothers, either becoming infected congenitally in utero or during passage throughout the birth channel during parturition (Messmer *et al*., 1998). No evidence for *C. abortus* and *C. pecorum* vertical transmission had been found in cattle (Jee *et al*., 2004). Consequently, the horizontal route of transmission continues to be the most likely and relevant path of infection for naive animals.

A high seroprevalence coupled with a high genomic DNA detection of these two chlamydial species, has been reported, often without any clinical signs, showing that the majority of infections arise in the absence of detectable disease, or occasionally along with severe manifestations (Kaltenboeck *et al*., 2005). Chlamydial species are often regarded as commensal microorganisms from several animals' gastrointestinal tracts (ruminants, birds, mice) (Rank & Yeruva, 2014). In cattle, these commensal microorganisms can be regularly shed by clinically asymptomatic animals (Reinhold *et al*., 2011). These bacteria can survive up to three years in the gastrointestinal tracts of animals not leading to an immune reaction by downregulating the immune system in the gut; histopathological inspection has corroborated the absence of inflammatory response (Perry & Hughes, 1999; Igietseme *et al*., 2001; Rank & Yeruva, 2014). A broad range of manifestations have been linked with infections of *C. pecorum* in livestock, ranging from asymptomatic to sporadic acute disease, involving conjunctivitis, polyarthritis, pneumonia, encephalomyelitis, and endometritis, and have also been connected to disorders of the urogenital and digestive tracts (Fukushi & Hirai, 1992; Polkinghorne *et al*., 2009; Walker *et al*., 2015). Beyond the clinical manifestation reported, possibly the most frequent consequence of *C. pecorum* infection is the absence of clinical signs (Jee *et al*., 2004). Even in asymptomatic animals, some evidence supports the existence of a subclinical negative impact of the infection. Nevertheless, infection, even as subclinical disease, affects for example, asymptomatic endemic infections by *C. pecorum*, resulting in decrease growth rates in calves at up to 48% (Poudel *et al*., 2012).

Evidence obtained in ewes showed that though animals develop immunity and do not repeat further abortions induced by *C. abortus*, they may possibly remain shedding bacteria at subsequent lambing or oestrus stages, thus leading to environmental contamination, and thus continuing to contribute to infection spread (Nietfeld, 2001; Da Silva *et al*., 2006). The detection of infected and carrier animals may be essential to diminish environmental bacterial contamination, consequently restricting the transmission of infection and the threat of spread to people. Aborted tissues and post-partum fluid discharges are thought to be the primary route of environmental contamination by *Chlamydia*, which is the principal source of transmission to naive animals (Aitken & Longbottom, 2007).

Abortion in cows is described as the loss of pregnancy within the period from day 42 to 260 of pregnancy (Peter, 2000). Gestation that ends before day 42 is usually described as early embryonic death, while a calf born dead between day 260 of pregnancy and full term is named a stillbirth (Hovingh,

2009). Abortions can have an infectious origin produced by agents such as bacteria, viruses, fungi, or protozoa, or be caused by a non-infection aetiology, including nutritional weaknesses, heat stress, trauma, or intoxications (Pal, 2006; Parthiban *et al*., 2015). As stated above, *C. abortus* is endemic in ruminants worldwide (Li *et al*., 2015), and this bacterium produces epizootic bovine abortion (Gokce *et al*., 2007). Abortions are deemed a relevant cause of economic failures experienced by bovine farmers because of extended calving intervals, calf loss, declines in milk production, costs of veterinary treatment, and external acquisition of cow replacements (Thurmond & Picanso, 1990).

Considering their intracellular nature, *Chlamydiae* need to go through a stage within cells during their life cycle. Thus, the isolation and propagation of these bacteria requires researchers to follow tissue culture procedures (Thejls *et al*., 1994). On well-growing isolates, results can be obtained within 48 – 72 hours, though in some samples, results may only be obtained after delays of 2 to 6 weeks (Sachse & Hotzel, 2003). Although egg inoculation and cell culture are the gold standard approaches for *Chlamydiae* diagnosis, the long incubation period necessary for *Chlamydia* isolation represents a considerable disadvantage (Condon & Oakey, 2007).

Methods such as complement fixation test (CFT) and enzyme-linked immunosorbent assays (ELISA) are commonly used for the serological investigation of *Chlamydia* infection. Although still widely employed, CFT presents low specificity, poor reproducibility between laboratories, and is generally quite laborious (Sachse & Hotzel, 2003). The antibody typically employed in ELISA is handled easily and is more sensitive and faster than CFT (Anonymous, 1996). Most of the validated serological tests for *Chlamydia* investigation are founded on the significant cross-reactive antigens common to all the chlamydial species, MOMP and LPS (Sachse & Hotzel, 2003). Although animal exposure can be confirmed by serology, serologic assays are not useful in distinguishing between *C. abortus* and *C. pecorum* because antigenic cross-reactivity has been identified (Griffiths *et al*., 1996, Rodolakis *et al*., 1998, Longbottom *et al*., 2001), so this method has limited value for when trying to differentiating between these bacterial infections.

The introduction of molecular methods for *Chlamydiae* detection, particularly polymerase chain reaction (PCR), has significantly improved the bacterium's identification in clinical samples and species' genetic differentiation. PCR presents several advantages when detecting *Chlamydia*, such as being easy to utilise, providing quick availability of outcomes, and being potentially standardisable and markedly safer than culture (Laroucau *et al*., 2001). Lately, conventional PCR and qPCR have been broadly utilised to evaluate clinical samples to detect *C. abortus*. Often, these PCR approaches are built on the amplification of the polymorphic membrane gene *pmp,* the outer membrane protein genes (*ompA, omp1* and *omp2*), genes encoding 16S-23S rRNA intergenic interval and 16S rRNA and helicase (Siarkou *et al*., 2002; Öngör *et al*., 2004; Berri *et al*., 2004; Greco *et al*., 2005; Marsilio *et al*., 2005; Güler *et al*., 2006; Berri *et al*., 2009)

Chlamydial infection has been investigated based on the nucleic acid detection in vaginal and cervical swabs from goats, cows, and sheep. For instance, the presence of *C. abortus* infection has been investigated in a flock of goats with a previously documented history of reproductive problems such as stillbirths, weak born kids, abortion, and persisting patterns of low reproductive performance (Marsilio *et al*., 2005). Additionally, a widespread *C. pecorum* infection was revealed by species-specific qPCR assays on vaginal swabs taken from healthy dairy cows belonging to herds located in South East Queensland (Anstey *et al*., 2019). Vaginal swabs were also evaluated at parturition of sheep to detect *C. abortus* following their experimental infection with variant strains LLG and POS (Livingstone *et al*., 2017). Though *C. suis* and *C. pecorum* do not appear to have central relevance in porcine abortions, *C. abortus* has been investigated and isolated from the genital tract and cervical swab from sows with abortions, repeated return to oestrus, and weak piglets in reduce litters (Thoma *et al*., 1997; Hoelzle *et al*., 2000; Camenisch *et al*., 2004). Furthermore, PCR tests on vaginal swabs were employed as an available and sensitive procedure for diagnosing chlamydial infections in human medicine (Shafer *et al*., 2003).

The utilisation of vaginal swabs presents some advantages in terms of risk of human contagion. The utilisation of swabs reduces the risk of infection significantly because of there being no close contact due to the direct handling of abortion material. Thus, the utilisation of vaginal swabs permits the collection of pathogens under relatively secure conditions (Ababneh *et al*., 2014). Typically, a small amount of the sample is sufficient for the assessment; however, sometimes the quantity of chlamydial DNA in the vaginal swabs may be reduced and thus, the detection limited (Livingstone *et al*., 2009). Also, high content levels of collagen in the placenta may ruin DNA quality, meaning that a vaginal swab would be a more suitable sample technique than using the placenta for PCR evaluation (Marsilio *et al*. 2005). Vaginal secretions, collected after abortion by swabbing, offer an appropriate sample for PCR assessment of abortifacient microorganisms and isolation.

6.1.1 The zoonotic impact of *C. abortus* and *C. pecorum*

Although *C. abortus* infection in people is infrequent, cases reporting the zoonotic transmission to humans have been informed. Clinical forms of human *C. abortus* infection range from being asymptomatic to influenza-like illness and pneumonia, sometimes infection may be devastating with serious complications, such as abortion (Pospischil *et al*., 2002b; Welder *et al*., 2005; Rodolakis & Mohamad, 2010; Sillis & Longbottom, 2011; OIE, 2018). In pregnant women, *C. abortus* produces PID and replicates in the trophoblasts cells leading to placental dysfunction and subsequent fetal death (Cohen & Brunham, 1999; Pospischil *et al*., 2002a; Walder *et al*., 2003). This scenario frequently makes necessary the hospitalisation of pregnant women in an intensive care unit (Meijer *et al*., 2004).

When *C. abortus* exposure occurs in pregnant women, it is hazardous and may lead to a life-threatening condition for mother and fetus. The outcome of infection seems to depend on the timing of the infection, and the disease may result in premature delivery, stillbirth, or abortion (Wong *et al*., 1985; Pospischil *et al*., 2002a). Infection also often produces renal function failure, hepatic disorders and disseminated intravascular coagulation, and could subsequently even cause death (Buxton, 1986). Moreover, the systemic complications that occur in the mother could be life-threatening if no emergency healthcare intervention is provided (Walder *et al*., 2005).

The zoonotic transmission of *C. abortus* is likely to arise by direct contact with infected animals (Wheelhouse & Longbottom, 2012). However, indirect contact, such as staying close or visiting a farm affected by this bacterium, has been reported (Cheong *et al*., 2019). The acquisition of *C. abortus* infection in humans may be similar to that which occurs in animals. The most frequent path is the inhalation of infected aerosol from fetal fluids, urine, or stools, and such aerosol may remain in a place where infected animals have been held. The early identification of symptoms and the implementation of laboratory evaluation that confirm human infection with *C. abortus* diagnosis, followed by suitable medical treatment can diminish the negative effects in pregnant women, and even reduce the risk of miscarriage. Unfortunately, no effective *Chlamydia* vaccines for humans are currently available (de la Maza *et al*., 2017).

Chlamydia pecorum's zoonotic potential is still unknown with no strong evidence supporting this (Berri *et al*., 2009). Further studies are needed to evaluate the zoonotic role of this bacterium.

6.1.2 *Coxiella burnetii* and *Chlamydia* spp. mixed infection in the reproductive tract

A study on ruminant flocks with problems of abortion-causing diseases has evaluated 149 vaginal swab samples by m-PCR to simultaneously investigate for *C. burnetii*, *C. abortus,* and *C. pecorum* (Berri *et al*., 2009). From the samples analysed, two vaginal swabs were m-PCR positive of both *C. abortus* and *C. burnetii*, but no simultaneous infection with the three bacteria was detected. Additionally, in an evaluation of vaginal swabs collected from 644 animals with a history of abortion within the last 12 months, clinical vaginitis or clinical endometritis, the presence of co-infections was not evidenced (Petit *et al*., 2008). Simultaneous infection by *C. abortus* and *C. pecorum* had also been demonstrated in vaginal swabs taken from small ruminants grazing in different areas of Algeria (Merdja *et al*., 2015).

6.1.3 Case-Control approach

This sort of epidemiological study has a retrospective nature, where the outcome of attention is initially specified and then subjects, in this case, animals, are chosen without (controls) and with (cases) suitable for the specified outcome. The researcher then looks back in the time to assess both groups for a risk factor, other exposure factor or any treatment of concern (Dupépé *et al*., 2019). These groups are then contrasted to determine the frequency of the outcome being evaluated. Studies with the case-control design are unable to answer questions about the incidence or the prevalence of a disease. However, they may test hypotheses about causation and therapeutic efficacy, thus having an analytic nature in contrast with other observational study designs, such as case-series or cross-sectional studies, that are more intrinsically descriptive.

A case-control study is a kind of observational investigation widely used in epidemiology in which two groups with differences in a specific outcome are compared on the basis of some presumed causal element. Moreover, these case-control studies are frequently utilised to find factors that may contribute to a specific condition by contrasting subjects with a condition (named as cases) with subjects who do not have that specific condition but are otherwise similar (controls).

Some criteria are essential when designing a robust case-control study. For instance, outcome, eligibility, and exposure criteria must be well specified (Dupépé *et al*., 2019), cases and controls should be chosen from one population (Schulz & Grimes, 2002), and controls should be specified by their outcome condition, for example, those that do not have the outcome being studied (Mayo & Goldberg, 2009). Case-control studies are, when design characteristics are rigorously carried out, effective epidemiological approaches to explore the relationship between a disease, in this case the abortion, and a particular factor of interest. Case-control studies estimate the strength of the association between a variable and the outcome being investigated in the form of an odds ratio (OR). A case-control study typically starts with a fixed number of diseased and non-diseased individuals so cannot calculate risk as don't know total population with/without disease. In case-control studies where the size of the population at risk is not known it is not possible to risks. When the outcome of interest being investigated is infrequent or rare, and cases and controls are at small risk for developing the outcome, relative risk (RR) can be approximated by the OR (Newman *et al*., 2013).

A case-control design has been commonly used in analytical research in veterinary epidemiology, specifically investigating the role of different abortifacient agents. For instance, several studies have been done using this approach to address the role of *Neospora caninum* in the frame of reproductive failures. Specific PCR for *N. caninum* were performed in aborted and non-aborted fetuses which came from healthy animals at the abattoir (Sager *et al*., 2001). Additionally, a case-control study was done to explore the connection between seropositivity and *N. caninum* and risk of abortion on cattle farms. This evaluation has revealed a substantial association between the seroprevalence against *N. caninum* and the incidence of abortion (Ghalmi *et al*., 2011).

Results obtained using case-control studies are expressed as odd ratios. The odds ratio in case control studies are determined as the odds of exposure in cases divided by the odds of exposure in controls. The OR illustrates the odds that an outcome will occur given a specific exposure, contrasted to the odds of the outcome occurring with no exposure (Szumilas, 2010). See the example in the below:

Table 6.1: Contingency table for case-control studies.

<table>
<tr><td colspan="2"></td><td colspan="2">Disease
(occurrence of abortion)</td></tr>
<tr><td rowspan="4">Exposure
(presence of Chlamydia)</td><td></td><td>Yes (cases)</td><td>No (controls)</td></tr>
<tr><td>Yes</td><td>a</td><td>b</td></tr>
<tr><td>No</td><td>c</td><td>d</td></tr>
<tr><td></td><td>a + c</td><td>b + d</td></tr>
</table>

Odds of exposure in cases = a / c

Odds of exposure in controls = b / d

Odd Ratio = Odds in cases / Odds in controls = (a / c) / (b / d) = ad / bc

The case-control approach was also used to investigate *C. burnetii* and the risk of abortion in the veterinary field and medicine. For instance, serologic markers of *C. burnetii* infection and spontaneous abortion were investigated in women. Blood samples were screened for IgG or IgM *C. burnetii* immunoglobulins using ELISA and IFA. No proof of a higher titer of antibodies against *C. burnetii* in patients that later experienced a miscarriage was found (Nielsen *et al*., 2012). Furthermore, serum samples from mid- or late gestation aborted cows on their first pregnancy and cows with similar age but with no history of abortion were analysed serologically. This study found no meaningful relationship between the occurrence of abortion and *C. burnetii* seropositivity (Changoluisa *et al*., 2019).

Similarly, epizootic abortion associated with *C. abortus* and *C. pecorum* infections was investigated in water buffalo (*Bubalus bubalis*) by case-control approach. Fourteen buffaloes which had aborted during the first pregnancy were utilised as cases, while another fourteen pregnant and healthy herd-mates were treated as a control (Greco *et al*., 2008). As mentioned previously in this thesis, detecting bacterial DNA in a clinical sample, in this case, finding *C. abortus* DNA by PCR does not confirm that the bacteria observed are infectious. For instance, *C. abortus* have been detected in swab samples from clinically healthy animals that had not aborted (Wang *et al*., 2001).

6.1.4 Objectives

This case-control study aims to estimate the association of *Chlamydia abortus* detection in vulvo-vaginal swabs and the occurrence of abortion. This case-control methodology will investigate whether the pathogen's presence predisposes the occurrence of abortion by analysing similarities and differences between the data collected on each group of animals. The chapter also aims to adopt a molecular protocol to have a rapid and efficient PCR test widely available for *Chlamydia* investigation.

6.2 Materials and Methods

6.2.1 Study Design and General Sampling Approach

Vets and farmers that sent bovine abortions to the Plataforma de Salud Animal "Veterinary Diagnostic Laboratory" at INIA were contacted for further on-farm sampling. After participation was agreed, the researcher's visit to the corresponding farm was coordinated to carry out the sampling of aborted and non-aborted dams. To achieve this purpose, two herd mates were chosen for each aborted dam (case). These herd mates animals were considered as "controls" in this case-control study. All cases dams had aborted within the last three weeks. In those situations where the sampling was delayed and exceeded this expected time due to external reasons, animals were not sampled.

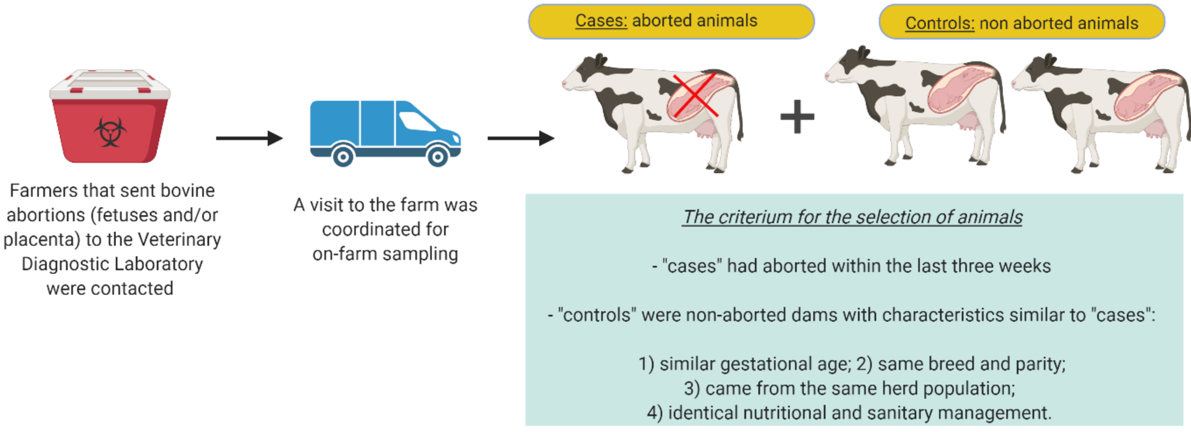


Figure 6.2: General sampling approach and criteria for the selection of animals.

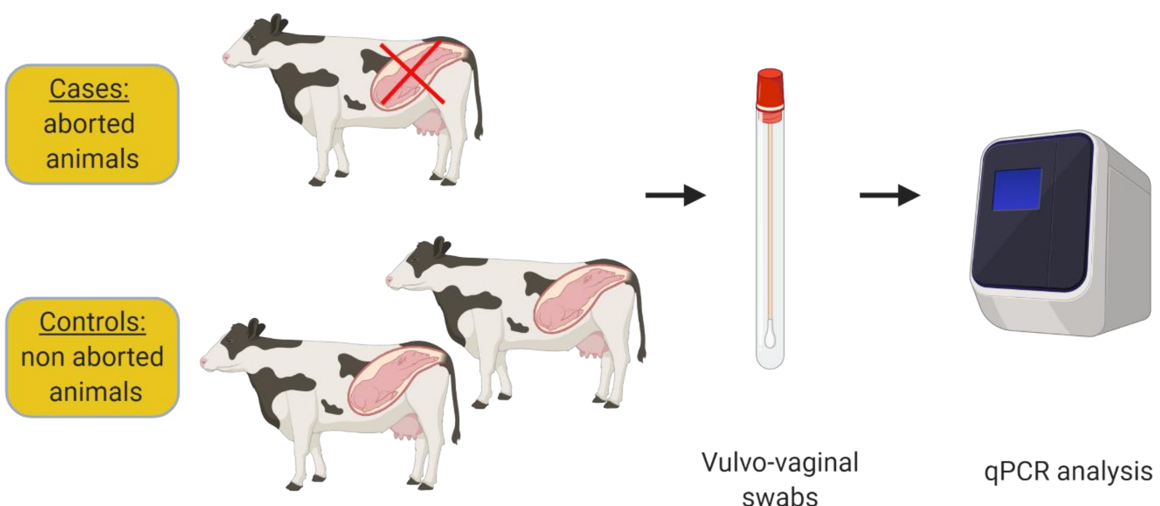


Figure 6.3: Aborted and non-aborted evaluation workflow.

Figure 6.4: Vulvo-vaginal swab sampling.

6.2.2 The criterium for the selection of control animals

Dams were required to match a strict inclusion criterion in order to be chosen as controls animals. These criteria comprised the following aspects: non-aborted animals, dams with a similar gestational age as the cases, same breed, and parity (primiparous or multiparous); and coming from the same herd population with identical nutritional and sanitary management. These criteria for selecting animals were established to ensure that all case and control dams were exposed to any potential risks to a similar degree. All control herd mates were visually healthy dairy cattle.

6.2.3 Vulvo-vaginal swab sampling

A vulvo-vaginal swab sample was collected from each case and each control animal using dry sterile cotton-tipped plastic swabs. First, the perineal/vulvar area was cleaned with a dry paper towel to minimise the sample's external contamination with urine, faeces, or dust. The swab stick was introduced into the vagina by rotating movements. Mild friction was applied in areas such as the lateral walls, the clitoris, and the vulvar lips' internal walls to complete sampling. The swab sticks were placed in sterile 15-mL Falcon tubes containing 5mL of phosphate-buffered saline (PBS, pH 7.2), and were kept refrigerated at 4 °C in ice-pack containers until they had arrived at the laboratory.

Once at the laboratory, swab sticks were first squeezed out against the Falcon tube wall, then each stick was removed and discarded. The samples were then concentrated by centrifugation at 14,000 g

for 20 minutes at 4°C. The resulting pellet was transferred to a 1.5-mL plastic microtube and kept stored at −20 °C until DNA extraction (Figure 6.5).

Gross alteration on the vaginal mucosa was visually investigated at the moment of sampling. Particular attention was taken when exploring the dorsal vaginal wall and the clitoral fossa because these areas are more commonly affected, and lesion usually are presented here. This examination was guided by the classification system of the vaginal lesion score (VLS) criteria proposed by Rae *et al*., 1993. A score 0 = no lesions, a score of 1 = mild inflammation with a low number of vesicles, a score of 2 = presence of vesicles and/or pustules accompanied by moderate inflammation, a score of 3 = marked tissue inflammation with pustules, and score 4 = severe inflammation, erosion, haemorrhage and/or purulent lesions (Figure 6.6).

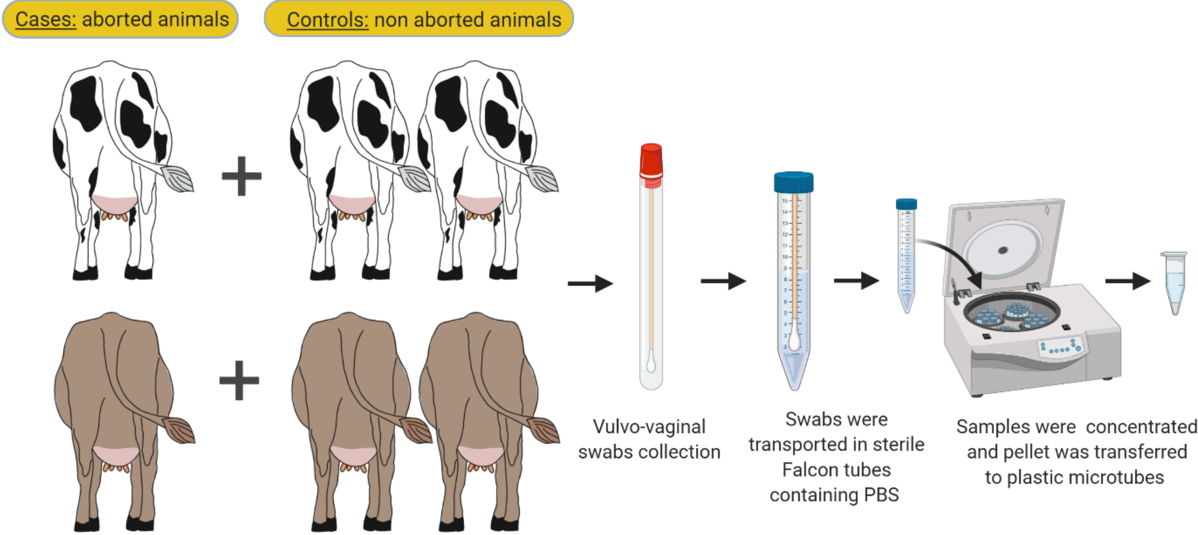


Figure 6.5: Vulvo-vaginal swabs sampling and workflow.

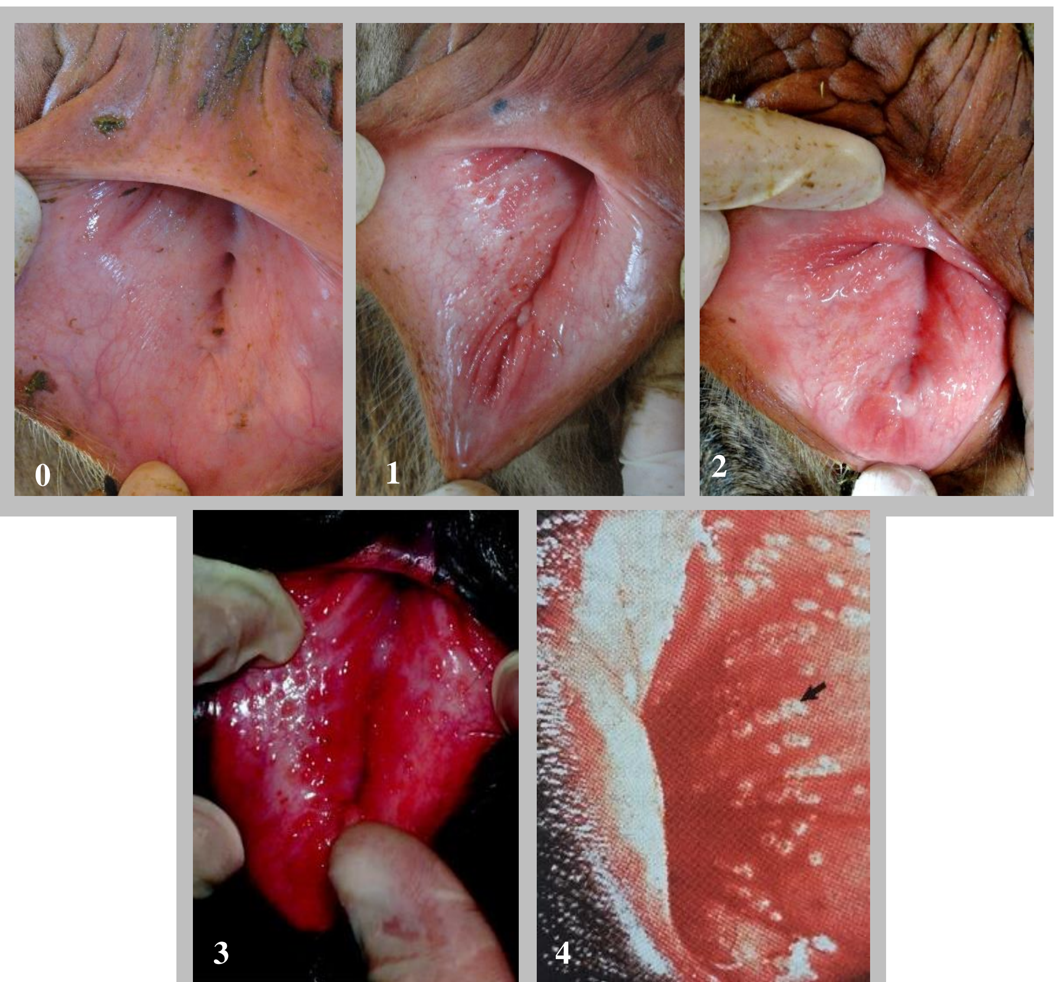


Figure 6.6: Vaginal lesion score. Criteria proposed by Rae *et al.,* 1993. Where 0 = no lesions, 1 = mild inflammation with a low number of vesicles, 2 = presence of vesicles and/or pustules accompanied by moderate inflammation, 3 = marked tissue inflammation with pustuled, and 4 = severe inflammation, erosion, haemorrhage and/or purulent lesions. Pictures were gently provided by Pescador C. A. (normal mucosa and lesion score 1 and 2), or obtained from published resources Gaeti *et al.,* 2014 (lesion score 3) and Zachary *et al*., 2013 (lesion score 4).

6.2.4 DNA extraction from vulvo-vaginal swabs

High-quality DNA was manually extracted from the resulting pellets obtain from the vulvo-vaginal swab samples and preserved in 1.5-mL plastic microtubes as mentioned above, utilizing a commercial

DNA extraction kit (QIAgen DNeasy Blood & Tissue DNA, GmbH, Hilden, Germany). This commercial kit was chosen because it had been used before for DNA extraction from vaginal swabs and had shown promising results in qPCR *C. abortus* amplification (Livingstone *et al*., 2017). The manufacturer's guidance was followed with slight modifications. The kit employed a column centrifugation system for DNA extraction and included the following steps:

1. 250 µl of the sample were added into the sterile microtube.

2. 200 µl of the lysis buffer (buffer AL) were added to the sample. Samples were mixed thoroughly by vortexing. *Buffer AL can form a precipitate upon storage. If needed, warm to 56°C until this precipitate had completely dissolved.

3. 200 µl ethanol (96–100%) was added to the sample and mixed again thoroughly by vortexing.

4. The mixture from the previous step was pipetted into the DNeasy Mini spin column placed in a 2 ml collection tube.

5. Centrifuged at 6000 x g (8000 rpm) for 1 min. Flow-through and collection tube discarded.

6. The DNeasy Mini spin column was placed in a new 2 ml collection tube. 500µl buffer AW1 was added. Buffer AW1 is a wash buffer that roles as a stringent wash with a low concentration of guanidine hydrochloride.

7. Centrifuged for 1 min at 6000 x g (8000 rpm). Flow-through and collection tube discarded.

8. The DNeasy Mini spin column was placed in a new 2 ml collection tube. 500µl buffer AW2 were added. Buffer AW2 is a Tris-based ethanol solution to remove salts.

9. Centrifuged at 20,000g for 3 minutes. Flow-through and collection tube discarded.

10. The DNeasy Mini spin column was placed in a sterile 1.5ml microtube. The microtube tube was furnished an identification label.

11. 200 µl Buffer AE were pipetted directly onto the DNeasy membrane and incubated at room temperature for 1 min. * Elution with a lower volume of buffer AE, instead of 200 µl, which improves the final DNA concentration but reduces the overall DNA yield.

12. Finally centrifuged for 1 min at ⩾ 6000 g (8000 rpm) to complete elution. DNeasy Mini spin column discarded.

Centrifugation was done always at room temperature (15–25°C). The recovered DNA was quantitatively and qualitatively evaluated by spectrophotometry using a Nanodrop (ND1000, NanoDrop Technologies, Inc, USA) before downstream application. After this, DNA samples were immediately stored at -20°C or -80°C, depending on the expected moment of further utilisation.

6.2.5 *Chlamydia abortus* reference strain

The *C. abortus* S26/3 strain was generously provided by PhD M. Livingstone and PhD D. Longbottom from the Moredun Research Institute, Edinburgh, United Kingdom.

6.2.6 Cloning into plasmid

The preparation of a plasmid harbouring the element *OmpA* as a template was required for quantifying this single-copy gene by qPCR. The element *OmpA* encodes the major outer membrane protein (MOMP). Standard curves were built for absolute quantification by employing serial dilutions of the plasmid containing the fragment of the *OmpA* element of *C. abortus*. First, the target sequence's 86-bp-length fragment was amplified by PCR final point using as template the genomic DNA extracted from *C. abortus* S26/3 strain, employing the same set of primers as had been used for the qPCR. Amplification was done by *Taq* polymerase to ensure 3′A-overhangs on the PCR product necessary for TA cloning. The non-template-dependent terminal transferase activity of the *Taq* polymerase integrates a single deoxyadenosine (A) to the 3´ ends of the PCR products. This aspect is critical for the correct ligation of the PCR inserts to the commercial vector because it has single, overhanging 3´ deoxythymidine (T) residues. The PCR product was examined by agarose gel electrophoresis, where a single band of the expected sized was detected. This PCR product (amplicons) was cloned into pCR®2.1-TOPO vector using the TOPO®TA cloning kit (Invitrogen, Carlsbad, CA, USA). After ligation, the recombinant vector was transformed into chemically competent *Escherichia coli* TOP10 cells (Invitrogen).

For the competent cells production, *E. coli* TOP10 cells were grown in Luria-Bertani (LB) broth medium at 37°C until an optical density of ~0.4 OD at 600 nm was reached. Then, successive centrifugation and resuspension stages of the pellet in 50 mM cold $CaCl_2$, occurred, followed by a final 30 min on-ice incubation to the prepare cells so that they would transform into competent cells. For the transformation, 2 µL of the TOPO® cloning reaction were added to 50 µL of competent *E. coli* cells. After a 20 min on-ice incubation, cells were subjected to a heat-shock (45 s at 42°C) without shaking. These transformed *E. coli* (100 µL) were plated on LB-Agar containing 50–100 µg/mL ampicillin and 40 mg/mL X-gal at 37°C for 24 hours. The enzyme beta-galactosidase (*LacZ*) uses the X-gal as a substrate, turning a deep blue colour when the enzyme effectively uses it. When the insert is adequately incorporated into the vector, the gen *LacZ* is disrupted, leading to the generation of white colonies. Thus, an efficient TOPO® cloning reaction would generate numerous white colonies; those colonies with a disrupted *LacZα* were selected. The recombinant plasmid DNA was recuperated using a DNA-spin

plasmid DNA purification kit, and the final plasmid was sequenced (Applied Biosystems, Foster City, CA, USA). The resultant plasmid was quantified spectrophotometrically at 260 nm by Nanodrop ND-1000 (Thermo Scientific, Fremont, CA, USA) and the 260/280 ratio measured its purity. The copy number of plasmids was estimated based on the plasmid quantity by using the next equation:

$$\text{Plasmid copies}/\mu l = [\ \text{plasmid DNA concentration (ng}/\mu l) \times (6.02\times10^{23})\ ] / [\ \text{plasmid length (bp)} \times 660]$$

The equation included the following components, the plasmid length (bp) which denoted the vector length (3.9 kb for the pCR®2.1-TOPO) and the length of the PCR product (86 bp), 660 which indicates the average molecular weight of one base pair (g mol^{-1} bp^{-1}), and 6.02×10^{23} which represents the Avogadro number. The number of molecules in the plasmid DNA preparation was established using the DNA concentration, the plasmid's molecular weight, and the Avogadro's number.

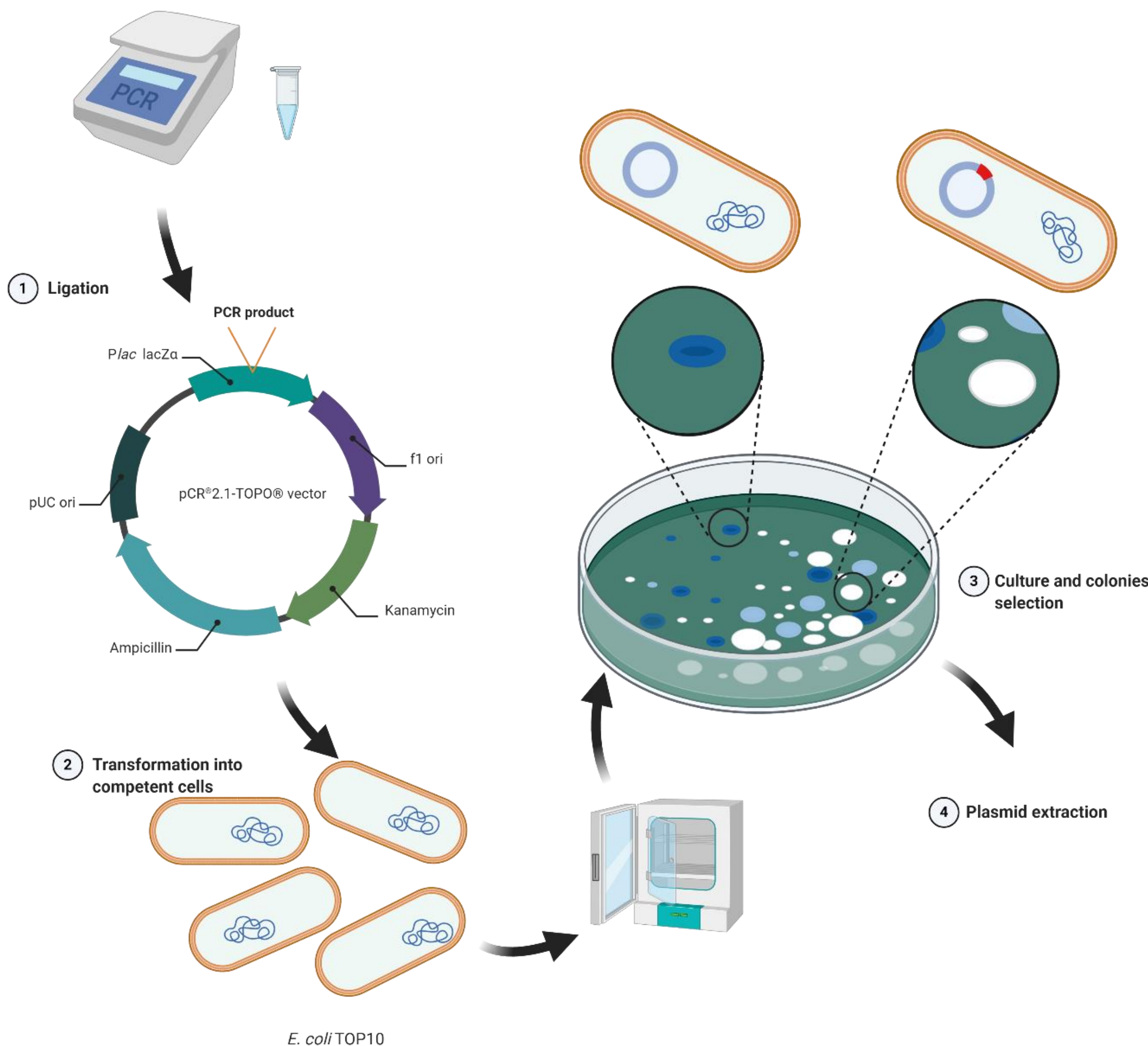


Figure 6.7: Plasmid cloning.

6.2.7 Construction of the qPCR standard curve

Ten-fold serial dilutions of the recombinant plasmid DNA were utilized to create the standard curves used for the quantitation assay. Five independent serial dilutions (ranging from 10^7 copies/µl to 10^2 copies/µl) of the plasmid were used to create a standard curve from which each reaction's sensitivity could be determined, and the amount of *C. abortus* DNA in the original sample was estimated. The dilution series was created to comprise all possible template quantities encountered in the unknown vulvo-vaginal swab samples. The dilutions were done with sterile dH_2O and were loaded in different PCR runs. Each dilution point was run in triplicate. Thus, three technical replicates and five biological replicates of each dilution point were evaluated. Whereas technical replicates referred to the same samples triplicated in the qPCR plate set up, which help adjust for potential errors during pipetting,

biological replicates implicated temporally separated experimental replicates to correct experimental error.

Standard curves were generated by plotting the linear regression of the plasmid copy number against the quantified Ct values [also named as cycle quantification (Cq) or crossing point (Cp)] for the 10-fold serial dilutions of the recombinant plasmid DNA employed as quantification standards. The Ct value indicates the cycle number at which the fluorescence signal generated within a reaction crosses the threshold. It is inversely correlated to the logarithm of the initial copy number, and the Ct value from a particular well indicates the point during the reaction at which enough amplicons have accumulated (Dorak, 2007). For each dilution point, eight Ct values were evaluated and averaged to construct the standard curve. The threshold is usually 10X the standard deviation of Rn for the early PCR cycles (baseline) set in the region of the PCR product's exponential growth (Dorak, 2007). The threshold is a numerical value provided for every run to calculate the Ct value for each amplification. Although some authors believe the lowest threshold as the best option, there is no single optimal threshold value (Dorak, 2007). Analytical software was used to correct the threshold to make the standard curve present the highest r^2 value.

Serial dilutions points were created to have an extended dynamic range; this involves the range of initial template concentrations over which Ct values are obtained (Dorak, 2007). As the dynamic range is large, there was greater opportunity to identify samples with high and low copy number in a single run. In absolute quantification, the interpolation within this range is precise, but the extrapolation beyond the dynamic range is not suggested as inaccurate estimations could result.

6.2.7.1 Threshold and baseline settings

The threshold was placed above any background amplification noise and within the exponential phase. Baseline settings were generated by the software auto-baseline function. The Ct values were generated when amplification curves crossed the specified threshold.

6.2.7.2 qPCR efficiency and sensitivity

The qPCR amplification efficiency (E) was calculated according to the following equation [$E = 10^{(-1/slope)} - 1$] or [$E(\%) = (10^{-1/slope} - 1) \times 100\%$] using the slope of the generated standard curve. The reaction's efficiency should be as close to 100% as possible, exhibiting a two-fold increase of amplicon at each cycle. The assay's precision and the variability between Ct values among technical replicates at each dilution were calculated. The mean Ct values, the standard deviation and CV (coefficient of

variation) (%) from each dilution were also estimated. The detection limit of the assay was settle based on the highest dilution point with a Ct value.

6.2.8 Selection of primers and probe for qPCR

A probe-based qPCR was carried out for the absolute quantification of the *C. abortus* in vulvo-vaginal swab samples from aborted and non-aborted dams. This quantification was done on a QuantStudio 5 Real-Time PCR System instrument (Life Technologies Inc.), based on the detection of *OmpA* as described (Livingstone *et al*., 2009) with some adjustments. For this purpose, the following primers and probes were employed, forward primer Cab-F, 5′- GCGGCATTCAACCTCGTT -3′, reverse primer Cab-R, 5′- CCTTGAGTGATGCCTACATTGG -3′, and TaqMan® probe Cab-R, 5'- GTTAAAGGATCCTCCATAGCAGCTGATCAG-3′. The probe was fluorescently labelled with a 6-carboxy-fluorescein (FAM) as the reporter particle attached at the 5'-end. Also, the probe had 6-carboxytetramethylrhodamine (TAMRA) as the 3'-end quencher.

The qPCR reactions contained 5,0 µl of 2X SensiFAST^{TM} probe No-ROX Kit (Bioline), 0.4 µl of primers (400 nM final concentration) and 0.25 probe (250 nM final concentration), 1.5 µl DNA template, and 2.45 µl of free-nuclease water to complete the total volume of the reaction (10 µl). The thermal cycling conditions comprised an initial denaturation for 2 min at 50°C, followed by 10 min at 95°C and finally 45 cycles of 15 s at 95°C and 1 min at 60°C. The total cycle lasted one h and 9 min. Each vulvo-vaginal sample was run in triplicate. The dilution points of the plasmid's serial dilutions containing *OmpA C. abortus* as a template were incorporated in each plate for the standard curve. Each PCR run also included an NTC that contained ultrapure distilled water as a template.

6.2.9 Data analysis

The relationship between the detection of *Chlamydia* in vulvo-vaginal swabs and the occurrence of abortion was evaluated using the χ^2-test. The strength of any association between the bacterium's molecular detection and abortion was acquired from the odds ratio (OR). The precision of the OR was estimated by the calculation of the 95% confidence interval (CI). The 95% CI were calculated using the formula: upper 95% CI = e ^ [ln (OR)+1.96√ (1/a+1/b+1/c+1/d)] and lower 95% CI = e^ [ln (OR)−1.96√ (1/a+1/b+1/c+1/d)] (Szumilas, 2010). Where a = number of exposed cases (aborted dam with a positive *Chlamydia* vulvo-vaginal swabs result), b = number of exposed controls (non-aborted dam with a positive *Chlamydia* vulvo-vaginal swabs result), c = number of unexposed cases (aborted dam with a negative *Chlamydia* vulvo-vaginal swabs result) and d = number of unexposed controls (non-aborted

dam with a negative *Chlamydia* vulvo-vaginal swabs result). Small CI suggests a higher precision of the OR, whereas a large CI shows a low precision of the OR. The $\chi2$-test was utilized to establish whether there was any meaningful difference in the frequencies of *Chlamydia* detection on vulvo-vaginal swabs between cases and controls.

6.3 Results

6.3.1 Descriptive data of vulvo-vaginal swab samples

Fifty-nine vulvo-vaginal swabs from aborted animals and 118 samples from herd mates were collected. A total of 177 vulvo-vaginal swabs were finally analysed. The samples were obtained from 16 different herds located in four different departments/areas as follow Colonia (8 farms), San José (6 farms), Soriano (1 farm) and Flores (1 farm). All these departments are situated in the central dairy region of Uruguay.

6.3.2 Establishment of the standard curve and its sensitivity

The recombinant plasmid DNA concentration was 759.51 ng/µl, the A260/A280 ratio was 1.98, and the A260/A230 ratio was 2.21. The conversion into copy number of plasmid DNA, using the formula introduced in the material and methods section, was 1.77×10^{11} copies/µl. The resulting plasmid was first diluted at 1:100. The standard curve was finally constructed employing the ten-fold serially diluted plasmid DNA from 10^9 to 10^0 copies/µl. The threshold was set above background amplification noise. The Ct values were plotted against the known copy number of each dilution point of the standard control. The slope of the calibration curve was -3.236, and the Y-intercept was 42.01. The samples were quantified by employing the formula Y=-3.236X+42.01 (Y=threshold cycle, X=log starting quantity). For all standard curves, r^2 values were high ($r^2 > 0.98$).

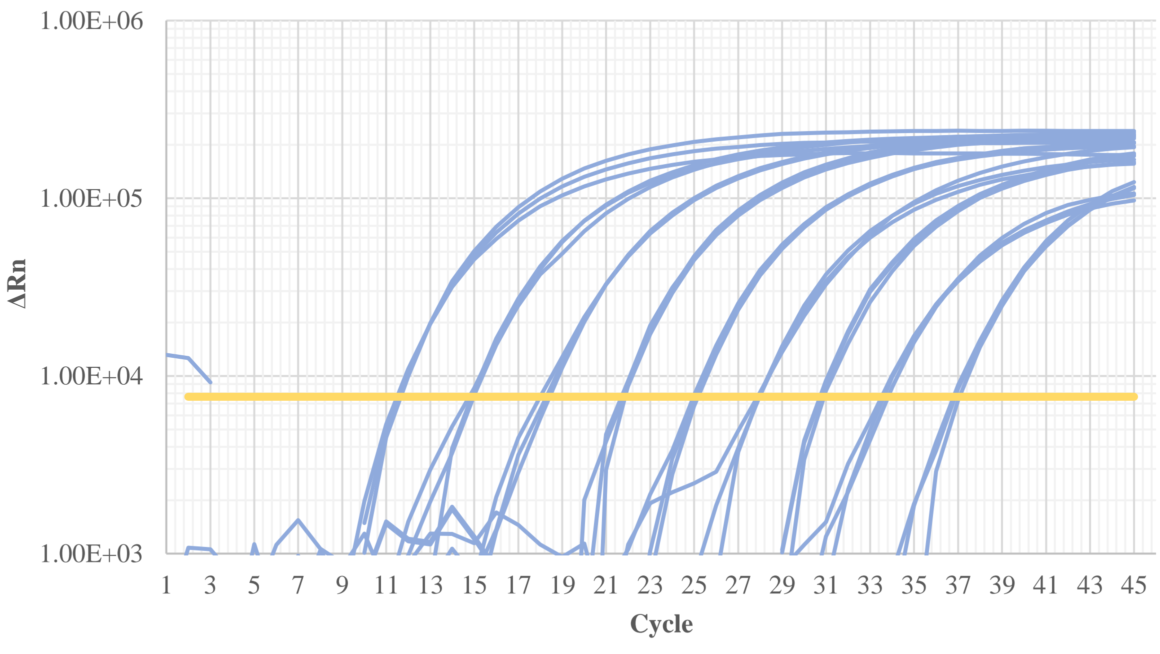


Figure 6.8: Amplification plot for *Chlamydiaceae*.

Serial dilutions of the recombinant plasmid DNA ranging from 10^9 to 10^0 copies/µl were evaluated by qPCR to assess the assay's sensitivity. The detection limit was 18 copies per reaction (10^1 copies/µl). The assay's precision was investigated by measuring Ct values for the eight replicates of each serial dilution point. Data from the eight replicates from each concentration were used to calculate the mean Ct, standard deviation and the coefficient of variation (CV). The CVs values showed the variability among results, giving a sense of how reproducible the assay was.

The cloned plasmid was used to create standard curves for *C. abortus* using the copy numbers and mean Ct values. Standard curves were linear when starting and plasmid copy numbers ranged from 10^1 to 10^9 (Figure 6.8). Linear regression of Cq value versus $\log_{10}$ mean concentration for corresponding ten-fold serial dilutions provided the equation later applied to Cq values obtained from vulvo-vaginal swabs to obtain copies/ul. Finally, the genome copy number of *C. abortus* from the vulvo-vaginal swabs was reliably estimated. Low variability, shown by reducing a CVs range, indicates efficient assay reproducibility. A slope of -3.236 can be estimated from the graph, which suggests a high amplification efficiency of the PCR of around 98%.

Table 6.2: Inter-assay variability of *Chlamydia abortus* amplification.

Nº of copies/µl	**Replicate 1**	**Replicate 2**	**Replicate 3**	**Mean Ct**	**SD[a]**	**CV[b] (%)**
1.77×10^9	10.050	9.647	9.913	9.870	0.205	2.08
1.77×10^8	14.578	14.785	14.120	14.494	0.340	2.35
1.77×10^7	19.249	18.353	18.862	18.821	0.450	2.39
1.77×10^6	21.970	21.864	23.182	22.339	0.732	3.28
1.77×10^5	25.673	26.212	27.388	26.424	0.877	3.32
1.77×10^4	28.756	29.548	30.589	29.631	0.919	3.10
1.77×10^3	31.241	31.749	32.952	31.981	0.879	2.75
1.77×10^2	36.422	36.812	36.898	36.711	0.254	0.69
1.77×10^1	38.203	36.360	UN	37.281	1.303	3.50
1.77	UN	UN	UN	NA	NA	NA

[a] SD: Standard deviation
[b] CV (%): Coefficient of variation
UN: undetermined. No template detected.
NA: not applicable, no target nucleic acid detected for calculation of mean, standard error or coefficient of variation.
A threshold value of 7.64E+0.3 was set for the determination of Ct values.

This table shows the amplification plot for *C. abortus*. The Cq values increased along the concentration of the starting template is reduced. The threshold was set at 0.01 above the background amplification signal and within the exponential phase.

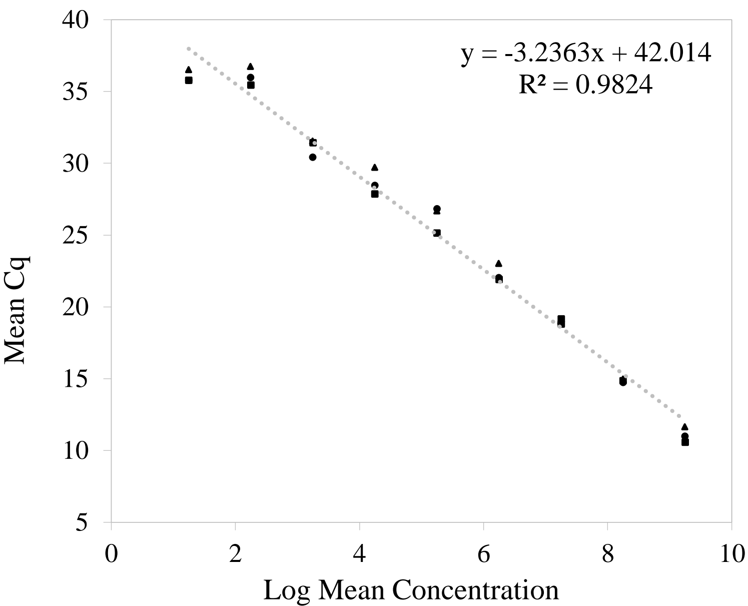


Figure 6.9 Amplification and standard curve construction. The amplification curves were constructed using ten-fold dilutions of recombinant plasmid DNA ranging from 10^9 copies/µL to 10^0 copies/µL. The standard curve equation was Y=−3.2367X+42.014. Concentration implies the template copy number per reaction. In x-axis: standards of DNA, y-axis: corresponding cycle threshold (Ct) values.

6.3.3 Descriptive data for the qPCR

Chlamydia abortus DNA was not detected in either vulvo-vaginal swabs samples from aborted or non-aborted dairy animals. Consequently, this finding limited the further statistical investigation of the association between the detection of *C. abortus* on the genital mucosa and the occurrence of abortion.

6.4 Discussion

Reproductive disorders and subfertility are significant causes of animals' premature culling in dairy herds worldwide because they severely decrease dairy farms' profitability. *Chlamydia abortus* has been thoroughly described as an abortifacient agent in ruminants, but the role of *C. pecorum* in losses in bovine gestations remains unclear. Some *C. pecorum* strains have been strongly linked to infertility and metritis in cows (Mohamad & Rodolakis 2010). Similar to what occurs with *C. burnetii*, *Chlamydiales* are not considered main infectious abortifacient causes in cattle; thus, they are frequently grouped under a common subheading named "other bacteria" or "minor or miscellaneous abortifacient bacterial agents" that produce bovine abortion. They are infrequently included in routine laboratory testing for diagnosis.

Accordingly, hitherto there has been no systematic investigation of chlamydial infection in cows in Uruguay, for instance, the prevalence of chlamydial infections, and their potential effect on the bovine reproduction of local cattle is generally unknown. This fact is not surprising, considering that other pathogens, typically deemed as frequent causes of bovine abortion, have dominated the field of local research and the available economic resources. The limited research done on this field comprises a serologic evaluation by ELISA of 318 bovine dams from two Uruguayan areas (Durazno and Florida) (Cattáneo *et al*., 2009). This investigation revealed that 28% of the samples analysed (89/318) were positive showing, for the first time, the significant presence of antibodies against *C. abortus*.

This study aimed to assess the presence of *C. abortus* DNA in vulvo-vaginal swab samples from aborted and non-aborted cattle from commercial herds in Uruguay. For this, a species-specific DNA-based qPCR approach designed to amplify the *OmpA* gene sequence was implemented. As the original idea was to quantify the bacterial burden, a fragment of the interest gene was first cloned into a plasmid vector to construct the standard curve for absolute quantification. Evidence gathered in this study may suggest that *C. abortus* is a pathogen not significantly associated to abortion in cattle in Uruguay. However, more investigation is needed to support this observation. *Chlamydia abortus* DNA was not detected in any samples analysed. The fact that all vulvo-vaginal swabs samples were *C. abortus* negative may not necessarily indicate the absence of the bacterium. This constraint in the detection may have been due to a reduced number of bacteria in the samples, a reduce bacterial concentration to the point of being insufficient for PCR detection. The bacterial investigation on a single vulvo-vaginal sample per animal could have conditioned the detection process, as it has been shown that the demonstration of *Chlamydiaceae* on the vaginal mucosa often needs repeated sampling, especially on heifers (DeGraves *et al*., 2003).

Evaluations conducted in small ruminants revealed that large amounts of chlamydial material could be detected in uterine secretions or vaginal exudate from ewes from roughly a day before the abortion up

to 2 to 3 weeks after it (Sanderson & Andersen, 1989; Papp *et al*., 1994; Aitken, 2000; Marsilio *et al*., 2005), while the secretions from goats may contain *Chlamydiae* from as early as nine days before the abortion to 2 weeks following it (Rodolakis *et al*., 1984). In the current study, the vulvo-vaginal swab samples were gathered up to 3 weeks after the abortion occurred. This time-frame was defined based on the data reported in small ruminants because no information about bacterial shedding dynamic in cattle is available. Reduced *Chlamydiae* excretion windows in cattle may have detrimentally affected the current study's bacterial detection and caused negative qPCR results.

In Uruguay, sheep and cattle frequently graze together; thus, they could be exposed to similar agents. Typically, *C. abortus* is endemic in the sheep population in most countries worldwide; however, infection in cattle is much more sporadic. Curiously, the *C. abortus* infection rate seems to be very low in Uruguayan sheep flocks. For instance, a concurrent investigation, where 62 aborted ovine fetuses from commercial flocks located throughout Uruguay were investigated, revealed no *Chlamydia* spp. infection (Dorsch *et al*., unpublished). This finding is in line with the low detection rate of *C. abortus* evidenced in dairy cattle. There is no genetic difference between strains affecting cattle and sheep (Seth-Smith *et al*., 2017). The sequencing of the whole genome of *C. abortus* strains obtained from goats, sheep, and cattle showed high conservation among strains found in different ruminant species. Reduced intraspecies variation among *C. abortus* strains was detected fundamentally in the PZ region (Longbottom *et al*., unpublished; Doull, 2016). Despite the absence of genetic differences between strains infecting cattle and sheep, *C. abortus* is an organism that is mainly transmitted at lambing/calving time when, while they graze together most of the time, sheep and cattle are likely to be separated.

Previous reports agree that the risk of transmission of *Chlamydiae* among cattle differed considerably depending on the location of the farm, whether it was situated in a rural, peri-urban or urban location; and the type of production systems used (Igayara-Souza *et al*., 2004; Jaouad, 2004). The probability of having an animal with a positive-*Chlamydia* PCR, based on the analysis of blood, milk or vaginal swabs, was shown to be higher in urban and peri-urban areas, contrasted with rural regions, and this seemed to be correlated with the intensification of the production as most intensive farming systems are located in urban areas where the demand for milk is high (Barkallah *et al*., 2018). Reproductive *Chlamydiae* infections found in urban areas, mainly abortions, occur mostly as enzootic problems, causing high economic costs (Igayara-Souza *et al*., 2004). Corresponding to what had been documented for *C. burnetii* infection, the risk of contracting *Chlamydia*, at the herd level, rises substantially as herd size grows (Al-Qudah *et al*., 2004; Yin *et al*., 2014; Merdja *et al*., 2015; Barkallah *et al*., 2018). Broadly speaking, larger herds may be connected to intensive management practices which are intrinsically more difficult to control, and which lead to closer contact between infected and susceptible animals. All

factors that rise animal-to-animal contact during an extended periods should be controlled. Uruguay has an extensive and mainly grazing-based dairy production culture. Unlike what occurs in other countries, in Uruguay, keeping cattle indoors during calving season is not common. Therefore, animals are not that closely congregated during the parturient period. This aspect should result in the lower contamination of pens, feed, and equipment. This may explain to some extent the lack of detection of *C. abortus* in dams and possibly the low incidence of *C. abortus* as a cause of abortion in Uruguay. *Chlamydial* shedding during parturition is the primary source of environmental contamination, enhancing the possible subsequent transmission to other animals (Papp & Shewen, 1996; Longbottom & Coulter, 2003). It has been reported that the risk of infection is likely to be decreased by using separate calving pens as this would minimise the contact of cows with contaminated materials (Jee *et al*., 2004).

A cross-sectional investigation conducted in the western region of Germany explored risk factors for *Chlamydia* spp. infection in dairy cows. This evaluation identified the following factors as most relevant to increased risk of *Chlamydia* spp. infection: the replacement of animals from outside suppliers, the utilisation of breeding bulls, the absence of separate calving pens, and inadequate cleanliness of walkways and bedding (Kemmerling *et al*., 2009). Farms can decrease the risk of *Chlamydia* spp. introduction to herds by limiting animal replacements from inside sources; however, it is not easy to accomplish this. Therefore, correct quarantine/confinement measures ought to be implemented when external replacement is the only available option. In a univariable analysis of related risk factors to *Chlamydial* infection, it was shown that the risk of infection was higher in ruminants in direct contact with soil than those animals in solid grounds (Barkallah *et al*., 2018). This point suggests that soil may supply contaminated residues (Coulon *et al*., 2012; Kebbi-Beghdadi & Greub, 2014). The presence of older animals within a herd has also been linked to elevated exposure risk to *C. abortus* (Sun *et al*., 2015).

In many species, *Chlamydia* spp. can be spread to the female genital tract by semen (Perez-Martinez & Storz, 1985). Previous findings showed natural mating as a risk factor for chlamydiosis, because of the frequent presence of *C. abortus* in clinically healthy bulls and animals experiencing vesiculitis (Storz *et al*., 1968; Kauffold *et al*., 2007; Kemmerling *et al*., 2009). Once bulls become infected, they behave as vectors for the bacterium. Recently the utilisation of artificial insemination was found to be a successful practice to diminish the risk of exposure to *C. abortus* (Djellata *et al*., 2020).

The utilisation of qPCR targeting the *OmpA* gene has been demonstrated to be a specific approach for *C. abortus* investigation (Sachse *et al*., 2009). The current results revealed no *C. abortus* detection in aborted dams or non-aborted herd-mates, suggesting this bacterium as of marginal significance in the dairy herds with reproductive problems analysed in this study. In an Austrian investigation where

cervical or vaginal swabs from 644 cows were evaluated, *C. abortus* was found in only 0.46% of the samples (3/644), whereas *C. pecorum* was detected more frequently, confirming previous studies (DeGraves *et al*., 2003), being detected in 9% of the swabs analysed (Petit *et al*., 2008). All positive-*C. abortus* samples corresponded to cervical swabs, with no bacterium detection in vaginal swabs (Petit *et al*., 2008). A later study also found *C. abortus* in bovine oviducts (Appino *et al*., 2015). The collection of swab samples from areas of the anterior reproductive tract may have helped to improve the likelihood of detecting *C. abortus*.

6.4.1 Limitations and further research

Positive control of amplification is an important component of quality assurance of the qPCR. The incorporation of an internal positive control of amplification would have helped to rule out inhibitors that could have negatively affected the amplification and ensured adequate DNA extraction.

Despite no *C. abortus* detection within the vulvo-vaginal swabs samples analysed here, serological evidence supporting *C. abortus* in Uruguayan dairy cattle is available. As part of the protocol, but not part of this thesis, all aborted and non-aborted herd-mates were bled, and serum samples stored for later serological assessment, possibly using ELISA. It would be interesting to investigate the proportion of seropositive animals within case dams and control dams.

Only a few evaluations had been carried out on *C. abortus* in dairy cattle in Uruguay, and the work presented here now comprises the majority of such research. More research is needed with enhanced sampling to determine that *C. abortus* as not relevant in local contexts. As mentioned by other authors, pair repeated samples in each animal could have been a good strategy to increase the likelihood of bacterial detection. This approach would be helpful fundamentally because the clearance of *C. abortus* from the genital tract is not well defined in cattle.

No local reports are available about the zoonotic role of *C. abortus*. Based on the results presented in this chapter, it can be estimated that *C. abortus* is not appreciably presented in dairy herds with a history of last-term abortion in Uruguay; however, considering the zoonotic potential of this bacterium, its deleterious effects on people should not be underestimated. The zoonotic risk, especially for pregnant women, comprises spontaneous abortion, stillbirths or preterm labour (Buxton, 1986; Longbottom & Coulter, 2003).

6.5 Conclusions

In Uruguay, only limited information is available about *C. abortus* in domestic production animals. This study has given insights into the evaluation of *C. abortus* in dairy cattle using a case-control approach which is the most frequent analytical epidemiological methodology. Aborted animals and their non-aborting herd mates from commercial dairy farms were assessed. Molecular investigation of *C. abortus* in vulvo-vaginal swab samples showed no evidence of *C. abortus* infection either in aborted or in control animals. Difficulties in identifying low-grade infection and the obtention of a single sample per animal would have limited the detection. The current findings lead the researcher to conclude that *C. abortus* is not a significant abortifacient agent in cattle in Uruguay.

Chapter 7: Serological evidence of **Coxiella burnetii** *infection in dairy farm and laboratory workers exposed to a bovine abortion outbreak in Uruguay*

The work presented in this chapter had been already published on the journal *Veterinary Sciences*. This publication can be founded in the Appendix A.

Rabaza, A., Giannitti, F., Fraga, M., Macías-Rioseco, M., Corbellini, L.G., Riet-Correa, F., Hirigoyen, D., Turner, K.M. and Eisler, M.C. Serological evidence of human infection with *Coxiella burnetii* after occupational exposure to aborting cattle. *Veterinary Sciences*, 2021, 8(9), p.196. https://www.mdpi.com/2306-7381/8/9/196/htm

7.1 Introduction

Q fever is a zoonotic disease produced by the broadly distributed, Gram-negative bacterium *Coxiella burnetii*. Many animal species can act as reservoirs of this pathogen, including birds, arthropods, companion, and production animals, but ruminants, notably goats, are the most frequent source of *C. burnetii* human infection (Eldin *et al.*, 2017). Tracing the sources of Q fever outbreaks is challenging as *C. burnetii* can be transmitted by at least six different routes. Human Q fever typically occurs after airway infection by the inhalation of aerosolised contaminated material from the placenta or birth fluids either during an abortion or the normal delivery of ruminants (Marrie, 1990c; Maurin & Raoult, 1999). Some authors proposed the oral route of transmission, with the ingestion of unpasteurised milk and raw dairy products as a risk factor for infection (Fishbein & Raoult, 1992; Gale *et al.*, 2015), although others dispute this, claiming the oral route as an infrequent transmission route (Krumbiegel & Wisniewski, 1970; Kazar, 1999). Likewise, transplacental (Stein & Raoult, 1998; Langley *et al.*, 2003), blood transfusional (Kersh *et al.,* 2013b), sexual (Milazzo *et al.*, 2001), and percutaneous (including tick-borne) transmission represent potential infection pathways, though these have been deemed rare (Raoult *et al.*, 1994; Raoult & Marrie, 1995). Furthermore, to trace back infections to their pathogen source, researchers must factor in how *C. burnetii* can be aerially dispersed across long distances (more than 5 km away) mostly in windy zones, which can make tracing the source of infection virtually impossible (Tissot-Dupont *et al.,* 2004). Additionally, *C. burnetii* presents a high resistance to environmental stressors, so the bacterium can reach places *a priori* thought to be beyond its reach, including urban areas (Schimmer *et al.*, 2010; Nusinovici *et al.*, 2015a).

Q fever is an occupational disease affecting people exposed directly or indirectly to livestock (Whitney *et al.*, 2009). The diversity of clinical outcomes is a major characteristic of human *C. burnetii* infection (de Alarcón *et al.*, 2003). The infection is asymptomatic in around 60% of cases (Hechemy, 2012). Clinical presentations can be either acute, including self-limiting febrile illness, hepatitis, pneumonia,

or central nervous system complications, or chronic, with endocarditis and chronic fatigue syndrome as the principal manifestations (Maurin & Raoult, 1999; Raoult *et al*., 2005). Acute Q fever is clinically characterised by a wide range of non-specific symptoms and signs, one reason why the disease is often misdiagnosed and underreported (Anderson *et al.*, 2013ab). Chronic Q fever results in higher mortality rates than the acute presentation (Sawyer *et al.*, 1987; Marrie & Raoult, 2002). Asymptomatic infection can progress to a chronic presentation, particularly in people with pre-existing conditions such as vascular grafts, cardiac valve disease, aneurysms, immunocompromise, pregnancy (Raoult *et al*., 2000; Fenollar *et al*., 2001; Landais *et al*., 2007).

The progression of Q fever will depend on the immunological status of the host and the biological characteristics of the bacterium (Camacho *et al*., 1995). The pathotype-specific virulence of *C. burnetii* is a hypothesis that might explain the proclivity of different isolates to trigger different outcomes of the disease. Analysis by molecular genetic methods of *C. burnetii* isolates classified the strains into six genomic clusters (groups I to VI) variously associated with either acute or chronic illness (Hendrix *et al.*, 1991). Despite this evidence supporting different genetic pathotypes, it is broadly accepted that host features are key factors in the chronicity of the disease, rather than traits of the *C. burnetii*-infecting strain itself (Leroy *et al*., 2011). For instance, people with chronic *C. burnetii* infection frequently present elevated production levels of IL-10 (Capo *et al*., 1996).

Due to variations of the lipopolysaccharides (LPS) of the bacterial outer membrane, *C. burnetii* has different antigenic phase variations (I and II) that determine diverse types of anti-*C. burnetii* immunoglobulins during infection (anti-phase I and anti-phase II IgM, IgG and IgA). The phase I *C. burnetii* strains have a full-length LPS with a complex O-antigen polysaccharide chain, whereas phase II strains have a severely truncated O-antigen. Most *C. burnetii* strains spontaneously switch between antigenic phase variations, with subsequent structural and compositional changes of the outer membrane (Ftáček *et al*., 2000). While the anti-phase I immunoglobulins reacted with the O-antigen, the anti-phase II immunoglobulins reacted with an ~3 kDa LPS (Hackstadt *et al*., 1985; Beare *et al*., 2018). The response facing phase I *C. burnetii* infection generates both anti-phase I and II immunoglobulins, and phase II infection only produces phase II immunoglobulins (Hackstadt *et al*., 1985). During infection, the human immunoglobulins are primarily reactive with phase II antigens, which appear to be the immunodominant antigen, although the reasons behind this have not been well-defined (Dupuis *et al*., 1985). The profile of serum immunoglobulins is commonly used to distinguish between acute and chronic exposure (Tozer *et al.*, 2011; Dal Pozzo *et al.*, 2017; Eldin *et al*., 2017). An immune reaction against phase II antigen characterises acute exposures (Figure 7.1), whereas anti-phase I immunoglobulins titres are characteristic of chronic exposures (Fournier *et al.*, 1998).

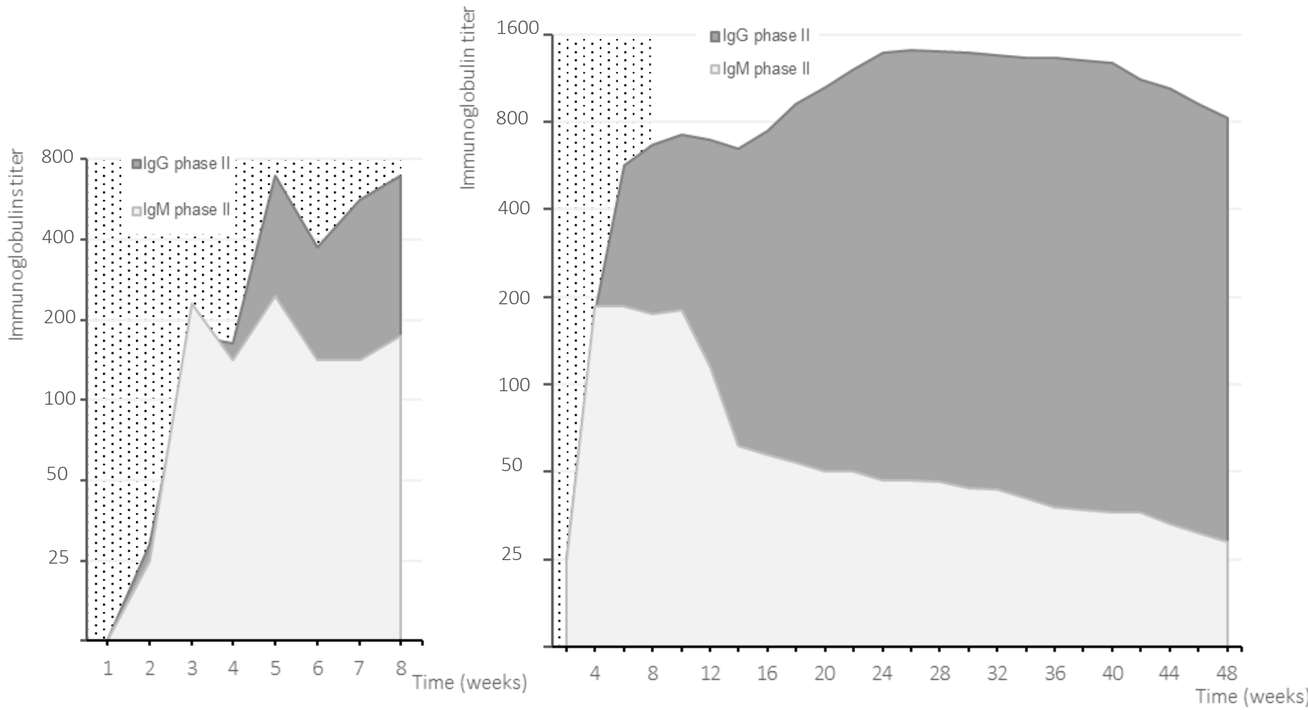


Figure 7.1: Schematic diagram of anti-phase II *Coxiella burnetii* IgG and IgM immunoglobulin kinetics overtime. The left-hand side panel represents the detailed variation during the first eight weeks; titres until week 48 are shown in the right panel. Adapted from Tissot-Dupont *et al*., 1994.

Coxiella burnetii is a broadly identified occupational hazard in abattoir and dairy farm workers in Uruguay. Since 1956 at least 18 human outbreaks involving abattoir and meat-processing workers have been identified in Uruguay by complement fixation, capillary agglutination test and layer microagglutination, and traced directly to cattle exposure (Salveraglio *et al.,* 1956; Somma-Moreira *et al.,* 1987; Ortiz-Molina *et al.,* 1987). The first local cases of Q fever epidemiologically linked to a dairy farm were diagnosed in 1988 using indirect fluorescent antibody testing (IFAT) for anti-*Coxiella* IgM assessment (Braselli *et al.,* 1989). The five cases, all of them adults, presented IgM titres of 1/20, without specifying antigenic phase variations. The infection was assumed to result from inhalation of contaminated dust; none of the five patients had ingested raw milk. A local Q fever outbreak linked to wildlife occurred during 2003-2004 (Hernández *et al.,* 2007). Workers from an experimental wildlife breeding station became infected, apparently through the inhalation of contaminated particles during grass mowing. The epidemiological investigation identified the pampas deer (*Ozotoceros bezoarticus*) as the presumed source of infection. To the best of our knowledge, none of the human Q fever outbreaks described in Uruguay or elsewhere have been related to bovine abortions caused by *C. burnetii*. This study presents a retrospective serological evaluation of laboratory and farm workers exposed to bovine placentas and fetuses aborted by *C. burnetii* in a dairy farm in Uruguay.

7.2 Materials and Methods

7.2.1 Bovine abortions and window of workers exposure

An outbreak of four cases of bovine abortion caused by *C. burnetii* was diagnosed in a dairy herd in Colonia, Uruguay. A detailed description of the diagnostic investigation conducted in this outbreak has been published elsewhere (Macías-Rioseco *et al*., 2019). Briefly, four cattle aborted full-term fetuses from April 10 to June 2, 2017. Placentas and fetuses from the aborted cattle were collected by farm workers and submitted to the local veterinary diagnostic laboratory for pathologic examination and diagnostic work-up. The diagnosis of bovine coxiellosis was confirmed based on typical placental lesions on histopathology, intralesional identification of abundant *C. burnetii* antigen by immunohistochemistry in trophoblasts, and PCR amplification of *C. burnetii* DNA in the placenta of all cases, while other abortifacient agents of cattle were ruled out by laboratory testing. The rest of the cattle herd was not evaluated.

The diagnosis of coxiellosis in the aborted cattle was notified/communicated to the local health authorities, which triggered an investigation by public health officials. Routine serologic testing was performed in August 2017 on 27 farm and laboratory workers that had been directly or indirectly exposed to the aborted cattle, fetuses and/or placentas. None of the workers had been vaccinated against *C. burnetii*. Estimated exposure window was defined based on the date when the index and last case of *C. burnetii* abortion occurred, comprising a timeframe of 54 days (7.7 weeks) (Figure 7.2).

There are some differences among published studies regarding the interval between exposure and symptomatology onset, as well as the time until the seroresponse activation. Based on the most recent literature, when clinical manifestation occurs, signs/symptoms begin approximately one to five weeks after exposure (Todkill *et al*., 2018), and seroconversion takes place roughly between two to three weeks after the beginning of the illness (Bae *et al*., 2019). Bearing in mind that the serologic assessment in people was done 127-134 days (18.1-19.1 weeks) after the index case and 74-81 days (10.6-11.6 weeks) after the last confirmed case, it can be presumed that workers were potentially coursing between 18-25 days (2.6-3.6 weeks) and 106-113 days (15.1-16.1 weeks) of the immune response at the time of the serologic evaluation (Figure 7.2).

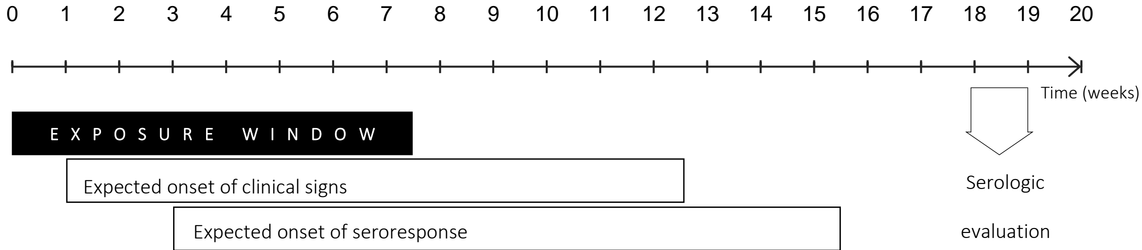


Figure 7.2: Timeline of the epidemiological and serologic investigation in workers after the Q fever outbreak in cattle. The index case (first case of bovine abortion) was reported at time 0 weeks, the window of exposure lasted for 7.7 weeks (April 10 to June 2, 2017). The time frame of the expected onset of symptomatology (Todkill *et al*., 2018) and seroresponse (Bae *et al*., 2019) were estimated based on the literature. The serologic evaluation of the workers was conducted between weeks 18 and 19 (August 14 and August 21, 2017).

7.2.2 Farm and laboratory workers' data and consent

Twenty-seven workers were involved in the study, of those fifteen were field workers, four laboratory workers and eight workers with activities in both field and laboratory contexts (Table 7.1). Written consent was obtained from all of them and the information was anonymised before analysis. The records comprised demographic background data such as age and sex, clinical information, (most of the workers self-reported symptoms), gathered during a medical examination, presence of pre-existing medical conditions, and the individual laboratory IFAT results. All these data contained in the official reports were made available to the authors, upon agreement, directly by the workers, and confidentiality was assured. Details about medical treatments could not be accessed. The study was granted ethical approval by the ethics committee of the University of Bristol (Ref.95382 / Id.342095).

7.2.3 Review of case records from the veterinary diagnostic laboratory

Records of diagnoses made by the local veterinary diagnostic laboratory between 10 April 2016 and 21 August 2017 were examined to rule out other potential exposures of laboratory workers to *C. burnetii*.

7.2.4 Indirect fluorescent antibody test

Serum samples were analysed for anti-*C. burnetii* phase II IgM and IgG antibodies using IFAT (Wegdam-Blans *et al*., 2012). The serology evaluation was performed at Mayo Clinic Laboratories (Focus Diagnostics, Inc., Cypress, CA, USA) following the method described by Edligner (1985).

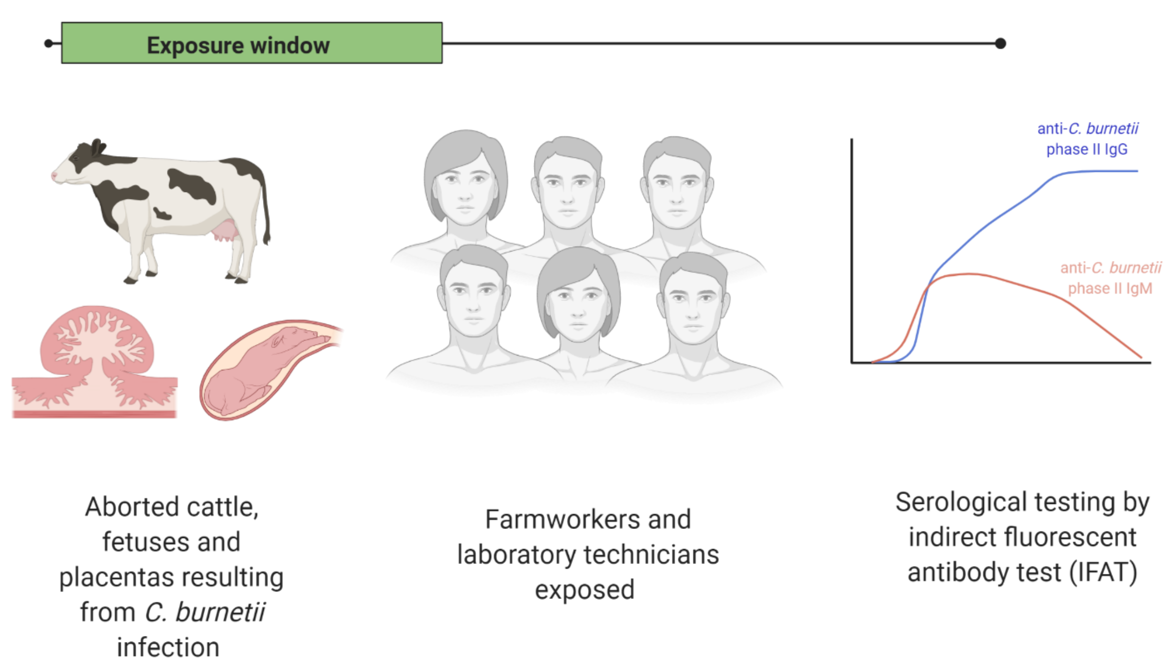


Figure 7.3: A retrospective evaluation of exposed workers to a Q fever outbreak in cattle.

7.2.5 Data analysis

Titres less than 1/16 in the IFAT for anti-*C. burnetii* phase II IgM and IgG antibodies were considered to be seronegative and those greater or equal to 1/16 were considered to be seropositive. The percentage of seropositivity was calculated as the number of seropositive individuals (titre $\geqslant$ 1/16) divided by the total number of workers tested. Phase II IgG to IgM ratios were calculated by dividing the IgG titre by the IgM titre. Univariable and multivariable analyses were conducted in which the IFAT status (seropositive or seronegative) was considered as the binary response variable. Gender (male and female), age group (21-30, 31-40 and >40) and work activity (farm and laboratory) were included as explanatory variables in univariable, and multivariable logistic regression models used to gain insight into factors (and their interactions) influencing *C. burnetii* seropositivity and to calculate odds ratios (OR) and their confidence intervals (CI95%). Statistical analysis was performed using RStudio software

7.3 Results

The study population comprised 27 individuals who worked either on the farm, in the laboratory, or both. Twenty-three individuals conducted at least some of their work on the farm, these comprising 13 farm workers, 2 veterinary practitioners and 8 laboratory workers. Twelve individuals conducted at least some of their work in the veterinary laboratory, these comprising the 8 laboratory workers who also conducted some farm work and 4 further laboratory workers who did not.

Ten of the 27 individuals had detectable titres of IgG antibody to *C. burnetii* phase II greater or equal to 1/16, and of these five also had detectable titres of IgM. Of the 23 conducting work on farm, 8 (34.8%) were IgG positive and 4 (17.4%) of these were also IgM positive (Figure 7.4). Seven of the 12 (58.3%) conducting lab work had detectable IgG titres, and 4 (33.3%) of these were also IgM positive, noting that 8 individuals undertook both types of work (Table 7.1 and 7.2). The univariable odds ratios for conducting laboratory work were 5.6 (CI95% 1.09–35.6, *P*=0.039) for IgG seropositivity and 7.0 (CI95% 0.853–150, *P*=0.071) for IgM seropositivity i.e., statistically significant for IgG and close to significance for IgM. The corresponding univariable odds ratios for conducting farm work were 0.533 (CI95% 0.055–5.13, *P*=0.566) for IgG seropositivity and 0.632 (CI95% 0.060–14.6, *P*=0.726) for IgM seropositivity i.e., not significant in either case.

The rate of seropositivity was twice as high in female workers (5/9, 55.6%; univariable odds ratio 3.25, CI95% 0.623–18.7; *P*=0.162) as in males (5/18, 27.8%) for IgG, but only slightly higher in females (2/9, 22.2%; univariable odds ratio 1.43, CI95% 0.161–10.7; *P*=0.729) than males (3/18, 16.7%) for IgM, in neither case statistically significant differences. Rates of IgG seropositivity in age groups 21-30 (4/8, 50%) and 31-40 (5/10, 50%) were identical and these were collapsed into a single category. Seropositivity in individuals less than or equal to 40 years old (9/18, 50.0%; univariable odds ratio 8.00, CI95% 1.12–165; *P*=0.037) was significantly higher than those greater than 40 (1/9, 11.1%) for IgG whereas for IgM, seropositivity in individuals less than or equal to 40 (4/18, 22.2%; univariable odds ratio 2.29, CI95% 0.275–48.9; *P*=0.468) was not significantly higher than those greater than 40 (1/9, 11.1%). Four of the five (80%) individuals seropositive for IgM were in the 31-40 year age group, which was significant (univariable odds ratio for age 31-40 compared to all other ages 10.7, CI95% 1.27–233, *P*=0.0283).Seropositivity levels were similar in symptomatic and asymptomatic individuals for both IgG (symptomatic 6/16, 37.5%; asymptomatic 4/11, 36.4%; univariable odds ratio for symptoms 1.05, CI95% 0.213–5.4; *P*=0.952) and IgM (symptomatic 3/16, 18.8%; asymptomatic 2/11, 18.2%; univariable odds ratio for symptoms 1.04, CI95% 0.143–9.12; *P*=0.970) (Figure 7.5). None of the seropositive workers had any of the predisposing conditions, such as pregnancy, low immune function, underlying cardiovascular disease, valvulopathy or valve prosthesis; thought to be of importance from the epidemiological perspective to prompt subsequent medical complications (Raoult *et al*., 2000; Fenollar *et al*., 2001; Landais *et al*., 2007).

Table 7.1: Anti-*Coxiella burnetii* phase II IgM and IgG titres, IgG to IgM ratio, demographic factors, background data of workers and potential exposure based on work activity.

Worker ID	Age range (years)	Gen der	Occupational Activity	Potential exposure	IgG phase II immunoglobulin titre	IgM phase II immunoglobulin titre	Phase II IgG /IgM ratio	Symptomatology*
1	41–50	M	Bacteriologist	Laboratory	1/256	1/512	0.5	Yes
2	21–30	F	Veterinary diagnostician	Field and	1/512	<1/16	-	Yes
3	21–30	F	Veterinary diagnostician	Field and	1/64	<1/16	-	Yes
4	31–40	F	Veterinary diagnostician	Field and	1/32	1/256	0.1	No
5	31–40	F	Veterinary practitioner	Field	1/64	1/128	0.5	Yes
6	31–40	M	Veterinary diagnostician	Field and	1/128	1/16	8	Yes
7	31–40	M	Laboratory technician	Field and	1/512	1/256	2	No
8	31–40	F	Veterinary diagnostician	Laboratory	1/16	<1/16	-	No
9	41–50	M	Farmworker	Field	<1/16	<1/16	-	No
10	21–30	M	Farmworker	Field	1/16	<1/16	-	Yes
11	31–40	F	Laboratory technician	Laboratory	<1/16	<1/16	-	Yes
12	61-70	M	Farmworker	Field	<1/16	<1/16	-	No
13	21–30	M	Farmworker	Field	<1/16	<1/16	-	Yes
14	21–30	M	Veterinary practitioner	Field	<1/16	<1/16	-	Yes
15	31–40	M	Farmworker	Field	<1/16	<1/16	-	Yes
16	51–60	M	Farmworker	Field	<1/16	<1/16	-	Yes
17	21–30	M	Farmworker	Field	1/16	<1/16	-	No
18	41–50	M	Farmworker	Field	<1/16	<1/16	-	Yes
19	31–40	F	Farmworker	Field	<1/16	<1/16	-	No
20	51–60	M	Farmworker	Field	<1/16	<1/16	-	No
21	41–50	M	Farmworker	Field	<1/16	<1/16	-	No
22	31–40	F	Veterinary diagnostician	Field and	<1/16	<1/16	-	Yes
23	31–40	M	Veterinary diagnostician	Field and	<1/16	<1/16	-	No
24	21–30	M	Veterinary diagnostician	Field and	<1/16	<1/16	-	Yes
25	51–60	M	Farmworker	Field	<1/16	<1/16	-	No
26	41–50	M	Farmworker	Field	<1/16	<1/16	-	Yes
27	21–30	F	Laboratory technician	Laboratory	<1/16	<1/16	-	Yes

Reference: for both antibody types titres of < 1/16 were considered negative; M: male; F: female; *: report of at least one suggestive symptom.

In the multivariable analysis for IgG seropositivity, addition of none of the terms farm work, age group, gender or symptoms improved upon the univariable model with laboratory work as the sole explanatory variable (likelihood ratio test $p > 0.4$ for all), suggesting this was already the minimum adequate model ($P = 0.039$). However, lab work was apparently confounded with age, 11 of 12 individuals conducting lab work being under 40 years of age. On collapsing age group to just two levels, as already noted, nine of 18 (50%) workers less or equal to 40 were seropositive for IgG, but just one of nine (11.1%) workers over 40 years of age was IgG seropositive (odds ratio and CI95% as above) and this was also the only individual in the over 40 age group conducting lab work; contrastingly, there were broadly similar numbers of IgG seropositives (6/9, 66.6%) and seronegatives (5/9, 55.6%) conducting lab work in the 40-and-under age group (Fisher's exact test $p \approx 1$).

There were too few IgM seropositive individuals (n = 5) for a meaningful multivariable analysis; it was however noteworthy that four of the five conducted farm work, four conducted lab work, with three conducting both farm and lab work, and that all four of those IgM positive individuals conducting field work were in the 31-40 age category, the remaining IgM seropositive individual who conducted only lab work being in the 41-50 age category.

When anti-*C. burnetii* phase II IgM and IgG titres were interpreted in conjunction, four distinct serological profiles could be identified among the workers (Figure 1). Five workers (IDs. 1, 4, 5, 6 and 7) had detectible titres (at least 1/16) of both IgM and IgG. Three of these five workers (IDs. 1, 4 and 5) whose IgM titres were higher than their IgG titres were classified as Profile 1, while the two workers (IDs. 6 and 7) whose IgG titres were higher than their IgM titres were classified as Profile 2. Five workers (IDs 2, 3, 8, 10 and 17) showed only IgG phase II titres with no detectable levels of IgM and were classified as Profile 3. Finally, 17 workers (IDs 9, 11-16, 18-27) in whom neither IgM not IgG titres were detected were classified at Profile 4.

Table 7.2: Frequency of anti-*Coxiella burnetii* phase II IgG and IgM titres in each subgroup of work.

Work	IgG phase II		
	+	-	
Field	3	12	15
Field + Laboratory and Laboratory	7	5	12
	10	17	27

Work	IgM phase II		
	+	-	
Field	1	14	15
Field + Laboratory and Laboratory	4	8	12
	5	22	27

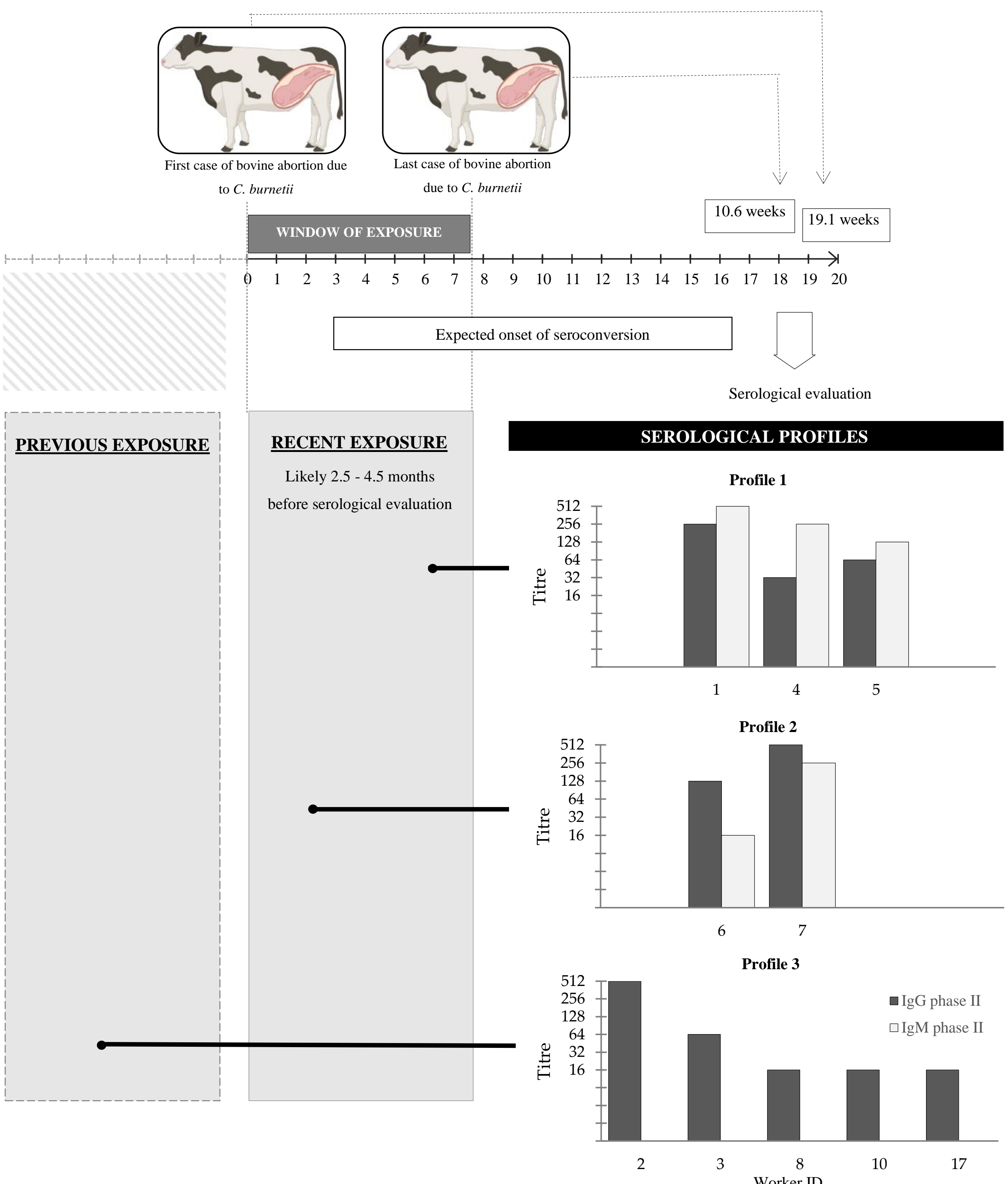


Figure 7.4: Timeline of the Q fever outbreak in cattle and serological investigations in farm and laboratory workers. Time zero was the date of the first case of bovine abortion. The window of exposure of farm and laboratory workers (when abortions occurred, and aborted materials were

collected and submitted to the local veterinary diagnostic laboratory for diagnostic work-up) lasted for 7.7 weeks (April 10 to June 2, 2017). The time course of the seroresponse was estimated based on published observations (Todkill *et al*., 2018). Serological sampling of humans was conducted on 14 and 21 August 2017 i.e., 18.1 and 19.1 weeks following the opening of the exposure window. Serological profiles are based on anti-*C. burnetii* phase II IgG and IgM levels measured by indirect fluorescent antibody test (IFAT). The profile of immunoglobulins was used to ascertain how recently were likely to have been infected: Profile 1: both isotypes detected, IgM titre > IgG titre – very recent; Profile 2: both isotypes detected, IgM titre < IgG titre; Profile 3: IgG detected but not IgM. Profile 4: neither IgM nor IgG detected (data not shown). Reciprocal titres are shown.

Worker ID	Thoracic pain	Myalgia	Abdominal pain	Headache	Fatigue	Odynophagia	Diarrhoea	Arthromyalgia	Dry cough	Sweating	Fever	Time of occurrence (months before serologic evaluation)
1		X			X		X	X		X	X	3
4												
5		X		X	X	X			X	X	X	3
6					X	X			X	X	X	3
7												
2	X	X	X	X	X		X			X	X	1
3						X			X	X	X	2
8												
10				X		X	X			X		1.5
17												
Frequency of signs	1/10	3/10	1/10	3/10	4/10	4/10	3/10	1/10	3/10	6/10	5/10	

Figure 7.5: Symptomatology shown by the ten dairy farm and laboratory workers with serological evidence of *Coxiella burnetii* infection. Workers are grouped by serological evidence of recent exposure, early and late in the current exposure window (IDs 1, 4, 5 and IDs 6 and 7; respectively); and past exposure (IDs 2, 3, 8, 10 and 17).

Six of the ten seropositive workers manifested a variety of non-specific symptoms, whereas the remaining four seropositive workers remained asymptomatic. Among those with clinical disease, sweating, fever, fatigue, and odynophagia were the most frequently reported. Most of the symptomatic workers (IDs 1, 3, 5 and 6) manifested clinically by middle-late May, i.e. three months before their serologic evaluation. Two workers (IDs 2 and 10) reported non-specific symptoms occurring around mid-late July (a month before serological examination). None of the seropositive workers had any medical condition known to predispose to subsequent medical complications. Review of the 17 sero-negative workers' medical records revealed that 10 presented some non-specific flu-like symptoms, whereas the other 7 of these seronegative workers remained asymptomatic. The proportion of symptomatic individuals was very similar among seropositive (6/10, 60.0%) and seronegative workers (10/17, 58.8%) (univariable analysis, $p \approx 1.00$).

The local veterinary laboratory examined submissions from 50 bovine and 5 ovine cases of abortion. Each case comprised either the fetus, the placenta or both. All cases were routinely examined for gross and histologic lesions, and cultured onto MacConkey and blood agar, Skirrow's medium, and Leptospira medium-based EMJH agar. Also, *Neospora caninum*, *Campylobacter* spp., *Tritrichomonas foetus*, Bovine parainfluenza virus 3, and Bovine viral diarrhea virus 1 were investigated by immunohistochemistry, direct immuno-fluorescence, dark-field microscopy examination, or PCR. None of these 55 fetuses presented any typical lesions leading to suspicion of coxiellosis. The cause of the bovine abortion was diagnosed in 25 of these cases (25/50). Most were diagnosed as infectious abortions (23/25) including agents such as *N. caninum* (11/23), *Campylobacter fetus* subsp. *venerealis* (1/23), Bovine parainfluenza virus 3 (1/23), as well as opportunistic agents (8/23). In two out of the five cases of ovine abortion, *Campylobacter jejuni* and *Campylobacter fetus* fetus were detected by PCR, while the other three cases remained undiagnosed.

7.4 Discussion

The focus of this discussion is to lay out the evidence supporting the zoonotic exposure and subsequent *C. burnetii* infection of workers. This takes up the first part of the section and following this is an acknowledgement and exploration of the possible constraints in the interpretation of the findings.

The chronology of serological responses and the immunoglobulin classes involved were investigated in a group of workers exposed to bovine abortions caused by *C. burnetii* to ascertain whether these aborted cattle were likely have been the source of human exposure. Surprisingly, given the importance assigned to Q fever as a zoonotic disease globally, there is an extreme paucity of evidence in accessible peer-reviewed literature associating Q fever with cattle. Most of the publications that investigated human Q fever outbreaks conducted serological assessments in people, followed by at most a description of the epidemiological link between people and cattle (such as visits to the affected herd, regular consumption of raw milk or unpasteurised dairy products, or occupational exposure); a few complemented this with PCR evaluations. Some studies also conducted serological evaluation in animals. However, serological approaches are not particularly informative in cattle, as seroconversion can occur without bacterial shedding, and animals can remain seropositive long after overcoming the infection; conversely cattle can shed *C. burnetii* before the development of detectable antibodies and may even shed the agent without ever seroconverting (McQuiston *et al.*, 2002). In contrast, our study provides more convincing evidence that exposure to aborted dairy cattle and their abortion materials is an occupational hazard for acquisition of Q fever.

In our study, based on the dates of the first and last known bovine abortion cases, the window of exposure to *C. burnetii* was estimated to comprise a timeframe of 54 days. On this basis, at the time of serological investigation, the workers had been exposed for no longer than 134 days (19.1 weeks) after contact with the first bovine case and no less than 74 days (10.6 weeks) after contact with the last bovine case. Thus, the workers were exposed to *C. burnetii* 74 - 134 days prior to serological examination. The incubation period of Q fever (exposure to disease onset) is pathogen dose-dependent, estimated at between 7 and 32 days (one to five weeks) (Todkill *et al.*, 2018), and seroconversion takes place roughly 14 - 28 days (two to four weeks) later (Dupuis *et al.*, 1985; Todkill *et al.*, 2018; Fournier & Raoult, 2003). Hence seroconversion may be expected after 21 days and almost certainly no later than 60 days (three to nine weeks) after exposure. On this basis, we estimate the seropositive workers in our study may have seroconverted between a theoretical minimum of 74 - 60= 14 days and maximum of 134 - 21= 113 days prior to serological examination. Hence serological investigation was conducted at least 1.6 weeks and possibly as

much as 16.1 weeks after seroconversion would be expected based on their exposure to bovine cases.

The profile of immunoglobulins reactive against *C. burnetii* antigens was used to provide insight into the timing of acquisition of infection, based on the known kinetics of antibody development in clinical Q fever (Tissot-Dupont *et al*., 2004; Wielders *et al*., 2015). In three cases (IDs 1, 4 and 5), IgM titres were higher than IgG titres, suggesting exposure had been recent, and coinciding with the latter stages of the known window of exposure to aborting cattle. Two other workers (IDs 6 and 7) were also seropositive for both antibody isotypes but had higher IgG titres than IgM; this may have reflected slightly less recent exposure, perhaps earlier on during the known window of exposure and possibly associated with the first bovine case. The minimum and maximum times between exposure to aborting cattle and the serological evaluation of workers (74–134 days) was entirely consistent with this abortion outbreak being the source of the human infections. The IgG anti-phase II concentration tends to exceed that of IgM anti-phase II on average about 4.5 days after the onset of the serological response, which equates to 25.5 days after exposure to *C. burnetii* (Wielders *et al*., 2015). Given that seroconversion may be expected 21–60 days after exposure, in our study we would expect seroconversion to have preceded serological testing by a minimum of 14 days. While this is a little longer than the estimated average time of 4.5 days from seroconversion to the point at which the IgG titre exceeds that of IgM, for some workers to have IgM titres higher than IgG at the time of testing was entirely consistent with the aborting cattle indeed being the source of exposure.

The IgG phase II antibody has a greater half-life than IgM phase II, with persistence up to 2.5 years making it an indicator of past infection (Wielders *et al*., 2015). In our study, five seropositive workers (IDs 2, 3, 8, 10 and 17) had IgG phase II antibody titres but no detectable IgM phase II, suggesting that exposure might have long before the known recent outbreak of bovine abortion, and those workers may have had a previous exposure that preceded the documented bovine outbreak.

In addition to the profile of immunoglobulins, the IgG/IgM ratio can be used as a rough estimator of the time after infection and can be used to discern between infection within three months and infection more than six months ago (Guigno *et al*., 1992). The IgG/IgM ratio is about 0.1 early after the onset of symptomatology, approximates to 1.0 within the first 100 days and is greater than 10 during the following 100 days. In our study, the IgG/IgM ratio ranged between 0.1 and 0.5 in worker IDs 1, 4 and 5, and between 2 and 8 in worker IDs 6 and 7. This evidence supports recent exposure and is entirely consistent with known exposure to aborting cattle 74–134 days

prior to serological analysis.

Two of the workers (IDs 2 and 10) had serological profiles suggestive of long past infection; they reported non-specific symptoms which were likely due to another aetiology, as they occurred long after probable exposure to *C. burnetii*. Likewise, symptoms reported by seronegative workers could be due to other seasonal illnesses and their responses on symptomatology could have been affected by their awareness of the investigation (Hawthorne bias).

The odds of *C. burnetii* seropositivity in laboratory workers, including those also undertaking occasional field activities, were greater than those for field workers for both anti-phase II IgG (OR 5.6 CI95%1.09-35.6) and anti-phase II IgM (OR 7.0 CI95% 0.853-150). Most of the farmworkers did not assist at calving and hence were exposed to *C. burnetii* infection indirectly e.g., through urine and faeces. Considering that shedding of *C. burnetii* by cows through these routes is scarce and intermittent (Guatteo *et al*., 2007), field workers would have faced a repeated but low-level bacterial challenge. In contrast, people engaged in laboratory activities, but without direct contact with farm animals might have been exposed to a high bacterial burden through the handling of abortion material infrequently or even on just a single occasion. Despite the suggestion of a protective role of female hormones such as *β*-estradiol (Leone *et al.*, 2004), infection rates were similar in male and female workers. Nor was an age-related increase in Q fever seropositivity observed in our study, as has been reported elsewhere (Pape *et al*., 2009). For IgG, there were a far greater number of seropositives in the 40-and-under age group (9/18) than in older individuals (1/9). Unfortunately, conducting lab work was confounded with age and it was difficult to be certain whether conducting lab work or being of age 40-and-under was the most important determinant of IgG seropositivity. The observation elsewhere that seropositivity tends to increase with age (Pape *et al*., 2009) work would indeed support lab work as being the more important of the two in this instance.

Other than the previously documented cases of bovine abortion due to coxiellosis (Macías-Rioseco *et al*., 2019), none of the bovine or non-bovine abortions routinely analysed by the local veterinary laboratory revealed macroscopic or histologic evidence suggestive of *C. burnetii* infection. Although other sources of *C. burnetii* exposure in laboratory workers beyond the analysed bovine outbreak cannot be altogether excluded, the known exposure to well-documented cases of bovine abortion caused by coxiellosis appears to be a far more likely and plausible source of infection for the human cases described in this study.

This study had a number of limitations that could be considered in future work aiming at furnishing further evidence for *C. burnetii* infection in humans exposed to infected bovines or

their abortion products. While the aetiology of the bovine abortions themselves was confirmed using by molecular methods (PCR) as well as histopathology and immunohistochemistry (Macías-Rioseco *et al*., 2019), the subsequent human infections documented here were confirmed only by serology; confirmation by molecular methods (Bae *et al*., 2019) would have strengthened this evidence. Furthermore, the extent to which the symptomatology described by the patients was related to Q fever is unclear. While the symptoms described and their chronology were consistent with acute infection with *C. burnetii* (Hartzell *et al*., 2020), we were unable to demonstrate a statistical association between symptoms and serological responses in the Phase II IFA for either IgG or IgM ($p > 0.95$). This might have been possible with a larger number of cases, but this was a study of a naturally occurring disease event and the sample size was not within our control. Lastly, in this study we used a titre of 1/16 or greater in the Phase II IFA as the seropositivity threshold for both IgG and IgM, as this was considered above the reference level by the testing laboratory (Mayo Clinic Laboratories), and indeed some authorities have used even lower IFA titres in epidemiological studies (Marrie & Pollak, 1995). We nevertheless performed a sensitivity analysis and re-analysed the data using a more conservative seropositivity threshold of 1/32, with little change in the overall implications of the results. Using this higher cut-off value, although there were fewer Phase II IgG positives overall (seven rather than ten) the association with lab work was even stronger, having an even higher odds ratio (14.0, CI95% 1.85–297) and a lower p-value ($p<0.01$); for IgM there were four rather than five positives overall and the revised odds ratio (4.67, CI95% 0.507–103) remained non-significant ($P=0.179$).

7.5 Conclusion

In conclusion this epidemiological investigation, the first closely linking Q fever to bovine abortion, provides novel serological evidence of *C. burnetii* exposure in people working in direct contact with either aborted cattle or their fetuses, placentas and vaginal discharges. Cattle aborting due to *C. burnetii* should not be underestimated as a potential hazard and possible source of human infection. Q fever should be considered in the spectrum of diseases in patients with an epidemiological link with animals, or with occupational-related exposure, especially those with fever of unknown origin. Vaccination should be considered for people at risk of Q fever through occupational exposure.

This thesis aimed to investigate *Coxiella burnetii* and *Chlamydiales* in commercial dairy herds in Uruguay, assess their association with abortion, and study their feasibility as a zoonotic threat in the local context. These agents had been typically considered as having a minor or relative marginal role in causing abortion in cattle and had never been systematically studied before. This work challenges the current routine diagnostic protocols used in cases of abortion, seeking evidence to support or dismiss the inclusion of these bacteria in diagnosis practices to help reduce the number of undiagnosed cases, especially those with histological changes suggesting an infectious aetiology. The specific aims of this thesis have broader impacts that are to improve the reproductive outcome and, consequently, the replacement of dairy cows on local farms by addressing these abortifacient agents for the first time. In turn, it will improve animal production efficiency and reduce the risk of zoonotic infections. The studies' critical findings described throughout the thesis are highlighted and discussed in this final chapter; further work suggestions and recommendations on this field are also given.

Abortion in dairy herds continues as a major concern with considerable economic failures for the sector. The aetiology of bovine abortion is frequently roughly divided into non-infectious and infectious causes. Non-infectious origin possibly accounts for many undiagnosed cases. Bovine abortions where there is no evidence for an infectious process can be the result of hormonal or metabolic imbalances, nutritional deficiencies (iodine, vitamin E, selenium), trauma (physical means), heat stress (high temperatures and humidity), genetic abnormalities, toxicities (poisonous plants, nitrate/nitrite poisoning), or poor management practices. Infectious abortions can be produced by bacterial, viral, protozoal and fungal agents. Bacterial agents include *Brucella abortus, Campylobacter fetus fetus, Leptospira hardjo, Arcanobacter pyogenes, Salmonella* Dublin*, Listeria monocytogenes*, *Bacillus licheniformis, Mycoplasma bovis, Mycoplasma bovigenitalium* and *Ureaplasma diversum.* The most frequently reported viruses causing abortion include Bovine virus diarrhoea and Bovine herpesvirus type 1. Protozoal and fungal agents have *Neospora caninum* and *Aspergillus fumigatus,* respectively.

Coxiella burnetii and *Chlamydia* spp. were selected from the infectious agents not previously investigated in Uruguay because some local reports about these bacteria were available despite never being systematically investigated. The original project initially aimed to study *Mycoplasma bovis, Mycoplasma bovigenitalium,* and *Ureaplasma diversum* together with the current bacteria

evaluated. Unfortunately, this investigation was not pursued due to time limitations and difficulties acquiring positive material.

Coxiella burnetii-DNA was detected and quantified in placentas from aborted dairy cattle. This finding expands the evidence supporting *C. burnetii* as an agent presented in dairy farms in Uruguay. *Coxiella burnetii* was identified for the first time as a bovine abortifacient pathogen on an artisanal cheese-producing farm that sells directly to consumers. The evidence supports *C. burnetii* as a relevant bacterium, which has probably been underestimated, causing abortions in dairy cattle from Uruguay. No molecular evidence for the presence of *C. abortus* or *C. pecorum* was gathered, and no co-infections of these pathogens were found. The status of *Chlamydiales* as a source of cattle abortion remains blurred. *Chlamydia abortus* and *C. pecorum* appear to have no substantial role in dairy cattle abortion in Uruguay.

Hitherto, the lack of diagnostic tools has restricted any epidemiological study of *C. burnetii* and *Chlamydiales* in the local context. This thesis represents the first attempt to simultaneously investigate *C. burnetii, C. abortus, and C. pecorum* in Uruguay (**Chapter Five**). For this purpose, a published protocol targeting well-evaluated genes was adapted to make available a tool to be used in local laboratories without sending samples abroad for analysis since this could be quite expensive. Therefore, the development of diagnostic tests to be used routinely in domestic laboratories is imperative to save costs and optimise the use of currently available facilities and enable work with greater autonomy.

Chapter Four is the first attempt of a large-scale epidemiological investigation of *C. burnetii* in collective milk samples from Uruguayan dairy herds. It represents a valuable and cost-saving methodological tool. This sampling approach, which indirectly evaluated 11.75% of the national dairy herd, showed that the bacterium is shed by clinically healthy cows from commercial farms, reaffirming the excretion of *C. burnetii* in milk from asymptomatic animals. A low occurrence (1.7%) of *C. burnetii* was partially revealed among the samples analysed, and this finding was in agreement with what was observed in similar dairy production systems (e.g. Chile) (Cornejo *et al*., 2020). As all samples came from commercial dairy farms, it can be inferred that milk produced by these herds entered the dairy industry, so sanitary-hygienic controls were conducted before human consumption. The trade and commercialization of raw milk for direct consumption by humans is currently banned in Uruguay. However, neither the partial on-farm industrialisation of milk for artisanal cheese production nor fluid raw milk consumption by the farmer and his family can be dismissed entirely. Thus, these uses of unpasteurised milk are difficult to estimate and

control. Consumers' preferences for raw milk products emerge as a growing global trend, and Uruguayan consumers are not an exception to this trend. Considering the survival of *C. burnetii* as a milk-borne pathogen in unpasteurised milk and raw dairy products, this report raises awareness of Q fever as potential food safety and public health concern. The results obtained broadly supports further surveillance investigations by bulk tank milk testing.

So far, the sole investigation of *C. abortus* was a serologic evaluation conducted in two convenience selected dairy herds in Uruguay in the 2000s. This thesis gave further insights into *C. abortus* in dairy cattle using molecular investigation and a case-control approach, the most frequent analytical epidemiological methodology (**Chapter Six**). Molecular analysis of *C. abortus* in vulvo-vaginal swab samples from aborted cows and their non-aborting herd mates from commercial dairy farms showed no evidence of *C. abortus* infection in none of the groups. This result may be interpreted as *C. abortus* displaying a non-relevant role as an abortifacient agent in cattle from Uruguay.

We have found serological evidence supporting that the local cattle population is a potential reservoir for *C. burnetii* infection in humans. This thesis reinforces the role of bovines as a source of Q fever by evaluating the timing of serological responses in a group of individuals exposed to abortions caused by *C. burnetii* in dairy cows. The immunoglobulin classes involved have been investigated to ascertain whether these animals or the aborting materials (placentas and fetuses) were likely to have been the source of human exposure. From the serological evidence presented in the thesis, it is concluded that *C. burnetii* aborted cattle should be considered a potential hazard and a possible source of human infection (**Chapter Seven**). The evidence set out in this research's findings highlights the need for clinicians and health professionals to give greater attention to local cases of Q fever. Q fever should be considered in the spectrum of diseases in patients with an epidemiological link with animals or with some sort of occupational-related exposure, especially those suffering from fever of unknown origin. Consequently, training in security and prevention measures focusing on biological risks should be implemented, and accurate information about zoonoses should be reinforced for farmers.

Two aspects emerged during the literature review about investigations of *C. burnetii* in bovine milk samples by molecular techniques. First, there were no publications on the topic in Uruguay that would serve as reliable data to contrast our results. Second, there was a wide range of *C. burnetii* molecular prevalence (ranging from 10.7% to 76.9%) on collective milk samples in studies conducted in different countries and regions. The latter was the seed for undertaking an ambitious investigation into the global meta-prevalence of *C. burnetii* in BTM samples, coupled

with the assessment of risk factors associated with infection and the analysis of moderators as sources of heterogeneity (**Chapter Three**). This assessment was done through a systematic review of the literature and meta-analysis of proportions. The meta-analysis described a high overall global prevalence of *C. burnetii* in BTM samples of 37.0% ($CI_{95\%}$ 25.2-49.5%), showing the widespread herd-level circulation of this bacterium in bovine dairy farms around the world. Meta-regression showed herd size as the most relevant moderator, with the odds of a BTM sample testing positive doubling with every unit increase. This finding makes sense as large-herd size may increase opportunities for transmission between infected and susceptible animals. This finding should be considered when planning preventive strategies based on management practices of animals. The geographic location, gross national income, and notification criteria for Q fever in the country where the study was conducted seemed to be no meaningful moderators. These results should be of interest to countries where the bacterium is a well-known health threat and also to countries like Uruguay, where epidemiological *C. burnetii* investigations have been limited or absent. The current systematic review and meta-analysis provided a global framework outlining the prevalence of *C. burnetii* in collective milk samples from commercial dairy farms. While this evaluation has uncovered the global herd prevalence of *C. burnetii* in dairy cattle to be high, in many countries, including some European high-income countries, the disease is not currently notifiable, and control is not mandatory. Further studies on this globally concerning zoonosis are widely justified by the high herd-level circulation of *C. burnetii* on bovine dairy farms revealed.

The epidemiology of Q fever in Uruguay is broadly unknown. Epidemiological studies have been limited by the lack of diagnostic tools and the impossibility of culturing the causative organism. These bacteria must be cultured under BSL-III laboratories because of their particular high infectivity, low infective dose, and aerosol transmission. This sort of laboratory setup was not operative in local facilities when conducting the study. Because of this, bacterial cultivation was not attempted in any of the studies conducted in this thesis. Beyond the results and insights obtained in this study about the two abortifacient bacteria, this thesis made available a battery of tools to be used locally. Three PCR assays were assessed, adapted and optimised on clinical samples such as aborted materials, vulvo-vaginal swabs or bulk-tank milk samples, thus providing valuable alternatives beyond bacteria isolation. The PCR assays are robust techniques and are a rapid and reliable alternative, able to quantify bacterial DNA from over an extensive range while merging the detection of these pathogens into a multiplex assay provides further benefits in saving time, cost and labour.

Despite the laboratory tools now available, some laboratory techniques are still pending. PCR assays cannot distinguish between infectious and non-infectious organisms, which could be seen as a limitation when investigating ongoing infections. DNA-based PCR *per se* does not allow any information about the organism's viability. Therefore, researchers using DNA-based PCR assays should ideally combine them with other alternative approaches when examining the viability. The uncovering of transcriptional activity is believed to be suggestive of viability (Keer & Birch 2003). Reverse transcriptase PCR has been employed to assess several pathogens' viability (Jenkins *et al*., 2003; Keer & Birch 2003; Ohashi *et al*., 2003). RNA examined using RNA-based RT-PCR is a promising target for viability studies due to its transitory nature. Additionally, laboratory tools for the bacterial identification within the lesioned tissue, such as FISH or IHQ, are still pending and are necessary when trying to show causality. There is still work to be done in this regard.

Serologic and molecular assays are complementing tools. While serologic assays detect immunoglobulins developed in the sample after a longer period, PCR can identify the microorganisms in samples such as milk immediately after contamination. The accessibility of an in-house serological test would be valuable. Because of limitations when interpreting results at the individual level, the serological assessment of collective samples (herd-level samples) is of choice instead of individual evaluations. Serologic approaches at the individual level are not informative enough. For instance, seroconversion can occur without detectable bacterial *C. burnetii* shedding; animals can persist as seropositive for some time after they have overcome infection; animals can shed *C. burnetii* before they generate detectable immunoglobulins; and even some animal shedders never seroconvert (Enright *et al*., 1971; Berri *et al*., 2001; McQuiston *et al*., 2002). Herd-level serological assessments would be more revealing and thus advantageous, contrasting to the ambiguous results obtained by individual serological tests.

The fact mentioned above that some PCR-positive cows stayed seronegative could suggest that some animals may develop an effective cellular immunity response with limited, and even untraceable, concentrations of immunoglobulins. Animals developing a cellular immune reaction in the absence of measurable immunoglobulins may be the way in which endemic coxiellosis remains in the herd. These cows could give birth to non-exposed calves without an immune response; they may become infected at pregnancy as primiparous cows and perpetuate endemic infection on the farm (Böttcher *et al*., 2011).

No *C. burnetii* and *Chlamydiales* genotypes have yet been identified in Uruguay. Genotyping is a laboratory-based genetic evaluation process with great value in distinguishing between temporally or geographically connected genotypes. This is an essential tool as it allows identifying the infection source and helps make decisions crucial to limiting the spread of infection (Roest *et al*., 2011b; Arricau-Bouvery *et al*., 2006). Genotyping work exceeds this thesis's targets and will be explored in a postdoctoral position (2022-2025). A recent promising investigation has genotyped *C. burnetii* directly from DNA extractions from BTM samples, obtaining satisfactory outcomes without the need for isolation or cultivation of the bacteria before genotyping (Chisnall, 2018). The *C. burnetii* DNA found in aborted placentas and pooled milk samples will be investigated by multiple-locus variable-number tandem repeats analysis (MLVA). The MLVA method was proposed to accurately genotype *C. burnetii* directly from clinical samples without bacterial culture. Also, it cannot be certain whether and to what extent the strains of *C. burnetii* causing abortions in cows are similar to those responsible for human Q fever outbreaks. Studying genotypic differences between the *C. burnetii* from the human Q fever outbreak, the PM samples, and the *C. burnetii* responsible for abortions would be highly informative to characterise genotyping profiles.

Some public health policies should be considered in light of the current results. Vaccination appears as one of the significant components of the Q fever prevention strategy. Countries where severe outbreaks were registered (Australia and the Netherlands) followed the policy of human vaccination. The vaccination of the local at-risk groups in Uruguay, such as people with direct contact to ruminants working in rural areas (farmers, vets practitioners, veterinary students), those employed in the industry (abattoirs), or those with indirect exposure to animals (laboratory technicians), should be highly recommended. Although Q fever is commonly an asymptomatic disease, in people with pre-existing conditions such as valvulopathy, immune suppression, vascular abnormalities, and pregnancy coxiellosis can lead to severe and life-threatening complications. Vaccination should be especially encouraged for those workers with these medical conditions predisposing them to develop heart valve infections and endocarditis. Particular emphasis should also be given to women (especially those at reproductive age) as *C. burnetii* infection could lead to severe consequences during pregnancy. Women play a relevant role in the local dairy sector and mainly engage in calving assistance and calf raising.

The only available vaccine for preventing Q fever in humans is licensed for Australia. This is a whole-cell formalin-inactivated *C. burnetii* vaccine (Q-VAX®) of the Henzerling strain (Marmion, 2007). The investigation of local strains of *C. burnetii* should precede any vaccination

program. There is no information about the genotypes of *C. burnetii* affecting humans and ruminants currently circulating in Uruguay, and this is an unexplored field of research that should be addressed. Any attempt to develop a local vaccine will be conditioned by the availability of an adequate BSL-III laboratory where living bacteria can propagate. Bacterial culture and isolation remain indispensable also for additional bacterial characterisation.

Other strategies based fundamentally on the education and training of at-risk workers and health clinicians are also crucial for preventing Q fever. The knowledge of the disease by the at-risk population helps them avoid high-risk behaviours. Preventing risky practices is particularly important in local conditions where on-farm raw milk utilisation is out of possible control. And where, often, the milking parlour and especially the calving paddock are located close to the family house for animal monitoring during calving season. Some basic measures for the prevention of workers include using special protective work clothes left on the farm before returning to the family home, a mask for respiratory and oral protection when calving assistance, a coat, gloves and rubber boots that must be disinfected after work. Effective prevention of Q fever is not possible without the joint work of medical and veterinary authorities. The knowledge of clinicians about the disease is fundamental to make them alert in particular because the inapparent course of Q fever challenges its diagnosis. A large-scale diffusion campaign about *C. burnetii* and its zoonotic risk and preventive behaviour should be carried out in Uruguay. People from at-risk exposure groups should be advised about detecting the bacterium in local herds and take the steps necessary to avoid infection.

The confirmation of a *C. burnetii*-abortion in a cow from a herd owned by an artisanal cheese manufacturer that commercialises raw cheese directly to consumers at small markets raises public health concerns considering *C. burnetii* milk-borne transmission. The pasteurisation of milk should be accentuated, particularly on farms with on-site industrialization of milk, such as artisanal cheesemakers.

The evidence showed by **Chapter Four** about clinically healthy animals shedding *C. burnetii* is particularly worrying. Asymptomatic animals would represent a relevant source of bacterial environmental contamination. The respiratory exposure to the bacterium through aerosols produced during milking of animals with silent bacterial shedding should not be underestimated. Environmental transmission of *C. burnetii* is believed to be the most frequent route of infection spread, considering that infective aerosols can cause infection far away from an infected farm (Schimmer *et al*., 2010). As air-borne *C. burnetii* transmission would seem to be a relevant route,

it would be worth investigating the small town's population in the vicinity of the affected farms, located within a 2 km radius, and workers from neighbourhood farms.

Abortion and reproductive losses are a perceptible economic and welfare burden to the Uruguayan dairy industry. However, several abortifacient agents are still unstudied. The lack of previous studies about pathogens such as *C. burnetii* and *Chlamydia* spp. is not entirely surprising as molecular approaches for veterinary diagnosis have recently been introduced in the country. Their use has only recently begun to spread. Even today, there is no local laboratory that investigates *C. burnetii* on human clinical samples by PCR. The work presented in **Chapters Four**, **Five** and **Six** have aided in bridging this gap of access to diagnostic tools.

Neither *C. abortus* nor *C. pecorum* were detected in the aborted placentas or the vulvo-vaginal swabs. Their role should be further investigated to conclusively discount their relevance in local contexts. It has been proposed that *Chlamydia* spp. might be carried in the vaginal mucosa of healthy animals and help somehow towards illness. However, it is not understood whether and to what extent these microorganisms can contribute to illness, and further research is required. Similar to other investigations of pathogens that colonise mucosae, the study presented in this thesis is vulnerable to sampling error. For instance, the *Chlamydial* colonisation may have persisted uncovered by the vulvo-vaginal swabs having been accidentally taken from an unsuitable place of the mucosa, with a not sufficiently sampling frequency or at the incorrect time. Indeed, the selection of the vulvo-vaginal swab sample, instead of a cervical swab, and obtaining them at a single sampling time may not have been optimal for these organisms' detection and should be revised in future studies.

The investigation of *C. burnetii* and *Chlamydia* spp. to better characterise bovine dairy abortions in Uruguay showed a relatively low prevalence of both bacterial species. Particularly for *C. abortus* and *C. pecorum,* where the lack of its detection in this study could indicate that these bacteria should be considered an unlikely cause of cattle abortion. This result indirectly evidenced that other agents not currently evaluated should contribute to abortions and further surveys are indeed necessary. The original project initially aimed to investigate *Mycoplasma bovis, Mycoplasma bovigenitalium,* and *Ureaplasma diversum* together with the current bacteria evaluated. Due to time limitations and constraints in acquiring positive material, this investigation was not concluded. Although these agents are normal inhabitants of the reproductive tract, reproductive failure has been reported (Doig, 1981). Because these mollicutes are often in healthy cattle's lower reproductive tract, the fetus can be contaminated when passing through the birth

canal. Then, the isolation of these organisms from an aborted fetus with no lesions is not conclusive evidence of causality (Kirkbride, 1990). The investigation of this group of bacteria might certainly be valuable to help determine the cause of infectious abortion that remain undiagnosed at present.

To sum up, this study confirmed the presence of *C. burnetii* in dairy herds in Uruguay and its negative impact. This confirmation was built on the molecular evaluation of collective milk samples and aborted placentas and the reaffirmation of the role of *C. burnetii* as an abortifacient agent in cattle. Additionally, findings provided robust evidence about aborted cattle's role in transmitting *C. burnetii* to humans. Therefore, the present thesis would be of interest not only from the livestock health perspective but also from human public health authorities. *Coxiella burnetii* should be brought into the range of pathogens affecting local livestock, preventive measures and public health policies contemplated.

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Appendix A

Publications

Rabaza, A., Fraga, M., Corbellini, L. G., Turner, K. M., Riet-Correa, F., & Eisler, M. C. Molecular prevalence of *Coxiella burnetii* in bulk-tank milk from bovine dairy herds: Systematic review and meta-analysis. One Health, (2021) 100208. https://pubmed.ncbi.nlm.nih.gov/33553561/

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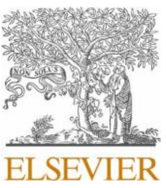

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Molecular prevalence of *Coxiella burnetii* in bulk-tank milk from bovine dairy herds: Systematic review and meta-analysis

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ARTICLEINFO

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ABSTRACT

urnetii is an obligate intracellular zoonotic bacterium that causes Q fever. Ruminants, including cattle, are broadly be reservoirs for this bacterium. Since 2006, many research groups have evaluated the herd-level prevalence of *C.* cattle by molecular techniques on composite milk samples. This study explored the global *C. burnetii* herd-level e from studies done on bovine bulk-tank milk (BTM) samples using PCR-based analysis. Also, moderators were ed to identify sources of heterogeneity. Databases (CAB Abstracts, Medline via Ovid, PubMed, Web of Science and holar) were searched for index articles on *C. burnetii* prevalence in BTM samples by PCR published between January-November-2018. Numerous studies (1054) were initially identified, from which seventeen original publications were n the meta-analysis based on the pre-defined selection criteria. These studies comprised 4031 BTM samples from

untries. A random-effects model was used because of considerable heterogeneity (I^2 = 98%) to estimate the herd-level e of *C. burnetii* as 37.0%($CI_{95\%}$25.2–49.5%). The average herd size appeared to account for a high level of the ieity. No other moderators (geographic location, gross national income or notification criteria for Q fever) seemed to inant. This systematic evaluation demonstrated a high molecular prevalence of *C. burnetii* in BTM samples both in and non-European countries, evidencing a widespread herd-level circulation of this agent in bovine dairy farms around . Meta-regression showed herd size as the most relevant moderator with the odds of a BTM sample testing positive with every unit increase.

1. Introduction

Coxiella burnetii the intracellular Gram-negative bacterium responsible for the zoonotic disease Q fever [1] has many reservoirs, including ruminants, that represent the primary source of environmental contamination and of infection in people [2]. This agent causes fertility disorders and metritis in cattle and is implicated in bovine abortion [3–5]. It often leads to abortion in small ruminants when a pregnant dam is infected, as *C. burnetii* exhibits a specific tropism for the trophoblast cells in placental cotyledons [6].

Coxiella burnetii has a complex epidemiological pattern and characteristics that make its control challenging. It is widely disseminated in nature and infects a large number of species, including mammals, birds, reptiles and fish [7]. There are two maintenance cycles in nature, one involving domestic species, and another including wild animal species and their ectoparasites. Ticks may be involved in the transmission of *C. burnetii* between wildlife and domestic species [8]. Additionally, the agent is extremely resistant remaining viable in the environment over extended periods [8]. *Coxiella burnetii* can also undergo air-borne transmission by contaminated dust particles, which can be facilitated by hot and dry weather conditions [9,10].

A large human outbreak of Q fever reported in the Netherlands (2007–2010), comprising more than 4000 cases, emphasised the need for robust surveillance campaigns and highlighted its importance as a threat to public health [9,11]. Transmission to people is principally by the inhalation of aerosolised contaminated animal placenta and birth fluids during abortions or the birth of normal offspring [12]. Practices such as the assistance of calving, handling of birth products, and manure spreading may present a high risk for *C. burnetii* transmission to humans [13–15].

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There is no consensus about the importance or effectiveness of the digestive route of infection by the consumption of raw milk and dairy product [6,16– The level of bacterial load by the different routes differs among ruminants [6]. While parturition products are the primary source of shedding in small ruminants, milk seems to play a central role as a shedding route of *C. burnetii* in dairy cattle [20,21]. Even asymptomatic animals [20] or seronegative cattle [22] have been identified as *C. burnetii* milk shedders. *Coxiella burnetii* can be excreted in milk for up- to 13 months [9,23], although this may be intermittent [6]. Two patterns of shedding have been identified in dairy cows which can be persistent heavy shedders or sporadic shedders [20].

18]. Nevertheless, respiratory exposure to aerosols produced during milking of animals should not be underestimated [19].

Based on these heterogeneous shedding patterns, composite samples such as bulk-tank milk (BTM) constitute useful and easily accessible specimens for large scale epidemiological investigation. A positive result provides robust evidence for the identification of infected herds. Bulk- tank milk testing is the preferred diagnostic approach for disease notification in many countries [24] and has epidemiological value for the

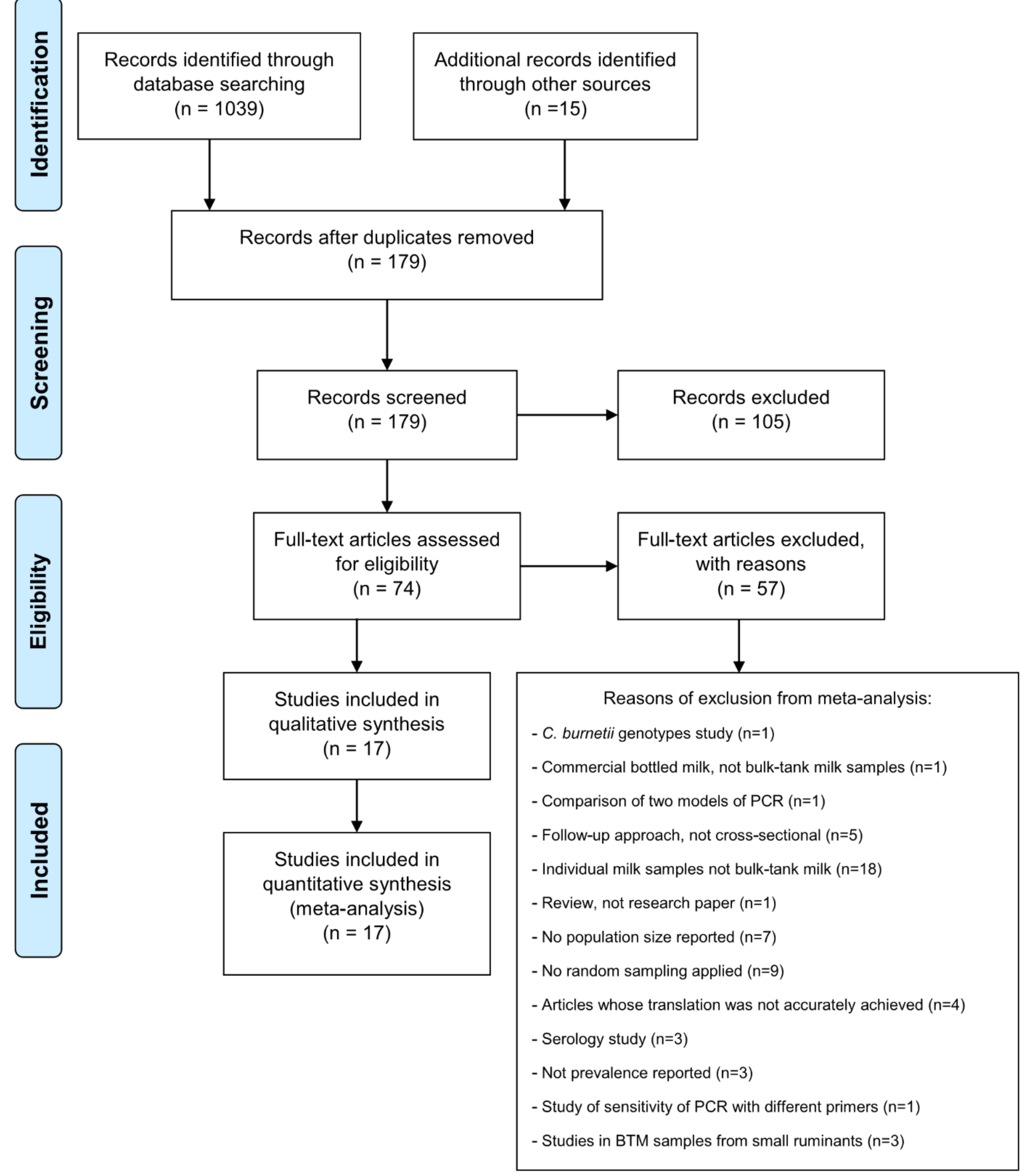


Fig. 1. PRISMA flow diagram describing the study design process for the systematic review and meta-analysis of the molecular prevalence of *Coxiella burnetii* in bulk- tank milk from bovine dairy herds.

monitoring of infection status over time in follow-up evaluations [25].
Recent large human Q fever outbreaks in the Netherlands, Spain, France and Germany have increasingly focussed attention on coxiellosis in many European countries where strategies including mandatory notification of the disease have been implemented. We systematically review studies of the herd prevalence of *C. burnetii* in dairy cattle using PCR on BTM samples, conduct a meta-analysis to determine the overall European and global prevalences and assess geographic region, average herd size, local legislation for coxiellosis and per capita income in each country where studies were conducted as potential moderators.

2. Material and methods

2.1. *Literature search and study selection*

The systematic review and meta-analysis followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines [26] (Fig. 1). The search strategy identified publications reporting the prevalence of *C. burnetii* on BTM samples analysed by molecular studies. The following electronic databases were used to identify studies published from January 1973 up to November 2018 (week 43 of 2018): CAB Abstracts, Medline, PubMed, Web of Science, Scopus, Science Direct and Google Scholar. The literature search comprised the terms: "*Coxiella burnetii"* or "Q fever" or "coxiellosis" and
"PCR" or "qPCR" or "real-time PCR" or "molecular diagnosis" and "BTM" or "milk", with no language restriction. No constraint in study designs was applied at this phase. Additional publications were identified by cross checking references included in the articles. Duplicates were identified by reference management software (Mendeley) and manually removed.

2.2. *Eligibility - inclusion criteria*

Publications on studies fulfilling all the following criteria were eligible for inclusion: (i) molecular investigation of *C. burnetii* by PCR, (ii) random sampling, (iii) composite single test-day samples obtained from the bulk storage tank located on a dairy cattle farm, (iv) primary studies, but not reviews, (v) cross-sectional studies reporting prevalence. Authors of articles not stating the total number of dairy cattle herds from which the sample was drawn were contacted to provide this missing data. Publications were examined by two independent reviewers (AR and MF) to ensure they matched the inclusion criteria. Discrepancies between the two reviewers on eligibility were discussed with the rest of authors until reaching agreement. *2.3. Data extraction and meta-analysis*

Studies were screened by title, and abstract and irrelevant publications were excluded. The remaining studies were full-text checked against the inclusion criteria described above. Articles that did not fulfil all these criteria were excluded. The number of publications excluded are shown in Fig. 1. Data were systematically extracted from all the studies that satisfied the inclusion criteria, including: the first author identity, year of publication, study title, journal title, country, study methodology (duration of sampling, herd size, sample size, the number of positives herds and/or prevalence, randomisation), molecular technique and target gene used. When available, information about the factors associated with the *C. burnetii* infection was also reported.
The *C. burnetii* herd prevalence determined in BTM samples (dependant variable) was considered as the effect size for the studies included in the meta-analysis. This meta-analysis of proportions was performed as outlined by Wang [27]. The heterogeneity among studies was first investigated by Cochran's *Q* (X^2) that tests the null hypothesis of homogeneity, and then quantified by the Higgins' I^2 statistic [28]. The heterogeneity was measured to select the model for the overall weighted *C. burnetii* herd prevalence estimation. As the level of heterogeneity was high, a random-effects model was first used to address both within-study variance (the sampling error) and the between-studies variance (τ^2). Possible sources of heterogeneity were investigated through the analysis of moderators. The evaluated moderators included: i) geographic region: Europe vs non-Europe; ii) average herd size; iii) local legislation for Q fever: mandatory notification vs non-mandatory notification [29–37], and iv) gross national income (GNI) per capita classification from the year the study was conducted, based on the Atlas method [38]. A subgroup analysis was performed for the categorical moderators. Categorical moderators were analysed using a mixed-effects model. The statistical significance of the moderators was evaluated by an omnibus test (*QM*) within the mixed-effects model [39]. The proportion of heterogeneity accounted for by each moderator was explored by the R^2 index. Meta-regression was also utilised to explore heterogeneity among the studies. All the moderators and their interactions were entered in the initial model and non-significant terms were then dropped stepwise (from lowest R^2 to highest R^2) [40]. The odds ratio (OR) for $\log_e$ average herd size was additionally investigated. Association among moderators was assessed by the Pearson correlation coefficient (r). Results from the meta-analysis with the corresponding 95% confidence intervals were summarized using forest plots. Egger's test was used to test for the possibility of a publication bias for studies with low or high effect sizes [41]. All the assessments were conducted using open RStudio software (Boston, MA) with metafor package, mvmeta package and metaprop commands [39,42]. **3. Results**

3.1. *Description of the studies*

After removal of duplicates, a total of 179 studies were identified initially (Fig. 1). Seventeen studies from twelve different countries (Belgium, Colombia, Hungary, Iran [2 studies], Italy [3 studies], Latvia, Netherlands [2 studies], Portugal, Spain, South Korea, UK and USA [2 studies]) were eligible for the meta-analysis based on the inclusion criteria. Six of those studies were conducted in non-European countries and 11 in European countries; 10 were conducted in countries where Q fever is a notifiable disease, while 7 were from countries where it is not. The study conducted in the Basque Country was included in the subgroup with mandatory notification, although this is the only Spanish province where the notification for Q fever is compulsory. Finally, 3 studies were conducted in upper-middle-income countries and 14 studies were in high income countries. The seventeen selected articles are summarized in Table 1 and included test results for a total of 4031 BTM samples collected over 9 years (2006 to 2015). Studies employed either conventional PCR (*n* = 5), quantitative PCR (*n* = 9) or nested PCR (*n* = 3). The transposon-like repetitive region of the bacterial genome (*IS1111*) was the gene most frequently used as the target in these PCRs
(*n* = 14), followed by *com1* (*n* = 2), *icd* (n = 1) and 16S rRNA genes (n = 1) (Table 1).

3.2. *The estimated overall meta-prevalence of* Coxiella burnetii *in BTM samples*

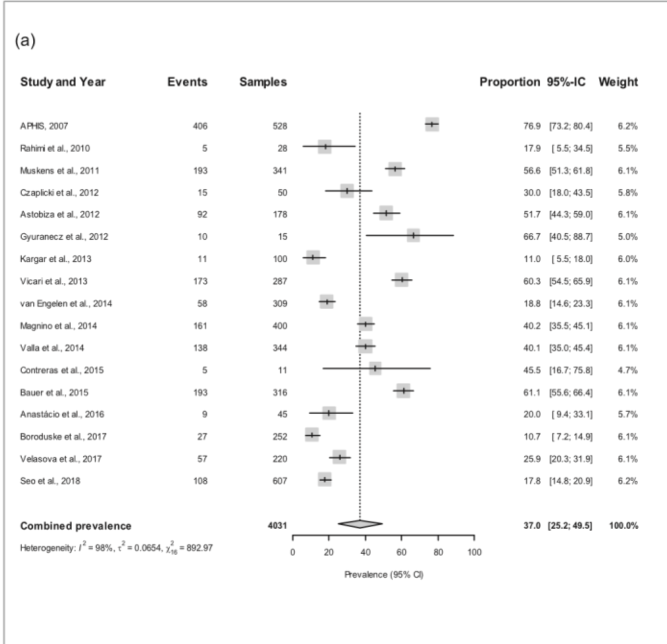
The median size of the eligible studies was 252 BTM samples. Of the total 4031 BTM samples, 1661 were diagnosed positive by molecular techniques. The percentages of positive BTM samples among the studies ranged from 10.7 to 76.9%. The overall weighted prevalence of *C. burnetii* in the random-effects meta-analysis was estimated at 37.0% ($CI_{95\%}$25.2–49.5%). The I^2 value of 98.0% ($CI_{95\%}$95.9–99.0) suggested high heterogeneity, with a τ^2 of 0.0654 ($CI_{95\%}$0.3296–1.4997), and an X^2 statistic of 892.97 (P <0.0001). The overall meta-analysis is shown in a forest plot (Fig. 2a). No obvious evidence of publication bias was detected in the meta-analysis on the basis of Egger's test (P = 0.599).

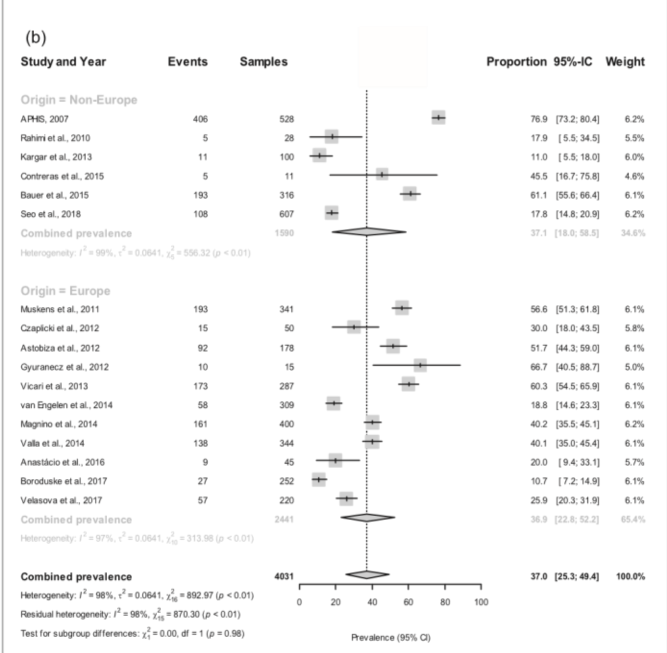
Table 1

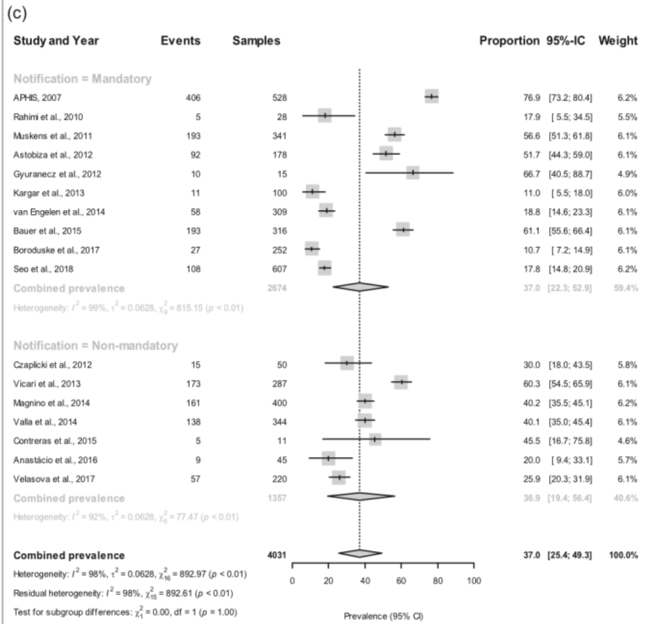
Characteristics and main results of the eligible studies ordered by molecular prevalence of *Coxiella burnetii* in bulk-tank milk samples.

Author	Year	Country	Study area	Average herd size	Period of study	Risk factor analysis	Gross national income per capita [38]	Is Q fever a mandatory notifiable disease?	Molecular approach	Target gene	N herds in study area	Percentage of herds sampled	BTM(i) samples tested	Positive BTM samples	Prevalence	95% CI	
Boroduske *et al.* [43]	2017	Latvia	Nationwide	8.6	2015	Yes	High- income	Yes	qPCR	*IS1111*	5040	5	252	27	10.7	7.2	14.9
Kargar *et al.* [23]	2013	Iran	Johrom	3.7	–	Yes	Upper-middle-income	Yes	nPCR	*com1*	3000	3.3	100	11	11	5.5	18.0
Seo *et al.* [44]	2018	South Korea	Gyeongsang	74	2015	No	High- income	Yes	nPCR	16S rRNA	869	69.9	607	108	17.8	14.8	20.9
Rahimi *et al.* [45]	2010	Iran	Chaharmahal and Bakhtiari	48	2008	No	Upper-middle-income	Yes	nPCR	*com1*	95	29.5	28	5	17.9	5.5	34.5
van Engelen *et al.* [46]	2014	Netherlands	Nationwide	71.7	2009–2011	Yes	High- income	Yes	qPCR	*IS1111*	20,746	1.5	309	58	18.8	14.6	23.3
Anastacio *et al.* [47]	2016	Portugal	Nationwide	21.7	2009–2013	Yes	High- income	No	PCR	*IS1111*	1712	2.6	45	9	20	10.9	33.8
Velasova *et al.* [48]	2017	UK	Nationwide	133	2014–2015	No	High- income	No	qPCR	*icd/ IS1111*	10,491	2.1	220	57	25.9	20.3	31.9
Czaplicki *et al.* [49]	2012	Belgium	Wallonia	28.5	2006	Yes	High- income	No	qPCR	*IS1111*	5086	1	50	15	30	8.7	51.3
Magnino *et al.* [50]	2009	Italy	Cremona, Montova and Pavia	180	2007–2008	No	High- income	No	PCR	*IS1111*	3550	11.2	400	161	40.2	35.5	45.1
Valla *et al.* [51]	2014	Italy	Nationwide	42.5	2011–2013	No	High- income	No	PCR	*IS1111*	30,000	1.1	344	138	40.1	35.0	45.4
Contreras *et al.* [37]	2015	Colombia	Monteria	150–600	2012	No	Upper-middle-income	No	PCR	*IS1111*	3341	0.3	11	5	45.5	16.7	75.8
Astobiza *et al.* [52]	2012	Spain	Bizkaia	46.1	2009–2010	No	High-income	No / Yes(ii)	qPCR	*IS1111*	178	100	178	92	51.7	44.4	59
Muskens *et al.* [25]	2011	Netherlands	Nationwide	65.7	2007	No	High- income	Yes	qPCR	*IS1111*	21,313	1.6	341	193	56.6	50.7	61.9
Vicari *et al.* [34]	2013	Italy	Lombardy	182	2011	No	High- income	No	PCR	*IS1111*	5750	5	287	173	60.3	54.5	65.9
Bauer *et al.* [53]	2015	USA	Indiana	145.3	2011	No	High- income	Yes	qPCR	*IS1111*	1225	25.8	316	193	61.1	55.6	66.4
Gyuranecz *et al.* [54]	2012	Hungary	Nationwide	14.5	2010–2011	No	High- income	Yes	qPCR	*IS1111*	17,172	0.1	15	10	66.7	40.5	88.7
APHIS [55]	2007	USA	18 states(iii)	162.6	2007	No	High- income	Yes	qPCR	*IS1111*	54,100	1	528	406	76.9	73.2	80.4

(i): BTM: bulk-tank milk samples, one per herd; PCR: conventional PCR; qPCR: real-time PCR; nPCR: nested PCR. (ii) mandatory notification in Basque Country. (iii) California, Idaho, Indiana, Iowa, Kentucky, Michigan, Minnesota, Missouri, New Mexico, New York, Ohio, Pennsylvania, Texas, Vermont, Virginia, Washington, Wisconsin.







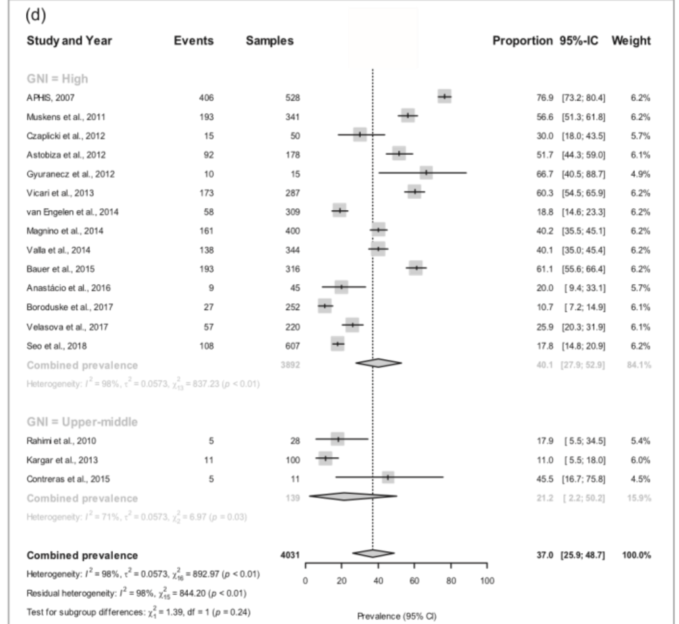


Fig. 2. Forest plot for the meta-analysis of herd-level *Coxiella burnetii* prevalence based on bulk-tank milk samples from the seventeen studies that matched the inclusion criteria in the systematic review. (a) All studies. (b) European and non-European country subgroups. (c) Grouped by mandatory and non-mandatory notification. (d) Grouped by the per capita Gross National Income (GNI) level.

3.3. *The meta-prevalence of* Coxiella burnetii *and moderator analyses*

The weighted average prevalence was similar within each of the two geographic subgroups (36.9% in European countries and 37.1% in non- European countries; ($I^2 = 98\%$; $X^2 = 870.29$, $P < 0.01$; *QM* (df = 1) = 0.002, P = 0.98), albeit with differing 95% confidence intervals of 22.8%– 52.2% in the former and 18.0%–58.5% in the latter group of countries (Fig. 2b). Similarly, countries with mandatory and non- mandatory notification of Q fever had a prevalence around 37.0% ($CI_{95\%}$22.3–52.9% and $CI_{95\%}$19.4–56.4%, respectively; ($I^2 = 98\%$; $X^2 = 892.61$, $P < 0.01$; *QM* (df = 1) = 0.010, P = 1.00) (Fig. 2c). In the subgroup analysis based on the GNI per capita (Fig. 2d), the prevalence was 40.1% ($CI_{95\%}$27.9–52.9%) in high-income countries and 21.2% ($CI_{95\%}$2.2–50.2%) in upper-middle-income countries ($I^2 = 98\%$; $R^2 = 3.10\%$; $X^2 = 844.20$, $P < 0.01$; *QM* (df = 1) = 1.39, P = 0.24). None of the three factors above appeared to contribute meaningfully to the observed level of heterogeneity based on the subgroup analysis. The meta-regression revealed that average herd size accounted for a significant proportion of the heterogeneity ($I^2 = 97\%$; $R^2 = 33.01\%$; $X^2 = 552.23$, $P < 0.01$; $QM = 4.55$, $P = 0.03$). As a significant moderator, high-size herds presented a higher herd-level *C. burnetii* BTM prevalence (Fig. 3). The odds ratio for the $\log_e$ of herd size was 2.00

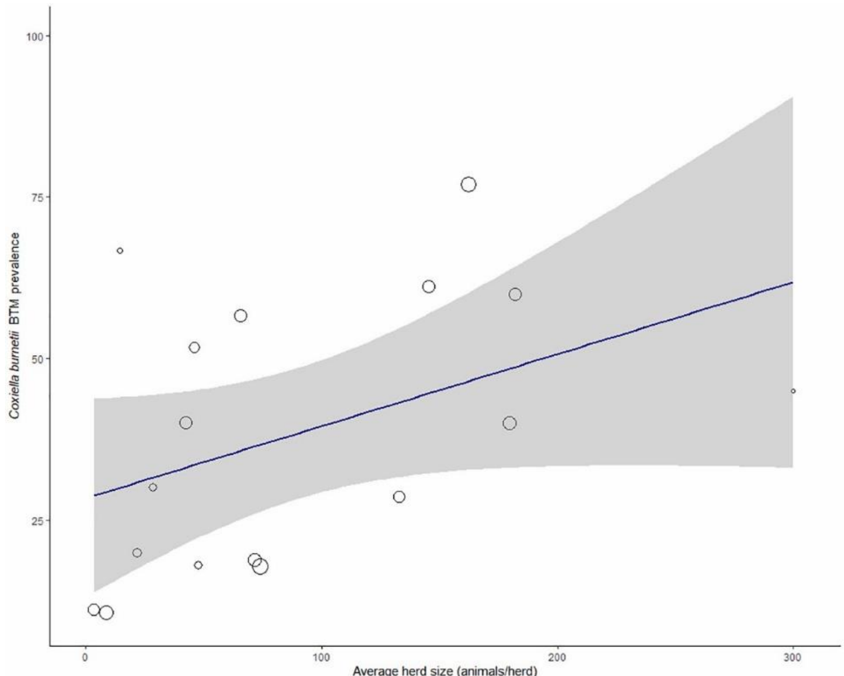


Fig. 3. Bubble plot for meta-regression of herd-level *Coxiella burnetii* prevalence based on bulk-tank milk with average herd size as continuous covariate. Points represent the seventeen studies that matched the inclusion criteria in the systematic review. Bubble size is in relation to the weight of each primary study.

(CI95%1.24–3.52; P = 0.02). A strong positive correlation was found between countries being located in Europe and high GNI per capita income (r = 0.633, P < 0.05), but between location in Europe and compulsory disease notification (r = − 0.239, P = 0.24), and between high GNI per capita and notification (r = − 0.076, P = 0.82) correlations were weak and negative. Herd size was not meaningfully correlated with the origin of the studies (r = − 0.468, P = 0.12), notification (r = − 0.428, P = 0.16), or with GNI per capita (r = − 0.444, P = 0.14).

4. Discussion

Global serological or molecular prevalences from pathogens as diverse as *Toxoplasma gondii* and *Helicobacter pylori* have been estimated by meta-analyses following a systematic review of the published body of studies [56,57]. We conducted a comprehensive keyword-based systematic review of the literature on the global molecular prevalence of *C. burnetii* in bovine BTM samples and data from those studies matching the inclusion criteria was extracted and included in a meta-analysis. For the purpose of this review, only adequately randomised studies with a cross-sectional design were included.

Heterogeneity among studies was first investigated by Higgins' I^2 statistic which indicates the proportion of heterogeneity not due to chance. A high level of heterogeneity (≥75%) indicates another source of variability besides the random error. The high I^2 value (98%) led to the choice of a random-effects model for estimating the overall weighted *C. burnetii* herd-level prevalence among eligible articles, which makes no assumption that the prevalence is constant across the studies. The meta-analysis shows that *C. burnetii* is widely distributed in dairy farms around twelve countries from 3 continents (America,

Europe, and Asia). The best estimate of global *C. burnetii* herd-level prevalence,
based on the studies matching the current inclusion criteria, was 37.0%. While there was no obvious evidence of publication bias based on Egger's test, this test has limited power and the possibility of bias cannot be altogether excluded [58].

Bulk tank milk samples are a widely used approach for studying infectious diseases of dairy livestock at the population level, despite that dry cows and unhealthy animals are not included and hence BTM only provides a partial representation of the herd sanitary status. The analysis of BTM samples represents a suitable and convenient approach for the investigation of *C. burnetii*, not only for initial farm-level screening in situations where their disease status is unknown, but also for repeated analyses during monitoring programmes or after sanitary interventions such as antibiotic administration [59] or vaccination [60,61]. A positive BTM result confirms herd exposure to *C. burnetii*.

The molecular diagnostic methods of studies included in this meta- analysis targeted different regions of the bacterial *C. burnetii* genome. The repetitive element *IS1111* was selected in most of the published studies as this multiple copy gene is presumed to increase the sensitivity of the test [62]. Other studies used PCRs targeting *com1*, *icd* and 16S rRNA genes. The *com1* element is frequently used for accurate quantification, as this is a single-copy gene [63]. Additionally, the analysis of 16S RNAs may reveal the prevalence of *Coxiella* as a genus, by the identification of both *C. burnetii* and *Coxiella*-like organisms [44].

The overall weighted *C. burnetii* prevalence found in bovine dairy herds was higher than the 5.1% to 22.1% range reported for BTM samples from sheep dairy flocks [47,64,65]. This difference could be explained by the primary route of bacterial transmission in each species. A higher *C. burnetii* prevalence might be expected in bovine milk, which is the predominant route of shedding for cows (and with a longer duration), whereas milk is less important for transmission from goats and sheep [9,23].

Two nationwide studies in Dutch dairy herds revealed markedly different prevalence levels in 2011 (56.6%) and 2014 (18.8%) [25,46], when using the same molecular approach in a similar number of herds. The lower prevalence in 2014 might be related to compulsory control measures applied in dairy goat farms after the large human Q fever outbreak in 2007–2010 [11,66]. There is some albeit limited evidence that the same outbreak strain may affect both cattle and goats in the Netherlands [67], and measures applied to goat farms might have indirectly helped to reduce prevalence in bovine herds. Similarly, three studies conducted in Italian herds in 2013 and 2014 also reported differences in *C. burnetii* prevalence. Valla *et al*. (2014) [51] revealed a nationwide prevalence of 40.0%, while Vicari *et al*. (2013) [34] found a higher prevalence of 60.0% in the northwest region of Lombardy, where almost half of Italian cows' milk is produced [68]. The molecular prevalence of *C. burnetii* found in Lombardy represented a marked increase compared to a previous two-year study (2007–2008) conducted in the same region (40.0%) [50].

Differences in the bacterial shedding patterns among ruminants and uncertainty about the importance of milk-borne infection may result in emphasis on different control measures depending on the species. In small ruminants, the identification of high-risk dams before parturition is important in avoiding zoonotic risk [69]. In cattle where milk is the primary shedding route, pre-partum monitoring may not be as appropriate [69]. Identification of chronic *C. burnetii* milk shedding cattle may be more effective in preventing environmental contamination, decreasing the risks of transmission among animals and preventing the spread of the bacterium.

Only five of the seventeen selected articles included analysis of factors associated with *C. burnetii* infection. Herd size, cattle density and purchasing replacement animals from external sources were all linked with *C. burnetii* infection [43,46]. Additionally, the presence of ticks on cattle was associated with BTM PCR positivity [46].

For both cattle and small ruminants, a positive correlation between herd size and herd prevalence of *C. burnetii* has been reported [70,71]. The association between herd size, density of animals and an enhanced risk of *C. burnetii* infection has been well demonstrated [10,72]. Close contact between cows is an intrinsic characteristic of dairy herd management systems, and larger herds offer even greater chances for contact and transmission. Densely populated farms are prone to a higher risk of transmission of the pathogen within the herd after *C. burnetii* is introduced into the farm. Additionally, high animal density leads to greater bacterial load and thus higher environmental contamination [73], which may represent an increased risk of transmission to either cattle or people. This meta-analysis showed that elevated prevalence of *C. burnetii* is associated with large-sized herds, where the odds of a BTM sample testing positive double with every unit increase in $\log_e$ herd size (odds ratio $CI_{95\%}$1.24–3.52). Accordingly, of the moderators analysed, average herd size had the largest effect, accounting 33.0% of the observed level of heterogeneity among studies.

While Q fever has been studied in both European and non-European countries, these two contexts have not previously been contrasted. The overall prevalence of *C. burnetii* infection was remarkably similar in European and non-European studies (both 37%). The greater variability among non-European studies ($CI_{95\%}$ 18.0%–58.5%) than among European studies ($CI_{95\%}$ 22.8%–52.2%) could be accounted for by the differences in the numbers of studies and herds investigated.

The mandatory notification of a disease should be helpful not only for early identification of outbreaks but also to enable evaluation of the effectiveness of control strategies. For instance, legislation implemented by the Dutch government in the face of the largest Q fever outbreak ever recorded included compulsory notification of coxiellosis [66]. In the current meta-analysis, a remarkable similarity was noted between overall weighted prevalence of *C. burnetii* in BTM samples from countries with mandatory (37.0%, $CI_{95\%}$22.3–52.9%) and non-mandatory (36.9%, $CI_{95\%}$19.4–56.4%) notification legislation.

In our meta-analysis, the GNI per capita seems to have a minor effect as a moderator of the prevalence of *C. burnetii* in BTM samples. When the studies were stratified according to this indicator of economic development, high-income countries had twice the overall weighted prevalence of upper-middle income countries, albeit that this difference was not statistically significant (P = 0.24). All publications matching the inclusion criteria were conducted in high and upper-middle income countries. None of the studies conducted in low-middle and low-income countries that were identified in the initial search fulfilled the inclusion criteria and were rejected from the meta-analysis. For instance, an ineligible study carried out in Egypt reported a 22% molecular prevalence of *C. burnetii* in individual milk samples [74] and one carried out in Bangladesh reported 15.6% seroprevalence in herd milk specimens [75]. These findings suggest that further field studies could prove rewarding.

The overall prevalence in low-middle and low-income countries remains unknown. There is evidence of extensive ruminant infection with

Some heterogeneity might have resulted from methodological variation among nine of the 17 studies that used qPCR to detect the *IS1111* target. Four of these [25,46,49,52] used the TaqVet *Coxiella burnetii* LSI kit and followed the same manufacturer's instructions for the amplification reaction and for the interpretation of the results. These four studies considered samples as positive with a cycle threshold (Ct) < 40. Two further studies used threshold Ct values of 36.5 [53] and 36.95 [54], while the remaining three studies using qPCR to detect *IS1111* did not report threshold Ct values.

Moreover, whereas the *IS1111* transposon-like element is a multi- copy gene [77], the 16S rRNA target used in South Korean study [44] and the *com1* target used in the two studies in Iran [23,45] are both single copy genes. The assays used in these studies might have had lower sensitivity and indeed, the studies using the single copy assays had three of the four lowest prevalence values. All three of these studies were in non-European countries where the disease is notifiable, and the two Iranian studies were in an upper-middle income country, which may have introduced a degree of bias in the analysis.

Although the moderator analysis identified average herd size as one source, most of the heterogeneity remained unexplained (residual heterogeneity $I^2 = 97.0\%$; $P < 0.01$). It is quite possible that other factors, not currently addressed, influence the *C. burnetii* herd-level prevalence. Unsurprisingly, two of the moderators were highly correlated; studies in European and in high-income countries showed a significant and positive correlation ($r = 0.627$, $P < 0.01$). Awareness of the relationships between moderators that may potentially induce bias in the analysis should be considered when drawing conclusions [78].

5. Conclusion

This meta-analysis reports a high overall global prevalence of *C. burnetii* in BTM samples of 37.0% ($CI_{95\%}$25.2–49.5%), showing widespread herd-level circulation of this agent in bovine dairy farms. These results should be of interest not only for European countries where *C. burnetii* is a well-known health threat, but also in countries where epidemiological investigations have been limited, its importance as a zoonosis may be underestimated and prevention strategies may need to be

C. burnetii throughout African countries where the threat of human exposure and significant economic impact are possibly underestimated [76].

implemented. Information on local biosecurity practices and environmental conditions would be valuable for a full understanding of *C. burnetii* prevalence globally, but such descriptions were lacking in most of the publications considered in the meta-analysis. While this study has shown the global herd prevalence of *C. burnetii* in dairy cattle to be high, in many countries, including high-income countries such as Belgium, Italy, Portugal and UK, the disease is not currently notifiable, and control is not mandatory. To make it so might represent an additional burden on dairy farmers and would require justification on economic or public health grounds for which further study might be required. The high herd-level circulation of *C. burnetii* in bovine dairy farms in several countries showed by this study reinforces the need for further investigations on this globally important zoonosis.

Author contributions

AR and MCE conceptualised the study. AR and MF performed the systematic review, including data collection and screening the retrieved records. AR, MCE and LGC conducted the data-analysis. All authors (AR, MF, LGC, KT, FRC and MCE) made contributions to the interpretation of results. All authors participated in the manuscript drafting and reviewing.

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Declaration of Competing Interest None declared.

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VETERINARY MICROBIOLOGY - REVIEW

Coxiella burnetii abortion in a dairy farm selling artisanal cheese directly to consumers and review of Q fever as a bovine abortifacient in South America and a human milk-borne disease

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Abstract *Coxiella burnetii* is a highly transmissible intracellular bacterium with a low infective dose that causes Q fever (coxiellosis), a notifiable zoonotic disease distributed worldwide. Livestock are the main source of *C. burnetii* transmission to humans, which occurs mostly through the aerogenous route. Although *C. burnetii* is a major abortifacient in small ruminants, it is less frequently diagnosed in aborting cattle. We report a case of *C. burnetii* abortion in a lactating Holstein cow from a dairy farm producing and selling artisanal cheese directly to consumers in Uruguay, and review the literature on coxiellosis as a bovine abortifacient in South America and as a milk-borne disease. The aborted cow had severe necrotizing placentitis with abundant intratrophoblastic and intralesional *C. burnetii* confirmed by immunohistochemistry and PCR. After primo-infection in cattle, *C. burnetii* remains latent in the lymph nodes and mammary glands, with milk being a significant and persistent excretion route. Viable *C. burnetii* has been found in unpasteurized milk and cheeses after several months of maturing. The risk of coxiellosis after the consumption of unpasteurized dairy products, including cheese, is not negligible. This report raises awareness on bovine coxiellosis as a potential food safety problem in on-farm raw cheese manufacturing and sales. The scant publications on abortive coxiellosis in cattle in South America suggest that the condition has probably gone underreported in all countries of this subcontinent except for Uruguay. Therefore, we also discuss the diagnostic criteria for laboratory-based confirmation of *C. burnetii* abortion in ruminants as a guideline for veterinary diagnosticians.

Keywords Abortion · Dairy production · Food safety · Milk-borne disease · Q fever · Zoonosis

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Introduction

Coxiella burnetii is a highly infectious, Gram-negative, obligate intracellular bacterium that causes Q fever (coxiellosis), a zoonosis described worldwide, deemed as re-emerging or emerging in various countries [1], and listed as a notifiable disease by the World Organization for Animal Health (OIE) [2]. Q fever has been regarded as one of the ten most important zoonotic diseases in terms of impact on human health and livestock production, and concern because of emergence or severity in developing countries [3]. Several vertebrate and invertebrate species can host *C. burnetii*; however, domestic ruminants are the major source of human infection [4, 5]. Reproductive losses, particularly abortion, are significant clinical consequences of coxiellosis in goats and sheep, although *C. burnetii* abortion has been infrequently confirmed in cattle [6–8], in which the infection is often subclinical [9]. In addition to abortion, clinical signs in ruminants may include premature delivery, stillbirth, and weak offspring [9], all of which result in economic losses to the livestock sector.

Q fever is mostly an occupational disease; workers in direct or indirect contact with ruminants are at increased risk of infection [10]. It is frequently either subclinical or clinically characterized by nonspecific symptoms, this being the reason why it is commonly undiagnosed [11]. However, *C. burnetii* can cause severe illness and abortion in people; the former is especially true in patients with immunodeficiencies or cardiopathies [12]. While Q fever has long been recognized in humans in most South American countries largely by serologic evaluation [13–18], the epidemiology, sources of infection, and eventual animal reservoirs involved in most cases remain largely unknown. Free-living and captive wildlife species [14, 19], ticks, ruminants [16, 17], and companion animals [20] have been suspected to play a role in transmission.

Coxiella burnetii is mainly transmitted aerogenously and has an extremely low infective dose by this route [21]. It can also be persistently shed in bovine milk and survive in unpasteurized dairy products [22, 23], which raises concerns about the possibility of foodborne transmission. Despite initial findings, when neither clinical Q fever nor antibodies were detected after the deliberate human consumption of unpasteurised milk contaminated with *C. burnetii* [24], the oral route of transmission has been confirmed experimentally in mice [25]. However, discrepancies remain among different research groups about the relevance of *C. burnetii* digestive transmission under non-experimental conditions.

Here, we report a case of bovine abortion caused by *C. burnetii* in a dairy farm in Uruguay that elaborated artisanal cheese which was directly sold to consumers. This prompted us to review the literature on coxiellosis as a cause of bovine abortion in South America and as a milk-borne disease for humans. Considering the few available publications on *C. burnetii* abortion in cattle in South America, we propose that the condition has gone undiagnosed or underreported in most countries of this subcontinent. Therefore, we also discuss the diagnostic criteria for laboratory-based etiologic confirmation of abortive coxiellosis, which could prove valuable as a general guideline for veterinary diagnosticians.

History and diagnostic investigation to identify *C. burnetii* abortion in the affected farm

In November of 2017, a lactating dairy cow from a herd of ~ 100 Holstein cows located in San José, Uruguay, had a spontaneous abortion in the second trimester of gestation. The herd's milk was used on-farm for artisanal

cheese manufacturing, and the cheese was regularly sold directly to consumers.

The aborted fetal tissues and placenta were submitted to the veterinary diagnostic laboratory of INIA for diagnostic workup. Samples of the placenta and tissues, including heart, trachea, esophagus, tongue, eyelid and conjunctiva, lymph nodes, intestines, forestomachs, kidney, liver, brain, synovial joint capsule, and skeletal muscle, were examined macroscopically and no lesions were observed. All samples were immersion-fixed in 10% buffered formalin, routinely processed, and stained with hematoxylin and eosin for histopathology. Microscopically, the chorion had severe diffuse neutrophilic and histiocytic placentitis with multifocal mineralization and necrosis of trophoblasts, as well as neutrophilic arteriolitis. Occasionally, the trophoblasts and infiltrating macrophages were swollen, rounded, and contained myriad intracytoplasmic, basophilic, ~ 1-µm-long coccobacilli (Fig. 1a); similar bacteria were found intralesionally in extracellular locations. No protozoa or fungi were identified in the chorion. The allantois showed lesions comparable to those described in the chorion, except for those involving the trophoblasts. No microscopic lesions or pathogens were found in any of the examined fetal tissues.

Based on the placental lesions, the intratrophoblastic bacteria were strongly suspected as the causative agents. Thus, serial sections of placenta were processed by immunohistochemistry for the detection of *Chlamydia* spp. and *C. burnetii* antigens, as previously described [8, 26], using placenta from two goats naturally infected by *Chlamydia* spp. and *C. burnetii*, respectively, as positive controls. Sections of placenta of the aborted cow, in which the primary antiserum was replaced by non-immune serum, were used as negative controls. The immunohistochemistry for *C. burnetii* showed strong positive immunoreaction, revealing abundant intralesional antigen, both in the cytoplasm of the trophoblasts and macrophages, and extracellularly (Fig. 1b), in the allantois and chorion. *Chlamydia* spp. immunohistochemistry was negative and so were the negative control sections.

For molecular confirmation, DNA was extracted from the placenta using a commercial kit (MagMAX Pathogen RNA/DNA kit, Life Technologies), and later used as a template for *C. burnetii* and *Chlamydia abortus* duplex PCR, based on the repetitive transposon-like region (*IS1111*) and *pmp 90/91* gene, following a previously described protocol [27]. The assay targeted two specific 687-bp and 821-bp long fragments for *C. burnetii* and *C. abortus,* respectively. The PCR was done in 25 µL final volume reactions, with a concentration of 0.8 µM of each primer (Trans-1: 5′-TAT GTAT CCACCG TA GCCA GT-3 ′, Trans-2: 5′-CCCA ACAAC ACC TCC TTA TTC-3′; pmpF: 5′-CTC ACC ATT GTC TCA

GGT GGA-3′, pmpR821: 5′-ACC GTA ATG GGT AGG AGG GGT-3′), 1.5 U of Taq polymerase (New England Biolabs®,

Ispwich, MA), 1 × PCR buffer (New England Biolabs®, Ispwich, MA), 3 mM of $MgCl_2$, 0.2 mM of dNTPs, and 2 µL of template. The PCR was run in a ProFlex™ PCR System (Applied Biosystems, Foster City, CA). The *C. burnetii* Nine Mile phase II strain and *C. abortus* reference strain S26/3 were used as positive controls. Ultrapure water was used as negative control. The PCR products were visualized by electrophoresis in 1.2% agarose gel stained with Good View® dye using a Bio-Rad GelDoc EZ imager (Bio-Rad Laboratories GmbH-Munich, Germany). Amplification revealed

C. burnetii DNA in the placenta, with negative results for *C. abortus*.

To investigate other possible causes of abortion, fetal liver and placenta were routinely cultured at 37 °C for 7 days aerobically on MacConkey and blood agars, as well as microaerobically on Skirrow agar (Oxoid, Basingstoke, Hampshire, England) using sealed

jars and commercial sachets (CampyGenTM, Oxoid, Basingstoke, Hampshire, England), for the simultaneous detection of *Campylobacter* spp. and *Brucella* spp. [28]. Kidney and liver were inoculated into Ellinghausen-McCullough-Johnson-Harris medium for *Leptospira* spp. culture [29]. No bacterial pathogens were isolated by these methods.

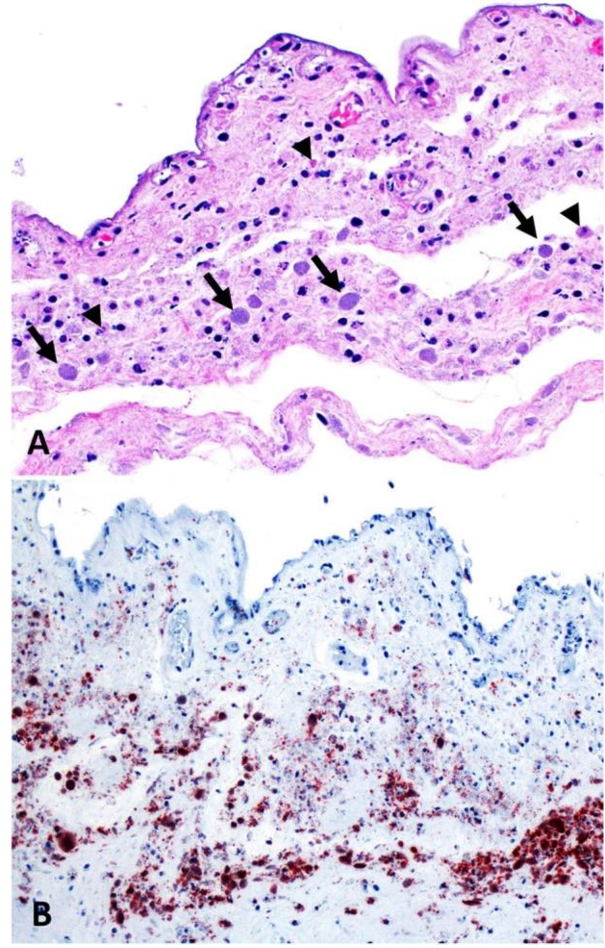

Fig. 1 Microscopic lesions in the placenta of the aborted Holstein cow. **a** The intercotyledonary chorionic stroma is infiltrated by neutrophils and macrophages that contain myriads of intracytoplasmic basophilic coccobacilli (arrows); pyknotic and karyorrhectic hypereosinophilic cellular debris (arrowheads) are indicative of necrosis. H&E. **b** In a serial section of **a**, the bacteria are strongly immunoreactive with *C. burnetii* antiserum, which is depicted as intracytoplasmic and extracellular granular brown chromogen deposition. Immunohistochemistry for *C. burnetii*, hematoxylin counterstain

Additionally, *Campylobacter fetus* and *Leptospira* spp. were investigated by direct immunofluorescence assays on acetone-fixed impression smears of liver and placenta (*C. fetus*), and kidney and liver (*Leptospira* spp.), using pure cultures of these bacteria as positive controls. Samples were incubated with a fluorescein isothiocyanate (FITC)conjugated anti-*C. fetus* antibody (Biotandil, Tandil, Buenos Aires, Argentina), and with a polyclonal rabbit FITC-conjugated antibody (LEP-FAC, NVSL, Ames, IA, USA) for *Leptospira* spp., and examined using a fluorescence microscope (AxioLab.A1, Carl-Zeiss, Germany). The placenta was also examined under dark-field microscopy to assess for trichomonads, spirochetes, or curved bacilli with darting motility. Lastly, the placenta was cultured on a medium for *Tritrichomonas foetus* (CM0161, Oxoid, Basingstoke, UK) supplemented with 1% chloramphenicol and inactivated bovine serum. *Leptospira* spp., *C. fetus*, and *T. foetus* were not detected by these methods.

In summary, we found placentitis with intratrophoblastic bacteria that were reactive with *C. burnetii* immunohistochemistry and identified *C. burnetii* DNA by PCR, while other abortifacients were not detected. Altogether, the results of the diagnostic investigation supported an etiologic diagnosis of *C. burnetii* placentitis and abortion.

Diagnostic criteria and challenges of laboratory-based diagnosis of *C. burnetii* bovine abortion

The examination of the placenta is the keystone in the diagnostic investigation of *C. burnetii* abortion [6, 8, 9]; thus, it is critical that the placenta is submitted to the laboratory when attempting to investigate coxiellosis. Obtaining placenta of aborted cattle under field conditions suitable for laboratory investigation, i.e., before significant autolysis and post-mortem contamination occur, is challenging, particularly in extensive pasture-based production systems such as those prevalent in South America. In fact, most submissions to veterinary diagnostic

laboratories include the aborted fetuses, but the placenta is much less frequently included [30–33], reducing the chances of reaching an etiologic diagnosis.

Placental lesions caused by *C. burnetii* can be severe enough to be appreciated grossly as intercotyledonary and cotyledonary placentitis, although in some cases the infection can induce subtle macroscopic placental alterations, while in others the placenta may look unremarkable [6, 8, 34]. Because *C. burnetii* targets mainly the placenta with high tropism toward trophoblasts, the histologic examination of this tissue is critical and perhaps the single most informative laboratory investigation. *Coxiella burnetii* colonization frequently induces a neutrophilic or mixed inflammatory reaction and necrotizing placentitis, which along with the visualization of abundant intracytoplasmic coccobacilli within distended trophoblasts, guide toward the diagnosis of coxiellosis [6, 8, 9]. Because *C. burnetii* does not usually cause lesions in the fetal tissues, even when severe placentitis is present, the examination of the fetal tissues is usually unrewarding [8]. Although fetal pneumonia has been described as an accompanying lesion in a few confirmed cases of *C. burnetii* abortion in cattle [6, 8], this is a non-specific lesion that can be caused by many bacterial, fungal, or protozoal infections, such as *T. foetus* [35]. As an association between lesions and the presence of the bacterium has been regarded as mandatory to confirm *C. burnetii* abortion in cattle [9], and lesions are mostly restricted to the placenta, laboratory submissions not including the placenta should be considered unsuitable for the assessment of *C. burnetii* abortion.

Once a histologic diagnosis of necrotizing placentitis with intratrophoblastic bacteria has been established, the identification of *C. burnetii* is the next step in the diagnostic investigation. This can be achieved through PCR-based tests, immunohistochemistry [6, 8], fluorescent *in situ* hybridization (FISH) [36], or combinations thereof. Besides *C. burnetii*, other intracellular bacteria that can cause placentitis and invade the trophoblasts including *C. abortus* and *Brucella abortus* [35] should be considered as differential diagnoses. In the case described here, abundant *C. burnetii* antigen was detected intralesionally by immunohistochemistry, and the presence of the agent was further confirmed by PCR, while *C. abortus* and *B. abortus* were ruled out by specific testing (immunohistochemistry and PCR for *C. abortus*, and selective culture for *B. abortus*). Thus, the identification of typical placental lesions in conjunction with the detection of *C. burnetii*, along with the exclusion of other abortifacients that can cause similar placental lesions, fulfilled the diagnostic criteria for etiologic confirmation of *C. burnetii* placentitis (Fig. 2) [9].

When attempting to identify *C. burnetii* infection and abortion either through direct or indirect laboratory methods, the use of single laboratory tests may be misleading. The mere detection of *C. burnetii* DNA in the placenta or fetal tissues does not necessarily imply disease causality, considering that subclinical infections are common [9] and the high molecular prevalence in dairy herds [37]. Similarly, serologic approaches at the individual level are not informative enough, as seroconversion can occur without detectable lesions or bacterial shedding, animals can remain seropositive long after they have overcome the infection, shed *C. burnetii* before the development of detectable antibodies, and even shed the agent without ever seroconverting [38]. Attempting the isolation of *C. burnetii* poses an unnecessary risk and requires level III biosecurity laboratories. Both PCR and immunohistochemistry are valuable tools for *C. burnetii* detection in diagnostic settings [6, 8]. PCR-based assays are sensitive, specific, and quick screening methods used in a wide variety of samples. Quantitative PCR targeting the *IS1111* gene has been used to quantify the bacterial load in placenta of aborted cattle [34,

39]. Immunohistochemistry enables the colocalization of *C. burnetii* antigen within lesioned tissues, which is a powerful indicator of causality [6]. Interestingly, in the case described here, although the chorion showed strong positive immunoreactivity by immunohistochemistry, the signal was even stronger in the allantois, which represents an unusual localization of bacterial antigen. FISH targeting the 16S ribosomal RNA of *C. burnetii* has been used experimentally for the intralesional identification of the agent in formalin-fixed paraffin-embedded placenta of aborted cattle, obtaining results comparable to those of immunohistochemistry [36], although this technique has not been broadly adopted in diagnostic settings. FISH targeting the 16S rRNA is a promising marker for intact and metabolically active bacterial cells, representing an alternative to evaluate *C. burnetii* viability when bacterial isolation or inoculation in experimental animals are not available options [40, 41]. The lack of veterinary diagnostic laboratories offering histology and validated immunohistochemical, FISH, and PCR-based tests for the identification of *C. burnetii* placentitis is a major limitation for the diagnosis of coxiellosis in cattle and other ruminants in South America.

Coxiella burnetii as a bovine abortifacient in South America

Scientific publications providing confirmatory evidence of spontaneous bovine abortions caused by *C. burnetii* are scarce not only in South America, where confirmed cases have only been reported in Uruguay [8, 42], but also globally [6, 7, 34]. *Coxiella burnetii* has been generally linked to sporadic abortion in cows, exhibiting infection rates that resemble those of opportunistic bacteria [6, 7, 9]. A recent study from Uruguay reported a cluster of four cases of abortion due to *C. burnetii* in Holstein cows in one dairy farm, based on gross and microscopic examination of the placentas, coupled with the identification of the agent by immunohistochemistry and PCR [8]. These four cases occurred between April and June of 2017; a fifth case was confirmed in August of the same year in the same farm [42]. This indicates that *C. burnetii* abortion in cattle can occur in clusters affecting several animals in a herd, as is usually the case in small ruminants. *Coxiella*

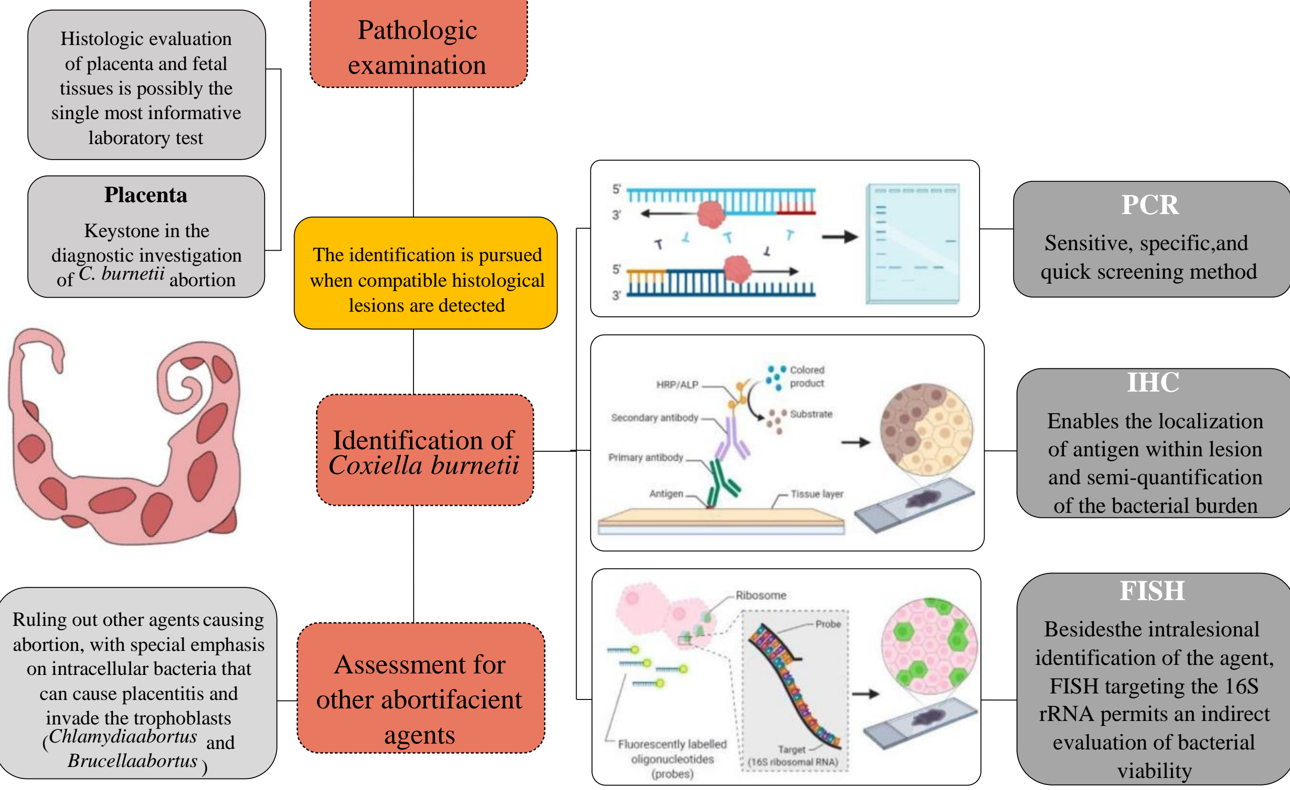

Fig. 2 Diagnostic workflow for laboratory-based confirmation of abortion caused by *Coxiella burnetii* in ruminants. PCR, polymerase chain reaction; IHC, immunohistochemistry; FISH, fluorescent *in situ* hybridization

burnetii was not identified as a cause of abortion in various case series aiming at assessing abortion causality in beef and dairy cattle in Argentina [30, 31], Brazil [32, 33], Uruguay [43], and Chile [44]. Collectively these studies analyzed 2080 aborted bovine fetuses, although none of them specifically tested for *C. burnetii* and only a minor subset of submissions included placentas; thus, the pathogen and disease may have been easily overlooked.

Other studies from South America aimed at investigating *C. burnetii* infection in aborted cattle. A retrospective survey conducted in Brazil, where pools of organs, gastric content, and brain from aborted bovine fetuses and stillborn calves were analyzed by PCR for the identification of *C. burnetii* DNA, found an infection rate of 10.7% (3/28) [45]. Whether these cases were examined histologically to assess for lesions of coxiellosis, as would have been required for attributing causality, was not reported.

In Ecuador, a case–control serologic study assessed the role of *C. burnetii* as a cause of bovine abortion in two large-scale dairy herds, each with approximately 2000 cows and abortion rates of 3–5%. Sera of 172 cows were screened for anti-*C. burnetii* antibodies using a commercial ELISA. The overall seroprevalence was high (52.9%), but no association with abortion was established as the seroprevalence was higher in the 77 non-aborted (57.1%) than in the 95 aborted (49.5%) cows [46].

The lack of scientific reports on bovine abortions caused by *C. burnetii* in other South American countries in which the agent is known to be present suggests that the disease may have gone undetected or underreported. This might in part reflect the difficulties and challenges associated with *C. burnetii* diagnostic confirmation and the limited availability of appropriate laboratory assays in veterinary laboratories in the region. However, the significance of *C. burnetii* as an abortifacient of cattle in the region should not be underestimated.

Q fever as a milk- and dairy-borne human disease and risk of Q fever through consumption of dairy products

The inhalation of contaminated aerosols, following normal parturition or abortion of domestic ruminants, is the major path of *C. burnetii* infection in people [9, 47]. However, after the initial infection in cattle, the bacterium remains latent in the lymph nodes and mammary glands, and bacterial shedding (presumably within macrophages) can occur in subsequent calving seasons and lactations, with milk shedding being a significant and persistent excretion route of *C. burnetii* [22]. As *C. burnetii* is an obligate intracellular bacterium, it is assumed that bacterial replication does not occur in milk and dairy products [48]; however, the agent is highly resistant to chemical and physical stressors [49], and can remain viable for long periods in the environment and in bovine milk at room temperature [50]. Studies that quantified *C. burnetii* shedding by qPCR have been conducted in individual milk samples of goats and cattle, although this molecular approach cannot distinguish between viable and non-viable bacteria. Goat samples presented concentrations in the range of 1×10^2 to 1×10^6 *C. burnetii* cells per ml when targeting the single copy gene *com1* [51], whereas cow samples showed similar concentrations varying from 1×10^1 to 1×10^4 *C. burnetii* cells per ml when targeting the *IS1111* [52, 53]. Differences on the *C. burnetii* load in milk among studies may suggest a heterogeneous bacterial shedding by this route. Further evaluation investigated the mean level of viable *C. burnetii* per ml of unpasteurized milk in shedding cows. This was estimated using the guinea pig (GP) intraperitoneal (IP) infectious dose (ID) 50% per ml (GP IP ID_{50}/ml), which is the dose intraperitoneally administered to all

members of a group of GP that results in 50% of them being infected. This mean level was approximately 98.8 GP IP I D_{50}/ml; each GP IP ID_{50} presumably representing between 2 and 112 bacteria per ml of milk [22, 48, 54]. Simulations based on these data suggest that the daily exposure to viable *C. burnetii* through unpasteurized milk in people can be high [48], although given the lack of dose–response data in humans, it is unknown whether this translates into a risk of infection through the oral route, which would have implications in food safety and public health.

Early studies conducted in people ingesting bovine milk naturally contaminated with undetermined concentrations of viable *C. burnetii* suggested that subjects exposed through the oral route did not develop clinical signs of Q fever [24, 55]. Results on post-ingestion serology were variable, while in one study all 34 exposed individuals remained seronegative [24], in another 35% (42 of 120) turned seropositive and 10% (12/120) showed a fourfold or greater increase in antibody titers (seroconversion) [55]. These different serologic outcomes were speculated to result from differences in the *C. burnetii* strains involved in the studies, although it should be considered that the load of viable bacteria may have also differed between studies. A recent experimental study in immunocompetent BALB/c mice (considered of intermediate sensitivity to *C. burnetii*) demonstrated that after gastric inoculation of 1×10^6 genome equivalents of *C. burnetii*, the agent can colonize and persist in the digestive tract, penetrate the intestinal barrier, colonize the mesenteric lymph nodes, and invade the blood and peripheral tissues including the liver and lungs [25]. More data are needed to understand the consequences of ingesting viable *C. burnetii* in people considering the infective doses and bacterial strains.

The risk of Q fever transmission through consumption of dairy products has been reviewed fairly recently [48]. While considered much lower than the risk of airborne transmission, the risk of oral transmission after the ingestion of contaminated raw milk or unpasteurized dairy products, including cheese, was regarded as not negligible [48]. Serological evaluations conducted in France linked the consumption of contaminated unpasteurized milk with seroconversion in people [56]. A serologic survey of a cohort of goat farmers, workers, and their contacts, involved in an outbreak of Q fever in the Canadian province of Newfoundland, identified the consumption of cheese made with pasteurized goat milk as a significant independent risk factor for infection [57]. Likewise, a 2-year epidemiological evaluation conducted in 1200 hospitalized children in Greece found that eating raw cheese coming from rural areas enhanced the risk of Q fever (p = 0.04, OR = 6, 95% CI = 1.1–33.2) [58]. Clusters of Q fever cases in which the ingestion of unpasteurized bovine milk was considered the most likely source of infection have been reported in the UK and USA [59, 60].

Numerous investigations revealed *C. burnetii* DNA in milk and derived products, including cheese, cream, butter, and yoghurt from cows, goats, and sheep [23, 61–63]. A molecular investigation performed on the most traditional and oldest type of raw-milk cheese in Brazil, known as Minas artisanal cheese and manufactured with bovine milk, revealed a high prevalence of *C. burnetii* in this ready-to-eat product, and estimated that 1.62 tons of cheese produced daily is contaminated with this bacterium [63]. *Coxiella burnetii* has been isolated from unpasteurized bovine milk [24, 55], including milk commercialized in the USA [64]. Molecular studies suggest that the *C. burnetii* genotypes predominating in dairy products are the same that infect dairy cattle [65]. However, only a few studies took a step further toward the investigation of its viability and hazard. Viable *C. burnetii* was proven in raw cheese by culture in Vero cells and inoculation in mice [23]. The potential inactivating effect of cheese ripening was

dismissed as viable *C. burnetii* was detected in samples of unpasteurized hard cheeses after 8 months of maturing [23]. There is little evidence that any of the processes used to produce butter or cream with unpasteurized milk would significantly inactivate the pathogen [48].

Coxiella burnetii in milk is successfully inactivated by pasteurization, which is fundamental for the prevention of milk-borne infectious illnesses, some of which, such as tuberculosis and brucellosis, are endemic in dairy cattle in South America. The oral route of infection and eventual foodborne transmission of *C. burnetii* should not be neglected and farmers, particularly those producing artisanal cheese on-farm instead of selling the milk to the dairy industry, as well as consumers, ought to be aware of the importance of pasteurization. In herd-level studies, *C. burnetii* was screened for by real-time quantitative PCR in bulk tank milk of 105 bovine dairy herds as part of the epidemiologic investigation of an outbreak of Q fever among dairy farm workers in Chile in 2017. Although only two farms tested positive, both sold milk directly to the local community that was consumed either raw or boiled, which was considered a potential source of infection to humans [66]. In 2017 in Brazil, *C. burnetii* DNA was found by the same technique in 4 of 112 samples of raw bovine milk that were being sold illegally for human consumption without official inspection at grocery stores, bars, farmers' markets, and small farms, which was identified as of public health concern [67]. In 2012, a random sampling conducted in Montería, Colombia, showed that 5 of 11 bulk tank milk samples collected from commercial cattle farms presented *C. burnetii* DNA, and 37 out of 61 (60.7%) apparently healthy farm workers at risk had specific IgG phase II antibody titers ≥ 1/64, suggestive of recent bacterial exposure [68].

In Uruguay, raw milk trade was first regulated in 1984, and its commercialization for direct consumption by humans is currently banned; however, the consumption of raw milk and milk products in rural areas is difficult to quantify, and therefore, to control. Of the nearly 18000 tons of cheese consumed yearly in the country, ~ 50% represents artisanal cheese produced in ~ 1000 dairy farms, most of which are in the departments of San José and Colonia. Artisanal cheese is largely commercialized internally directly to consumers at the manufacturing farms or farmers' markets, or at larger scales through intermediaries, but international contraband of Uruguayan artisanal cheese has also been documented [69]. It has been estimated that up to 50% of artisanal cheesemakers produce under informal conditions, implying that they do not necessarily comply with regulations established by the Uruguayan Ministry of Livestock, Agriculture, and Fisheries [69]. A survey conducted among local artisanal cheesemakers revealed that only a minority use pasteurized milk [69]. This practice may embody a hazard for Q fever transmission to consumers, considering the high stability of *C. burnetii* in final dairy products even with acidic pH or reduced water activity [23]. Due to its indigenous microbiota, the cheeses made with unpasteurized milk have specific organoleptic characteristics of gastronomic value, such as a strong flavor and a peculiar texture, much appreciated by consumers [23, 70]. The consumers' preferences toward raw milk products are emerging as a growing global trend, which could be of public health concern as this implies a higher risk of acquiring milk-borne diseases.

Conclusions

Our investigation expands the evidence supporting *C. burnetii* as a significant cause of bovine abortion in Uruguay and represents the first report of *C. burnetii* abortion in a dairy farm producing and selling artisanal cheese directly to consumers. The scant scientific literature on *C. burnetii* abortion in cattle from South America suggests that this notifiable and

zoonotic disease may have gone undetected or underreported in most countries of this subcontinent. Laboratory investigations for the etiologic confirmation of *C. burnetii* abortion should rely on the observation of typical placental lesions in aborting dams, coupled with the identification of *C. burnetii* by immunohistochemistry, FISH, and/or PCR. The existing literature supports that raw milk and derived dairy products represent potential sources of *C. burnetii* transmission to humans, although further investigations are needed to assess the risk of digestive transmission to humans considering exposure, infective doses, and bacterial strains. The threat to public health posed by *C. burnetii* through dairy products should not be neglected, and the need for on-farm milk pasteurization by artisanal cheesemakers should be emphasized. Further epidemiologic investigations are needed to better understand the role of *C. burnetii* as a cause of abortion in cattle in South America, and the risk and impact of Q fever transmission through the ingestion of unpasteurized dairy products in the region.

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Author contribution Ana Rabaza conceptualized the study, conducted laboratory work, wrote the initial manuscript draft, reviewed, edited, and approved the manuscript. Melissa Macías-Rioseco conducted laboratory work, reviewed, edited, and approved the manuscript. Martín Fraga conducted laboratory work, reviewed, edited, and approved the manuscript. Francisco A. Uzal conducted laboratory work, reviewed, edited, and approved the manuscript. Mark C. Eisler reviewed, edited, and approved the manuscript. Franklin Riet-Correa acquired funding, reviewed, edited, and approved the manuscript. Federico Giannitti conceptualized the study, conducted laboratory work, acquired funding, wrote, reviewed, edited, and approved the manuscript.

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Data availability Data and material are available (without disclosing the farm information) from the corresponding author on reasonable request.

Conflict of interest The authors declare no competing interests.

Code availability Not applicable

Consent to publish Not applicable.

Ethics approval The study was not conducted on live animals; therefore, no ethical approval is required.

Consent to participate Not applicable.

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Article

Serological Evidence of Human Infection with *Coxiella burnetii* after Occupational Exposure to Aborting Cattle

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Abstract: Cattle are broadly deemed a source of *Coxiella burnetii*; however, evidence reinforcing their role in human infection is scarce. Most published human Q fever outbreaks relate to exposure to small ruminants, notably goats. Anti-phase II *C. burnetii* IgG and IgM were measured by indirect fluorescent antibody tests in 27 farm and veterinary diagnostic laboratory workers to ascertain whether occupational exposure to cattle aborting due to *C. burnetii* was the probable source of exposure. Four serological profiles were identified on the basis of anti-phase II IgG and IgM titres. Profile 1, characterised by high IgM levels and concurrent, lower IgG titres (3/27; 11.1%); Profile 2, with both isotypes with IgG titres higher than IgM (2/27; 7.4%); Profile 3 with only IgG phase II (5/27; 18.5%); and Profile 4, in which neither IgM nor IgG were detected (17/27; 63.0%). Profiles 1 and 2 are suggestive of recent *C. burnetii* exposure, most likely 2.5–4.5 months before testing and, hence, during the window of exposure to the bovine abortions. Profile 3 suggested *C. burnetii* exposure that most likely predated the window of exposure to aborting cattle, while Profile 4 represented seronegative individuals and, hence, likely uninfected. This study formally linked human Q fever to exposure to *C. burnetii* infected cattle as a specific occupational hazard for farm and laboratory workers handling bovine aborted material.

Keywords: coxiellosis; indirect fluorescent antibody test (IFAT); occupational hazard; Q fever; zoonosis

1. Introduction

Coxiella burnetii causes the zoonosis Q fever, a disease which typically occurs after the inhalation of aerosolised contaminated material from the placenta or birth fluids of ruminants following either abortion or normal delivery [1,2]. Evidence for transmission of *Coxiella burnetii* by ingestion of contaminated raw dairy products is equivocal; infection was reported after consumption of raw cow's milk [3] and contaminated goat cheese [4,5], but neither clinical Q fever nor antibodies were detected following deliberate human consumption of unpasteurised contaminated milk [6]. Although there is a high global prevalence of *C. burnetii* in cattle [7] and cattle are widely considered a risk for Q fever, there is little, if any, formal evidence for the contribution of bovines to human infection. Most published reports of human outbreaks in Europe relate to exposure to small ruminants, notably goats, as the main source of human infection [8–11]. Evidence for the association of an outbreak

of Q fever affecting 1300 people in southeast Poland with cattle was limited to the demonstration of specific antibodies in bovines [12,13]. Only weak serological evidence supported *C. burnetii* as the cause of bovine abortions that were epidemiologically associated with Q fever outbreaks in Germany and Poland, but other common bovine abortifacient agents such as *Neospora caninum* and *Brucella abortus* were not investigated [14,15]. We are not aware of any published data firmly linking Q fever to *C. burnetii*-positive bovine abortions.

Q fever is asymptomatic in approximately 60% of cases [16]. Acute clinical presentation includes a wide range of non-specific symptoms, while endocarditis and chronic fatigue syndrome are the principal chronic manifestations [2,17], particularly in people with pre-existing conditions (cardiopathies, aneurysms, immunocompromise or pregnancy) [18–20].

Variation in the lipopolysaccharides of the bacterial outer membrane results in antigenic phases that determine diverse types of anti-*C. burnetii* immunoglobulins (anti-phase I and anti-phase II IgM, IgG and IgA). During infection, phase II antigens appear to dominate immunoglobulin responses [21] and immunoglobulin profiling is used to characterise chronicity of exposure [22–24]. Immune responses to phase II antigens are characteristic of acute exposure, whereas anti-phase I titres characterise chronicity [25,26].
While no previous human Q fever outbreak has been firmly related to bovine abortion caused by *C. burnetii*, this study presents more substantial laboratory evidence for a specific occupational hazard for workers exposed to aborted cattle or handling material from bovine abortions.

7.6 2. Materials and Methods

7.6.1.1 *2.1. Bovine Abortions and Window of Workers Exposure*

Following an outbreak of bovine abortion in a dairy herd in Colonia Department, Uruguay, placentas and full-term foetuses from four aborting cattle were collected by farmworkers and submitted to the local veterinary diagnostic laboratory between 10 April and 2 June 2017. Bovine coxiellosis was confirmed on the basis of typical placental lesions on histopathology, with identification of intralesional *C. burnetii* antigen in trophoblasts by immunohistochemistry and PCR amplification of DNA, and other abortifacients of cattle were ruled out by comprehensive testing [27]. The outbreak of coxiellosis was notified to the local health authorities, which triggered an investigation by public health officials. Serological sampling of humans was conducted on 14 and 21 August 2017, i.e., 18.1 and 19.1 weeks following exposure. Serological testing was performed on 27 farm and laboratory workers directly or indirectly exposed to the aborting cattle, foetuses

and placentas. None of these workers had been vaccinated against *C. burnetii*.

7.6.1.2 *2.2. Farm and Laboratory Workers' Data and Consent*

Written consent was obtained from all patients and their information was anonymised. Records comprised demographic data such as age, gender, clinical findings obtained during a medical examination, pre-existing medical conditions and the individual laboratory indirect fluorescent antibody test (IFAT) results for anti-*C. burnetii* phase II IgM and IgG antibodies. Data were made available by explicit agreement of the workers who were assured of confidentiality. Details about medical treatments could not be accessed. No animal or human samples were collected or analysed expressly for this study and results of laboratory testing were evaluated for this study as a secondary analysis. The study was granted ethical approval by the ethical committee from the University of Bristol (Ref.95382/Id.342095).

7.6.1.3 *2.3. Review of Case Records from the Veterinary Diagnostic Laboratory*

Records of diagnoses made by the local veterinary diagnostic laboratory between 10 April 2016 and 21 August 2017 were examined to rule out other potential exposures of laboratory workers to *C. burnetii*.

7.6.1.4 *2.4. Indirect Fluorescent Antibody Test*

Serum samples were analysed for anti-*C. burnetii* phase II IgM and IgG antibodies by the Mayo Clinic Laboratory (Rochester, MN, USA) using the indirect fluorescent antibody test (IFAT) for anti-*C. burnetii* phase II IgM and IgG antibodies [28,29].

7.6.1.5 *2.5. Statistical Analysis*

Titres less than 1/16 in the IFAT for anti-*C. burnetii* phase II IgM and IgG antibodies were considered to be seronegative and those greater or equal to 1/16 were considered to be seropositive. The percentage of seropositivity was calculated as the number of seropositive individuals (titre ≥ 1/16) divided by the total number of workers tested. Phase II IgG to IgM ratios were calculated by dividing the IgG titre by the IgM titre. Univariable and multivariable analyses were conducted in which the IFAT status (seropositive or seronegative) was considered as the binary response variable. Gender (male and female), age group (21–30, 31–40 and >40) and work activity (farm and laboratory) were included as explanatory variables in univariable and multivariable logistic regression models used to gain insight into factors (and their interactions) influencing *C. burnetii* seropositivity and to calculate odds ratios (OR) and their confidence intervals ($CI_{95\%}$). Statistical analysis was performed using RStudio software [30].

7.7 3. Results

The study population comprised 27 individuals who worked either on the farm, in the laboratory or both. Twenty-three individuals

conducted at least some of their work on the farm, these comprising thirteen farm workers, two veterinary practitioners and eight laboratory workers. Twelve individuals conducted at least some of their work in the veterinary laboratory, these comprising the eight laboratory workers, who also conducted some farm work, and four further laboratory workers who did not.

Ten of the 27 individuals had detectable titres of IgG antibody to *C. burnetii* phase II greater or equal to 1/16, and, of these, five also had detectable titres of IgM. Of the

23 conducting work on the farm, eight (34.8%) were IgG positive and four (17.4%) of these were also IgM positive. Seven of the twelve (58.3%) conducting lab work had detectable IgG titres, and four (33.3%) of these were also IgM positive, noting that eight individuals undertook both types of work (Table 1). The univariable odds ratios for conducting laboratory work were 5.6 ($CI_{95\%}$ 1.09–35.6, $p = 0.039$) for IgG seropositivity and 7.0 ($CI_{95\%}$ 0.853–150, $p = 0.071$) for IgM seropositivity, i.e., statistically significant for IgG and close to significance for IgM. The corresponding univariable odds ratios for conducting farm work were 0.533 ($CI_{95\%}$ 0.055–5.13, $p = 0.566$) for IgG seropositivity and 0.632 ($CI_{95\%}$ 0.060–14.6, $p = 0.726$) for IgM seropositivity, i.e., not significant in either case.

The rate of seropositivity was twice as high in female workers (5/9, 55.6%; univariable odds ratio 3.25, $CI_{95\%}$ 0.623–18.7; $p = 0.162$) as in males (5/18, 27.8%) for IgG, but only slightly higher in females (2/9, 22.2%; univariable odds ratio 1.43, $CI_{95\%}$ 0.161–10.7; $p = 0.729$) than males (3/18, 16.7%) for IgM, in neither case statistically significant differences.

Rates of IgG seropositivity in age groups 21–30 (4/8, 50%) and 31–40 (5/10, 50%) were identical and these were collapsed into a single category. Seropositivity in individuals less than or equal to 40 years old (9/18, 50.0%; univariable odds ratio 8.00, $CI_{95\%}$ 1.12–165; $p = 0.037$) was significantly higher than those greater than 40 (1/9, 11.1%) for IgG whereas for IgM, seropositivity in individuals less than or equal to 40 (4/18, 22.2%; univariable odds ratio 2.29, $CI_{95\%}$ 0.275–48.9; $p = 0.468$) was not significantly higher than those greater than 40 (1/9, 11.1%). Four of the five (80%) individuals seropositive for IgM were in the 31–40 year age group, which was significant (univariable odds ratio for age 31–40 compared to all other ages 10.7, $CI_{95\%}$ 1.27–233, $p = 0.0283$).

Table 1. Anti-*Coxiella burnetii* phase II IgM and IgG titres, IgG to IgM ratio, demographic factors, background data of workers and potential exposure based on work activity.

Worker ID	Age Range (Years)	Gender	Type of Work	Exposure Category	IgG Phase II Titre *	IgM Phase II Titre *	Phase II IgG/IgM Ratio	Symptomatic †
1	41–50	M	Bacteriologist	Laboratory	1/256	1/512	0.5	Yes
2	21–30	F	Veterinary diagnostician	Farm and laboratory	1/512	<1/16	-	Yes
3	21–30	F	Veterinary diagnostician	Farm and laboratory	1/64	<1/16	-	Yes
4	31–40	F	Veterinary diagnostician	Farm and laboratory	1/32	1/256	0.1	No
5	31–40	F	Farm veterinarian	Farm	1/64	1/128	0.5	Yes
6	31–40	M	Veterinary diagnostician	Farm and laboratory	1/128	1/16	8	Yes
7	31–40	M	Laboratory technician	Farm and laboratory	1/512	1/256	2	No
8	31–40	F	Veterinary diagnostician	Laboratory	1/16	<1/16	-	No
9	41–50	M	Farmworker	Farm	<1/16	<1/16	-	No
10	21–30	M	Farmworker	Farm	1/16	<1/16	-	Yes
11	31–40	F	Laboratory technician	Laboratory	<1/16	<1/16	-	Yes
12	61–70	M	Farmworker	Farm	<1/16	<1/16	-	No
13	21–30	M	Farmworker	Farm	<1/16	<1/16	-	Yes
14	21–30	M	Farm veterinarian	Farm	<1/16	<1/16	-	Yes
15	31–40	M	Farmworker	Farm	<1/16	<1/16	-	Yes
16	51–60	M	Farmworker	Farm	<1/16	<1/16	-	Yes
17	21–30	M	Farmworker	Farm	1/16	<1/16	-	No
18	41–50	M	Farmworker	Farm	<1/16	<1/16	-	Yes
19	31–40	F	Farmworker	Farm	<1/16	<1/16	-	No
20	51–60	M	Farmworker	Farm	<1/16	<1/16	-	No
21	41–50	M	Farmworker	Farm	<1/16	<1/16	-	No
22	31–40	F	Veterinary diagnostician	Farm and laboratory	<1/16	<1/16	-	Yes
23	31–40	M	Veterinary diagnostician	Farm and laboratory	<1/16	<1/16	-	No
24	21–30	M	Veterinary diagnostician	Farm and laboratory	<1/16	<1/16	-	Yes
25	51–60	M	Farmworker	Farm	<1/16	<1/16	-	No
26	41–50	M	Farmworker	Farm	<1/16	<1/16	-	Yes
27	21–30	F	Laboratory technician	Laboratory	<1/16	<1/16	-	Yes

* For both antibody isotypes, titres of <1/16 were considered negative; M: male; F: female. † At least one suggestive symptom reported.

Seropositivity levels for IgG were similar in symptomatic and asymptomatic (4/11, 36.4%) individuals for both IgG (symptomatic 6/16, 37.5%; asymptomatic 4/11, 36.4%; univariable odds ratio for symptoms 1.05, $CI_{95\%}$ 0.213–5.4; $p = 0.952$) and IgM (symptomatic 3/16, 18.8%; asymptomatic 2/11, 18.2%; univariable odds ratio for symptoms 1.04, $CI_{95\%}$ 0.143–9.12; $p = 0.970$).

In the multivariable analysis for IgG seropositivity addition of none of the terms farm work, age group, gender or symptoms improved upon the univariable model with laboratory work as the sole explanatory variable (likelihood ratio test $p > 0.4$ for all), suggesting this was already the minimum adequate model ($p = 0.039$). However, lab work was apparently confounded with age, with 11 of the 12 individuals conducting lab work being under 40 years of age. On collapsing the age group to just two levels, as already noted, nine of the eighteen (50%) workers less or equal to 40 years of age were seropositive for IgG, but just one of the nine (11.1%) workers over 40 years of age was IgG seropositive (odds ratio and $CI_{95\%}$ as above), and this was also the only individual in the over 40 age group conducting lab work; contrastingly, there were broadly similar numbers of IgG seropositives (6/9, 66.6%) and seronegatives (5/9, 55.6%) conducting lab work in the 40 and under age group (Fisher's exact test $p \approx 1$).

There were too few IgM seropositive individuals (n = 5) for a meaningful multivariable analysis; it was, however, noteworthy that four of the five conducted farm work, four conducted lab work, with three conducting both farm and lab work, and that all four of those IgM positive individuals conducting field work were in the 31–40 age category, the remaining IgM seropositive individual, who conducted only lab work, being in the 41–50 age category.

When anti-*C. burnetii* phase II IgM and IgG titres were interpreted in conjunction, four distinct serological profiles could be identified among the workers (Figure 1). Five workers (IDs. 1, 4, 5, 6 and 7) had detectible titres (at least 1/16) of both IgM and IgG. Three of these five workers (IDs. 1, 4 and 5) whose IgM titres were higher than their IgG titres were classified as Profile 1, while the two workers (IDs. 6 and 7) whose IgG titres were higher than their IgM titres were classified as Profile 2. Five workers (IDs 2, 3, 8, 10 and 17) showed only IgG phase II titres with no detectable levels of IgM and were classified as Profile 3. Finally, 17 workers (IDs 9, 11–16, 18–27), in whom neither IgM nor IgG titres

were detected, were classified at Profile 4.

Six of the ten seropositive workers manifested a variety of non-specific symptoms, whereas the remaining four seropositive workers remained asymptomatic. Among those with clinical disease, sweating, fever, fatigue and odynophagia were the most frequently reported. Most of the symptomatic workers (IDs 1, 3, 5 and 6) manifested clinically by

middle–late May, i.e., three months before their serologic evaluation. Two workers (IDs 2 and 10) reported non-specific symptoms occurring around mid–late July (a month before serological examination). None of the seropositive workers had any medical condition known to predispose them to subsequent medical complications [18–20]. A review of the seventeen seronegative workers' medical records revealed that ten presented some non-specific flu-like symptoms, whereas the other seven of these seronegative workers remained asymptomatic. The proportion of symptomatic individuals was very similar among seropositive (6/10, 60.0%) and seronegative workers (10/17, 58.8%) (univariable analysis, $p \approx 1.00$).

The local veterinary laboratory examined submissions from fifty bovine and five ovine cases of abortion. Each case comprised either the foetus, the placenta or both. All cases were routinely examined for gross and histologic lesions, and cultured onto MacConkey and blood agar, Skirrow's medium and *Leptospira* medium-based EMJH agar. Additionally, *Neospora caninum*, *Campylobacter* spp., *Tritrichomonas foetus*, Bovine parainfluenza virus 3 and Bovine viral diarrhoea virus 1 were investigated by immunohistochemistry, direct immunofluorescence, dark-field microscopy examination or PCR. None of these 55 foetuses presented any typical lesions leading to suspicion of coxiellosis. The cause of the bovine abortion was diagnosed in 25 of these cases (25/50). Most were diagnosed as infectious abortions (23/25) including agents such as *N. caninum* (11/23), *Campylobacter fetus* subsp. *venerealis* (1/23) and Bovine parainfluenza virus 3 (1/23), as well as opportunistic agents (8/23). In two out of the five cases of ovine abortion, *Campylobacter jejuni* and *Campylobacter fetus* were detected by PCR, while the other three cases remained undiagnosed.

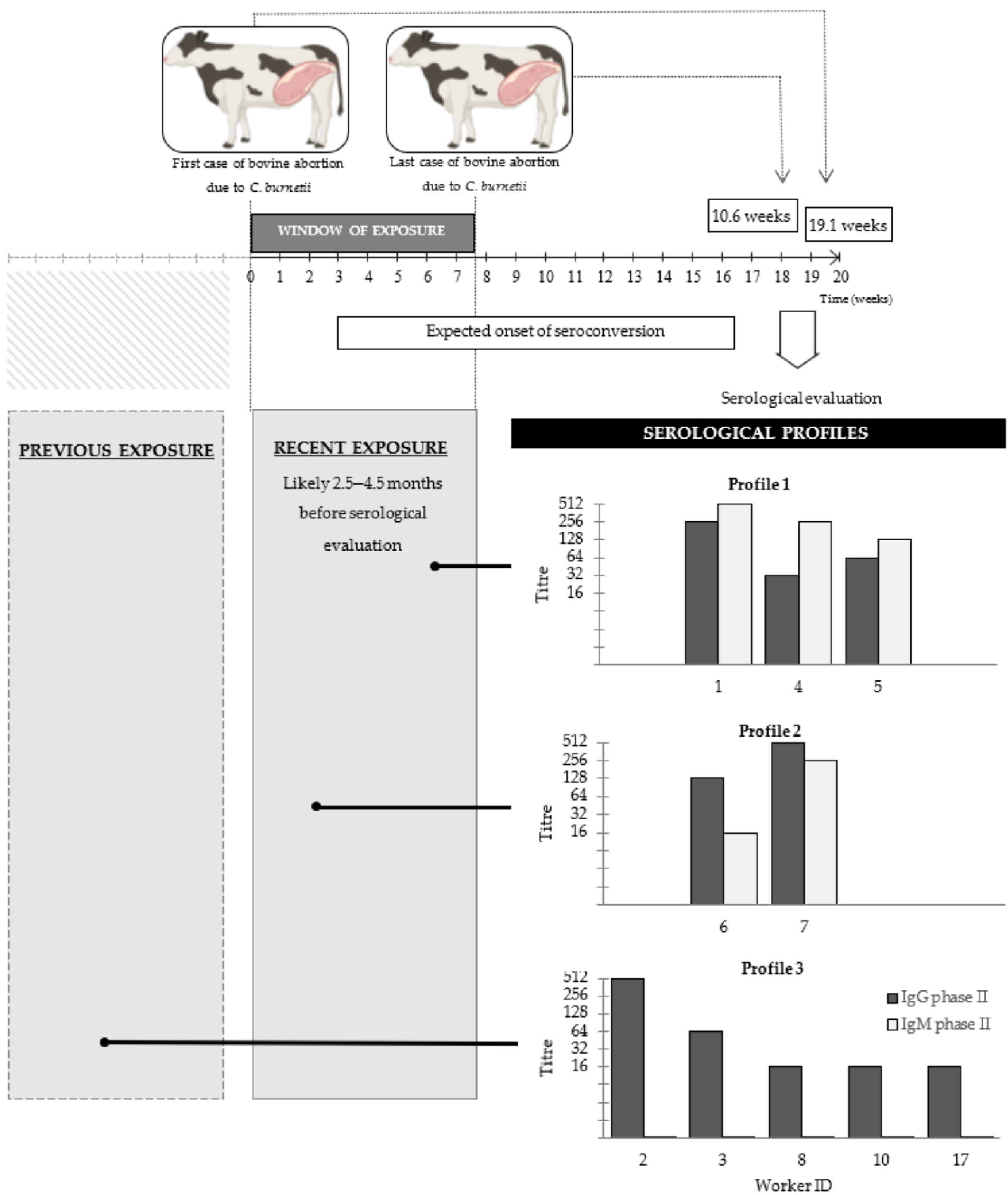

Figure 1. Timeline of the Q fever outbreak in cattle and serological investigations in farm and laboratory workers. Time zero was the date of the first case of bovine abortion. The window of exposure of farm and laboratory workers (when abortions occurred, and aborted materials were collected and submitted to the local veterinary diagnostic laboratory for diagnostic work-up) lasted for 7.7 weeks (10 April to 2 June 2017). The time course of the seroresponse was estimated based on published observations (Todkill *et al.*, 2018). Serological sampling of humans was conducted on 14 and 21 August 2017, i.e., 18.1 and 19.1 weeks following the opening of the exposure window. Serological profiles are based on anti-*C. burnetii* phase II IgG and IgM levels measured by indirect fluorescent antibody test (IFAT). The profile of immunoglobulins was used to ascertain how recently they were likely to have been infected: Profile 1: both isotypes detected, IgM titre > IgG titre—very recent; Profile 2: both isotypes detected, IgM titre < IgG titre; Profile 3: IgG detected but not IgM. Profile 4: neither IgM nor IgG detected (data not shown). Reciprocal titres are shown.

7.8 4. Discussion

The chronology of serological responses and the immunoglobulin classes involved were investigated in a group of workers exposed to bovine abortions caused by *C. burnetii* to ascertain whether these aborted cattle were likely to have been the source of human exposure. Surprisingly, given the importance assigned to Q fever as a zoonotic disease globally, there is an extreme paucity of evidence in accessible peer-reviewed literature associating Q fever with cattle. Most of the publications that have investigated human Q fever outbreaks conducted serological assessments in people, followed by, at most, a description of the epidemiological link between people and cattle (such as visits to the affected herd, regular consumption of raw milk or unpasteurised dairy products, or occupational exposure); a few complemented this with PCR evaluations. Some studies also conducted serological evaluation in animals. However, serological approaches are not particularly informative in cattle as seroconversion can occur without bacterial shedding, and animals can remain seropositive long after overcoming the infection; conversely cattle can shed *C. burnetii* before the development of detectable antibodies and may even shed the agent without ever seroconverting [31]. In contrast, our study provides more convincing evidence that exposure to aborted dairy cattle and their abortion materials is an occupational hazard for the acquisition of Q fever.

In our study, based on the dates of the first and last known bovine abortion cases, the window of exposure to *C. burnetii* was estimated to comprise a timeframe of 54 days (Figure 1). On this basis, at the time of serological investigation, the workers had been exposed for no longer than 134 days (19.1 weeks) after contact with the first bovine case and no less than 74 days (10.6 weeks) after contact with the last bovine case. Thus, the workers were exposed to *C. burnetii* 74–134 days prior to serological examination. The incubation period of Q fever (exposure to disease onset) is pathogen dose-dependent, estimated at between 7 and 32 days (one to five weeks) [32], and seroconversion takes place roughly 14–28 days (two to four weeks) later [21,32,33]. Hence, seroconversion may be expected after 21 days and, almost certainly, no later than 60 days (three to nine weeks) after exposure. On this basis, we estimate that the seropositive workers in our study may have seroconverted between a theoretical minimum of 74 − 60= 14 days and a maximum of 134 − 21= 113 days prior to serological examination. Hence, serological investigation was conducted at least 1.6 weeks, and possibly as much as 16.1 weeks, after seroconversion would be expected based on their exposure to bovine cases.

The profile of immunoglobulins reactive against *C. burnetii* antigens was used to provide insight into the timing of the acquisition of infection based on the known kinetics of antibody development in clinical Q fever [34,35]. In three cases (IDs 1, 4 and 5), IgM titres were

higher than IgG titres suggesting exposure had been recent and coinciding with the latter stages of the known window of exposure to aborting cattle. Two other workers (IDs 6 and 7) were also seropositive for both antibody isotypes but had higher IgG titres than IgM; this may have reflected slightly less recent exposure, perhaps earlier on during the known window of exposure and possibly associated with the first bovine case. The minimum and maximum times between exposure to aborting cattle and the serological evaluation of workers (74–134 days) was entirely consistent with this abortion outbreak being the source of the human infections. The IgG anti-phase II concentration tends to exceed that of IgM anti-phase II on average by about 4.5 days after the onset of the serological response, which equates to 25.5 days after exposure to *C. burnetii* [35]. Given that seroconversion may be expected 21–60 days after exposure, in our study we would expect seroconversion to have preceded serological testing by a minimum of 14 days. While this is a little longer than the estimated average time of 4.5 days from seroconversion to the point at which the IgG titre exceeds that of IgM, for some workers to have IgM titres higher than IgG at the time of testing was entirely consistent with the aborting cattle indeed being the source of exposure.

The IgG phase II antibody has a greater half-life than IgM phase II, with persistence up to 2.5 years, making it an indicator of past infection [35]. In our study, five seropositive workers (IDs 2, 3, 8, 10 and 17) had IgG phase II antibody titres but no detectable IgM phase II, suggesting that exposure might have been long before the known recent outbreak of bovine abortion, and those workers may have had a previous exposure that preceded the documented bovine outbreak.

In addition to the profile of immunoglobulins, the IgG/IgM ratio can be used as a rough estimator of the time after infection and can be used to discern between infection within three months and infection more than six months ago [36]. The IgG/IgM ratio is about 0.1 early after the onset of symptomatology, approximates to 1.0 within the first 100 days and is greater than 10 during the following 100 days. In our study, the IgG/IgM ratio ranged between 0.1 and 0.5 in worker IDs 1, 4 and 5, and between 2 and 8 in worker IDs 6 and 7. This evidence supports recent exposure and is entirely consistent with known exposure to aborting cattle 74–134 days prior to serological analysis.

Two of the workers (IDs 2 and 10) had serological profiles suggestive of long past infection; they reported non-specific symptoms that were likely due to another aetiology as they occurred long after probable exposure to *C. burnetii*. Likewise, symptoms reported by seronegative workers could be due to other seasonal illnesses, and their responses on symptomatology could have been affected by their awareness of the investigation (Hawthorne bias).

The odds of *C. burnetii* seropositivity in laboratory workers, including those also undertaking occasional field activities, were greater than those for field workers for both anti-phase II IgG (OR 5.6 $CI_{95\%}$ 1.09–35.6) and anti-phase II IgM (OR 7.0 $CI_{95\%}$ 0.853–150). Most of the farmworkers did not assist at calving and, hence, were exposed to *C. burnetii* infection indirectly, e.g., through urine and faeces. Considering that shedding of *C. burnetii* by cows through these routes is scarce and intermittent [37], field workers would have faced a repeated but low-level bacterial challenge. In contrast, people engaged in laboratory activities but without direct contact with farm animals might have been exposed to a high bacterial burden through the handling of abortion material infrequently or even on just a single occasion. Despite the suggestion of a protective role of female hormones such as *β*-estradiol [38], infection rates were similar in male and female workers. Nor was an age-related increase in Q fever seropositivity observed in our study, as has been reported elsewhere [39]. For IgG, there were a far greater number of seropositives in the 40 and under age group (9/18) than in older individuals (1/9). Unfortunately, conducting lab work was confounded with age and it was difficult to be certain whether conducting lab work or being of an age 40 and under was the most important determinant of IgG seropositivity. The observation elsewhere that seropositivity tends to increase with age [39] would indeed support lab work as being the more important of the two in this instance.

Other than the previously documented cases of bovine abortion due to coxiellosis [27], none of the bovine or non-bovine abortions routinely analysed by the local veterinary laboratory revealed macroscopic or histologic evidence suggestive of *C. burnetii* infection. Although other sources of *C. burnetii* exposure in laboratory workers beyond the analysed bovine outbreak cannot be altogether excluded, the known exposure to well-documented cases of bovine abortion caused by coxiellosis appears to be a far more likely and plausible source of infection for the human cases described in this study.

This study had a number of limitations that could be considered in future work aiming at furnishing further evidence for *C. burnetii* infection in humans exposed to infected bovines or their abortion products. While the aetiology of the bovine abortions themselves was confirmed using molecular methods (PCR) as well as histopathology and immunohistochemistry [27], the subsequent human infections documented here were confirmed only by serology; confirmation by molecular methods [40] would have strengthened this evidence. The bovine outbreak is the most probable source of infection for laboratory workers and veterinarians, but other sources cannot be fully excluded. Furthermore, the extent to which the symptomatology described by the patients was related to Q fever is unclear. While the symptoms described and their chronology were consistent with acute infection

with *C. burnetii* [41], we were unable to demonstrate a statistical association between symptoms and serological responses in the Phase II IFA for either IgG or IgM ($p > 0.95$). This might have been possible with a larger number of cases, but this was a study of a naturally occurring disease event and the sample size was not within our control. In this study, we used a titre of 1/16 or greater in the Phase II IFA as the seropositivity threshold for both IgG and IgM, as this was considered above the reference level by the testing laboratory (Mayo Clinic Laboratories), and, indeed, some authorities have used even lower IFA titres in epidemiological studies [42]. We, nevertheless, performed a sensitivity analysis and re-analysed the data using a more conservative seropositivity threshold of 1/32, with little change in the overall implications of the results. Using this higher cut-off value, although there were fewer Phase II IgG positives overall (seven rather than ten), the association

with lab work was even stronger, having an even higher odds ratio (14.0, $CI_{95\%}$ 1.85–297) and a lower p-value ($p < 0.01$); for IgM there were four rather than five positives overall and the revised odds ratio (4.67, $CI_{95\%}$ 0.507–103) remained suggestive but non-significant (p = 0.179). Lastly, the persistence of phase II IgM must be considered in the interpretation of results when investigating acute Q fever, particularly in endemic, post-epidemic and late epidemic contexts [29]. This is not likely to be a significant limitation to the present study as, although there has been no centralised system of recording and few data are available, to the best of the authors' knowledge, Q fever cases have been reported only sporadically since the first local outbreak was documented in 1956 [43].

7.9 5. Conclusions

In conclusion this epidemiological investigation, the first closely linking Q fever to bovine abortion, provides novel serological evidence of *C. burnetii* exposure in people working in direct contact with either aborted cattle or their foetuses, placentas and vaginal discharges. Cattle aborting due to *C. burnetii* should not be underestimated as a potential hazard and possible source of human infection. Q fever should be considered in the spectrum of diseases in patients with an epidemiological link with animals, or with occupational-related exposure, especially those with fever of unknown origin. Vaccination should be considered for people at risk of Q fever through occupational exposure.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the ethical committee from the University of Bristol (Ref.95382/Id.342095).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data available upon request due to restrictions e.g., privacy or ethical.

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Conflicts of Interest: The authors declare no conflict of interest.

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Appendix B

Formulario de consentimiento informado
Reporte de casos de Fiebre Q en INIA La Estanzuela 2017

Por medio de la presente, quien suscribe funcionario/a____ declaro que la Dra. ________________ y la investigadora __________________ me ha informado sobre su intención de divulgar mi caso con fines puramente académicos, haciendo uso de los datos que verídicamente le he referido y exámenes de laboratorio pertinentes. Me ha informado también que mi identidad no será revelada y velará por que mi pudor se mantenga intacto. Me ha informado que los datos recabados serán manejados con absoluta confidencialidad y sólo con fines científicos. Mi participación es absolutamente voluntaria. Mi participación no implica beneficios económicos ni para mí ni para los investigadores responsables. Recibí una copia de este formulario de consentimiento.

Por lo anterior convengo en participar y permitir divulgar mi caso en la investigación sobre Fiebre Q en INIA La Estanzuela en 2017.

Fecha:	Fecha:
Firma participante:	Firma investigador:
Aclaración:	Aclaración:

www.inia.uy

English translation of the informed consent.

Informed consent form
Report of cases of Q Fever in INIA La Estanzuela 2017

Hereby, the undersigned ____ declares that Dr. _______________ and the researcher _________________ have informed me of their intention to disclose my case details for purely academic purposes, making use of the data that I have truthfully provided and relevant laboratory tests. She has also informed me that my identity will not be revealed and that she will ensure that discretion will be maintained. She has informed me that the data collected will be handled with absolute confidentiality and for scientific purposes only. My participation is absolutely voluntary. My participation does not imply financial benefits either for me or for the researchers responsible. I have received a copy of this consent form.

Therefore, I agree to participate and allow my case details to be included in the Q Fever investigation at INIA La Estanzuela in 2017.

Date:	Date:
Participant's signature:	Investigator's signature: