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Coxiella burnetii in Central Portugal as an emerging disease in a One Health perspective

Tese de Doutoramento em Ciências Veterinárias

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Declaration

I Rita Marisa da Silva Cruz, confirm that the work present in this thesis is my own. Where the information has been derived from other sources, I confirm that this has been indicated in this thesis.

Signed:

To my Family, Friends and co-Workers

To Professor Doutor João Mesquita

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ABSTRACT

Q fever is a zoonotic disease with a huge impact on public and animal health. It has a practically global distribution and is caused by *Coxiella burnetii*, which is capable of infecting several animal species, among them ruminants, which are the major reservoir of the agent and consequently the largest source of infection for humans. With unique morphological characteristics, there are several studies carried out worldwide, either related to the agent itself or related to the impact of this zoonosis in public and animal health. Despite the fact that it has been considered, in Portugal, a notifiable disease since 1999, very few cases have been reported, and consequently there is little knowledge about the circulation of this agent. At the same time, there are few studies that infer the prevalence of small and large ruminants in continental Portugal, as well as the impact on productive and public health level.

The main objective of this dissertation is to evaluate the circulation of *Coxiella burnetii* in portuguese sheep, as well to study in detail the effective presence and circulation of this agent in Serra da Estrela sheep (a autoctonous portuguese sheep breed) in the central region of Portugal.

This work is divided in 6 chapters. Chapter 1 presents a general review on *Coxiella burnetii* biology, morphological features, epidemiology, animal and humal pathogenesis as well as its clinical manifestations, diagnostic methods, the situation of Q fever in Portugal either in humans and in animals, and the characterization of Serra da Estrela region. Chapter 2 indicates the major objectives of this thesis.

Chapter 3 focusis on the situation of Q fever in small ruminants in continental Portugal through the realization of an epidemiological survey to estimate the seroprevalence of Q fever antibodies in sheep of all portuguese regions. For this, IgG anti-*C. burnetii* IgG was evaluated. We found that anti-*C. burnetii* antibodies were present in 122 sheep, which represents a 11.4% IgG anti-*C. burnetii* seroprevalence in portuguese sheep. We also found that this prevalence was higher in the Centre region when compared to the other portuguese regions.

Chapter 4 aimed at estimating the presence of IgG anti-*C. burnetii* in a population of sheep in the central region of Portugal. A prospective serosurvey was setup based on blood collection from a representative sample of 168 animals, collected both in 2015 and 2016, and

sera were tested for IgG anti-*C. burnetii*. Of the 2015 sample collection, 7.7% (13/168) animals tested positive for IgG anti-*C. burnetii* while of the 2016 collection 17.3% (29/168) tested positive, showing a statistically significant ($p = 0.008$) increase. This supports the notion that Q fever might be an emerging disease in central Portugal.

Chapter 5 describes two Q fever outbreaks affecting sheep and goat flocks, as well as a serological survey in bulk tank milk samples assessing *C. burnetii* circulation in a population of Serra da Estrela in two consecutive years. In this study it was found that 10.2% of the 78 bulk tank milk samples collected in 2015 presented IgG antibodies against *C. burnetii*. The same farms, visited and sampled in 2016 showed that 25.6% (95%CI) of them were positive. This steep increase in the number of anti-*C. burnetii* farms between the 2015 and 2016 collections showed to be statistically significant ($p = 0.020$) and is strongly suggestive of Q fever emergence in Central Portugal.

Chapter 6, indicates the main conclusions achieved through the realization of this work, as well as the future work to be done. The study carried out throughout this dissertation was the first to be performed establishing the seroprevalence based on a seroepidemiological study in small ruminants at national level, also establishing distribution of seropositive animals in the different regions of Continental Portugal. The present thesis has shown that despite an apparent low seroprevalence there is an increase in the prevalence of Q fever, which suggests that the agent not only seems to be distributed and circulating throughout the country but seems to be also emerging.

KEYWORDS: *Coxiella burnetii*; Zoonosis; Public health; Serra da Estrela; Prevalence; Seroepidemiology; Epidemics.

RESUMO

A febre Q é uma doença zoonótica com enorme impacto ao nível da saúde pública e animal. Esta tem uma distribuição praticamente global e é causada pela *Coxiella burnetii*, que é capaz de infectar várias espécies de animais de entre os quais se destacam os ruminantes, sendo estes o maior reservatório do agente e consequentemente a maior fonte de infeção para o homem. Com características morfológicas únicas, são inúmeros os trabalhos realizados a nível mundial, quer sobre o agente em si, quer sobre o impacto desta zoonose em termos de saúde publica e animal. Os trabalhos realizados a nível mundial levaram a um maior conhecimento deste agente das suas características filogenéticas, assim como da sua metodologia de atuação.

Apesar de ser considerada, ao nível da saúde pública, uma doença de declaração obrigatória desde 1999, são poucos os casos notificados, sendo, consequentemente, escasso o conhecimento sobre a endemicidade desta infeção. Simultaneamente são poucos os estudos que inferem a prevalência. Desta infeção ao nível dos pequenos e grandes ruminantes em Portugal continental, assim como o seu impacto a nível de produtivo e da saúde pública.

O principal objetivo desta dissertação é avaliar a presença de febre Q nos pequenos ruminantes em Portugal Continental, mais especificamente no centro de Portugal, bem como a incidência desta infecção nesta região.

Este trabalho está dividido em 6 capítulos. O Capítulo 1 apresenta uma revisão geral da biologia da *Coxiella burnetii* assim como, das características morfológicas, da sua epidemiologia, da patogenia em animais e nos humanos assim como as suas manifestações clínicas, os métodos de diagnóstico, a situação atual da febre Q em Portugal, tanto em humanos como em animais, e, por ultimo a caracterização da Serra da Região da Estrela, região onde incidiu, por fatores diversos parte deste trabalho. O capítulo 2 refere os principais objetivos desta tese.

O Capítulo 3 centrou-se na situação atual da Febre Q em pequenos ruminantes em Portugal continental, através da realização de um estudo sero-epidemiológico para avaliar a prevalência de Febre Q em pequenos ruminantes de Portugal Continental. Para isso, foi avaliada a presença de IgG anti- *C. burnetii* em amostras de sangue de pequenos ruminantes de todas as regiões de Portugal Continental. Foi identificada uma prevalência de 11.4%, o que significa que 122 individuos da amostra total foram seropositivos para Ig G anti-*C. burnetii*. Com este estudo

constatámos que esta prevalência foi maior na região Centro, quando comparada com as demais regiões portuguesas.

O Capítulo 4, teve objetivo determinar a presença de IgG anti-*C. burnetii* numa população de ovelhas na região centro de Portugal. Desta forma, foi realizado um estudo serológico prospectivo numa amostra serológica de 168 animais em 2015 e 2016. Os soros foram testados para IgG anti-*C. burnetii*. Os resultados revelaram que das amostras colhidas em 2015, 7,7% (13/168) animais foram positivos para IgG anti-*C. burnetii*, enquanto das amostras colhidas em 2016, 17,3% (29/168) apresentaram resultado positivo, revelando, desta forma um reultado estatisticamente significativo ($p = 0,008$). Desta forma, conclui-se que ocorreu um aumento na seroprevalência de *C. burnetii*, apoiando o facto de que a febre Q pode ser uma doença emergente no centro de Portugal.

O Capítulo 5, descreve os dois surtos de febre Q que afetaram 2 efetivos de ovelhas e cabras, bem como a sua caracterização epidemiológica e estudo epidemiológico efetuado em amostras de leite do tanque de forma a avaliar a presença e circulação de *C. burnetii* numa população de ovelhas da Serra da Estrela, em duas épocas de produção leiteiras consecutivas, relativas aos anos de 2015 e 2016. Dos resultados obtidos verificou-se que 10.2% das 78 amostras de tanque recolhidas em 2015 apresentaram resultados positivos para IgG anti-*C. burnetii* e que as mesmas explorações, quando testadas em 2016, foram positivas em 26.5 % dos casos. Este incremento na prevalência revela um aumento na seropositividade de explorações para a *C. burnetii*. Os resultados obtidos mostraram-se estatisticamente significativas ($p = 0,020$) e são fortemente sugestivos de a febre Q ser considerada uma doença emergente no centro de Portugal.

O Capítulo 6 indica as principais conclusões alcançadas através da realização deste trabalho, bem como o trabalho futuro a ser feito. O estudo realizado ao longo desta dissertação foi o primeiro a ser realizado estabelecendo a seroprevalência a partir de um estudo soroepidemiológico em pequenos ruminantes a nível nacional, estabelecendo também a distribuição de animais seropositivos nas diferentes regiões de Portugal Continental. A presente tese mostrou que, apesar de uma baixa seroprevalência, há um aumento na prevalência de febre Q, o que sugere que o agente parece estar distribuído e a circulando em todo o país, sendo isso é necessário alertar para a possibilidade de transmissão zoonótica, principalmente na região centro de Portugal, uma vez que a relação de proximidade entre os agricultores e ovelhas é considerável. A implementação de programas de monitorização e um plano de vigilância

epidemiológica em efetivos sentinelas podem ajudar a prevenir ou mitigar os efeitos de potenciais epidemias.

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PALAVRAS-CHAVE: *Coxiella burnetii;* Zoonose; Saúde Pública; Serra da Estrela; Prevalência; Seroepidemiologia; Epidemia.

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LIST OF ACRONYMS AND ABBREVIATIONS

ACCM	Acidified Citrate Cysteine Medium
AHAW	Animal Health and Welfare
ANCOSE	National Association of Serra da Estrela Sheep breed
BHK-21	Hamster Kidney Fibroblast
BSL3	Biosafety Level 3
BTM	Bulk Tank Milk
CDC	Centers for Disease Control and Prevention
CEVDI/INSA	Centre for Vectors Infectious Diseases of Ricardo Jorge National Health Institute
CFSPH	Center for Food Security and Public Health
CFT	complement fixation test
CHO	Chinese hamster ovary fibroblast
CI	Confidence Interval
DGS	General Directorate of Health
DNA	Deoxyribonucleic acid
DOP	Denominated Origin Protected
ECDC	European Centre for Disease Prevention and Control
EFSA	European Food Safety Authority
ELISA	Enzyme-Linked Immunosorbent Assay
EU	European Union
FISH	Fluorescent in-situ Hybridisation
HEL	Human Embryonic Lung Fibroblast
HeLa	Human Cervical Epithelial
IAP	Integrin-Associated Protein
IFA	Indirect Immunofluorescence Assay
IFAP	Institute for Financing Agriculture and Fisheries
IFN-ϒ	Gamma Interferon
IgG	Immunoglobulin G
IHC	Immunohistochemistry

L-929	Murine Fibroblast
LAI	Laboratory Acquired Infection
LAMP	Loop Mediated Isothermal Amplification
LCV	Large Cell Variant
LPS	Lipopolysaccharides
MLVA	Multilocus variable-number tandem repeat analysis
MST	Multispacer Sequence Typing
NC	Negative Control
NIAID	National Institute of Allergy and Infectious Diseases
NUTS	Nomenclature des unités territoriales statistiques
OD	Optical Density
OIE	OIE-World Organisation for Animal Health
PC	Positive Control
PCR	Polymerase Chain Reaction
PFGE	Pulsed-Field Gel Electrophoresis
qPCR	Real-Time Polymerase Chain Reaction
R	Rough
RFLP	Restriction Fragment Length Polymorphism
rRNA	Ribosomal Ribonucleic Acid
S	Smooth
S/P	Sample to Positive Control ratio
SCV	Small Cell Variant
SE	Serra da Estrela
TLR4	Toll-like receptor 4
TNF	Tumour Necrosis Factor
VERO	African Green Monkey Kidney Epithelial

CHAPTER 1 - GENERAL INTRODUCTION

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1.1 Q fever history

1.1.1. Discovery of *Coxiella burnetii*

Q fever was first described in 1935 by Edward Holbrook Derrick, the director of the Laboratory of Microbiology and Pathology of the Queensland Health Department, in Brisbane, Australia after the regular occurrence of sporadic cases since 1933 (McDade, 1990; Maurin & Raoult, 1999). He was assigned to investigate an outbreak of an unknown disease that occurred among the local abattoir workers, with fever as the predominant symptom (Derrick, 1937; Chmielewski & Tylewska-Wierzbanowska, 2012).

Derrick was first decided to determine the characteristics of the illness and carefully monitored the clinical features of the disease. The symptoms described included fever, lasting from seven to twenty-four days, headaches, depression, anorexia and pain in the limbs. The patient blood cultures performed, were negative and there was no detection of any antibodies for the known agents, at the time, in their sera. Because of this, it was concluded that the outbreak was the result of a new disease of unknown aetiology and called it Q fever, Q for query, English term that means doubt (McDade, 1990).

Several experiments and studies were performed, in order to determine the cause of the disease. Derrick inoculated guinea pigs with blood and urine from infected patients and observed that the animals developed fever. He also verified transmission of the disease, when the guinea pigs were inoculated with tissue suspensions prepared from infected animals. However, he failed to detect bacteria in infected tissues. As a consequence of this result, Derrick obtained the wrong conclusion that the etiologic agent of this disease was a virus (Burnet & Freeman, 1937; Chmielewski & Tylewska-Wierzbanowska, 2012).

In an attempt to clarify the aetiology of the disease, Derrick sent the livers of infected guinea pigs to Frank Macfarlane Burnet, a virologist who worked at the Walter & Eliza Hall Institute in Melbourne. After further studies, where infected tissues were inoculated in other animal species, in 1937, Burnet and his assistant Mavis Freeman were finally able to isolate organisms, from guinea pigs, of bacterial origin with similar characteristics to the members of the genus Rickettsia (Burnet & Freeman, 1937; Chmielewski & Tylewska-Wierzbanowska, 2012). The Q fever agent was then designated as *Rickettsia burnetii* (McDade, 1990).

In 1935, independently of Derrick's work, Gordon Davis at the Rocky Mountain Laboratory in Montana, United States of America was investigating the ecology of Rocky Mountain spotted fever, when isolated an infectious agent from Dermacentor andersoni ticks,

collected at the Nine Mile Creek (Maurin & Raoult, 1999). He verified that guinea pigs from where ticks were feed, developed an indeterminate fever syndrome and therefore suspected that had isolated *Rickettsia rickettsi*, the etiological agent of Rocky Mountain fever. However, the following *in vivo* inoculations shown the absence of lesions usually associated with spotted fever diseases (McDade, 1990). One year later, Herald Rea Cox joined Davis to continue the studies for identify and characterize the Nine Mile agent (Maurin & Raoult, 1999). They observed properties not identified before: the agent could pass filters, it was Gram-negative, and had an extracellular and intracellular pleomorphic rickettsia-like appearance (Davis *et al*., 1938; McDade, 1990). The name proposed for this new etiological agent was *Rickettsia diasporica* (Cox, 1941; McDade, 1990). A major advance was obtained when Cox was succeeded in cultivated this new agent in chicken embryos (Maurin & Raoult, 1999).

Only few years late, the importance of this discovery and its pathogenicity for humans become clear. In May of 1938, Rolla Dyer, director of National Institute of Health, visited Cox at the Rocky Mountain Laboratory and about ten days later became ill. The symptoms described were retro-orbital pain, fever, chills and cold sweats. At the sixth day of symptoms, a blood sample was collected, inoculated into guinea pigs and the animals developed fever. Subsequent studies have shown that this agent was the same that had been isolated by Davis from *Dermacentor andersoni* ticks. Dyer did know the Australian reports and suggested the link between this new agent and the "Q fever" agent (McDade, 1990).

The etiological agent of Q fever disease was discovered almost simultaneously in Melbourne, Australia, by Burnet and Freeman and in Montana, United States of America, by Cox and Davis (Maurin & Raoult, 1999).

In 1948, Cornelius Philip proposed the reclassification of the etiological agent of Q fever into *Coxiella burnetii*, honoring simultaneous Burnet and Cox, to their contributions to a better understanding of the agent (Philip, 1948; McDade, 1990).

Coxiella burnetii was originally classified as rickettsia-like, in the genus *Coxiell*a, family Rickettsiaceae, order Rickettsiales (Bergey *et al*.,1984). However, phylogenetic studies, based on 16S rRNA gene sequence and genome analysis, shown a high homology with *Legionella pneumophila* and was reclassified into a separate genus *Coxiella* of the gamma subdivision of Proteobacteria (Stein et al., 1993; Kazar, 2005) (Figure 1.1.).

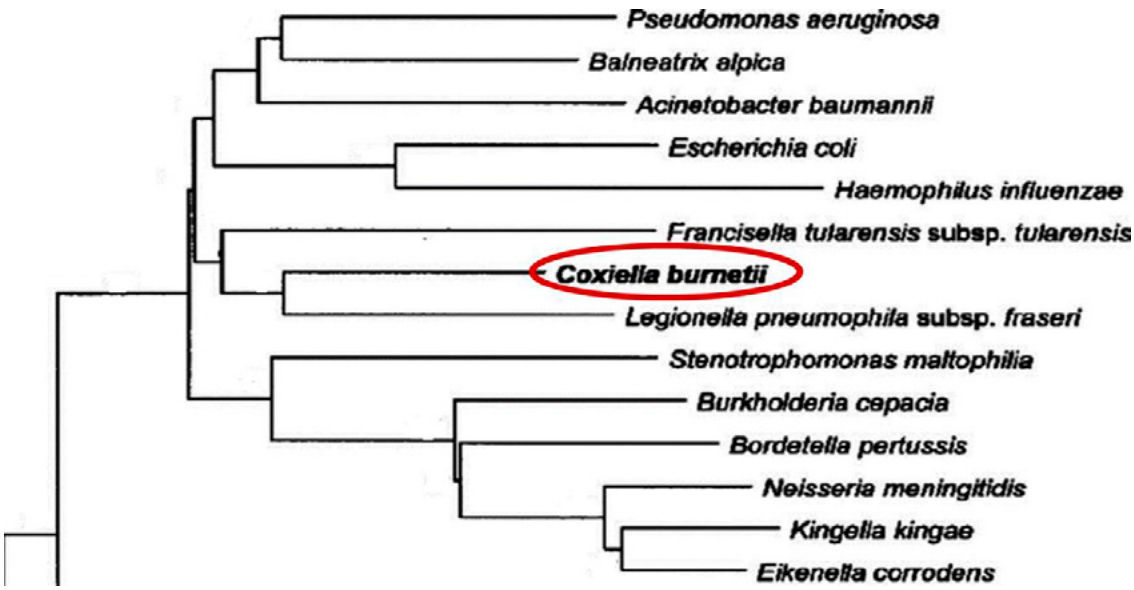

Figure 1.1 Phylogenetic relations within the phylum Proteobacteria, based on 16s rRNA sequence comparison of *C. burnetii* with its closest member of the γ-proteobacteria phylum (adapted from Drancourt & Raoult, 2005).

1.2 *Coxiella burnetti* bacteriology

1.2.1. Phenotypic features

Coxiella burnetii is a small, nonmotile, obligate intracellular, Gram-negative bacterium that replicates within the phagolysosome of host cells (Howe & Mallavia, 2000; Seshadri *et al.*, 2003) (Figure 1.2.).

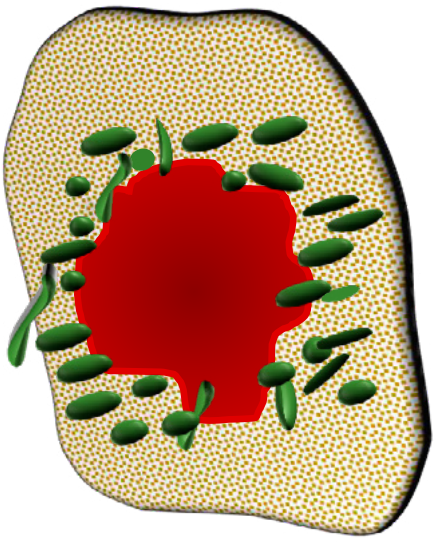

Figure 1.2. Schematic representation of pleomorphic cocobacilli shape *C. burnetii* inside a host cell.

It has pleomorphic coccobacilli shape (0.2–0.4 mm wide, 0.4–1.0 mm long) and produces two morphologically distinct cell types, that comprises a bi-phasic developmental cycle (Hechemy, 2012) a small cell variant (SCV) and a large cell variant (LCV), also called dormant SCV and active LCV (Howe & Mallavia, 2000). The SCV has a characteristic condensed chromatin, has a thicker peptidoglycan layer and, as the name implies, is smaller in size (Howe & Mallavia, 2000). SCV has highly resistance to environmental stress (dissecation, heat, UV radiation and osmotic pressure), allowing *C. burnetii* to survive in the extracellular environment, maintaining its infectivity: LCV is the metabolically active and replicative entity, formed after the invasion of the host by the SCV and is more sensitive to environmental stresses than the SCV. During the stationary phase of the organism's growth cycle, LCV undergoes sporogenic differentiation to produce resistant, spore-like forms, the small-cell variants (SCV) (Angelakis & Raoult, 2010).

These are released when the cells lyse and can survive for long periods in the environment (Howe & Mallavia, 2000; Angelakis & Raoult, 2010; Hechemy, 2012), maintaining the developmental cycle of *C. burnetti* (Howe & Mallavia, 2000; Hechemy, 2012).

Coxiella burnetii has an extracellular matrix, similar to that of other Gram-negative bacteria (Skultety *et al*., 2011) and like several other Gram-negative species. *C. burnetii* can display two different lipopolysaccharide (LPS) phenotypes (Roest *et al*., 2013), a virulent phase I and an avirulent phase II. *C. burnetii* transition from virulent-to-avirulent involves LPS truncation known as phase variation (Beare *et al*., 2018).

The virulent phase I form of *C. burnetii* has been isolated from both, lower and higher animals, and is extremely infectious, being capable to replicate in immunocompetent hosts (Roest *et al*., 2013). The extracellular matrix fraction of phase I cells comprises a smooth (S) lipopolysaccharide (LPS), I with an O-specific chain. Phase II is not infectious, occurs only in laboratory strains after many culture passages in either embryonated hen eggs or cell cultures and can be easily distinguished serologically from native isolates (Skultety *et al*., 2011). Contrasting with phase I cells, the extracellular matrix of phase II cells has an incomplete LPS II, structure that appears rough (R), and lacks the O-antigenic region (Skultety *et al*., 2011; Roest *et al*., 2013) (Figure 1.3.).

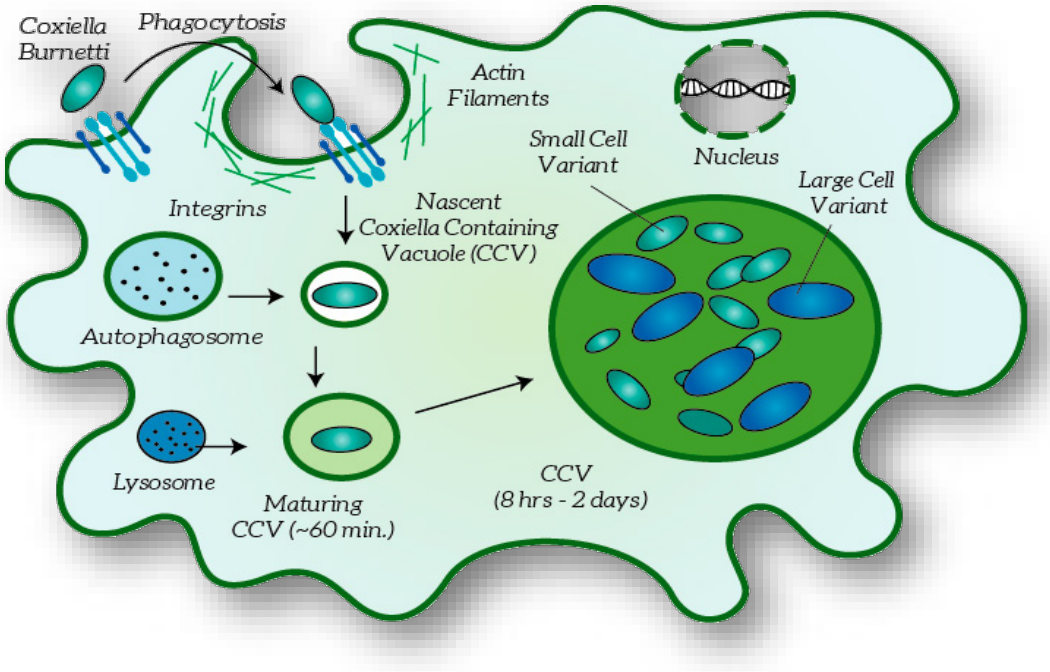

Figure 1.3. *C. burnetii* produces two morphologically distinct cell types, that comprises a bi-phasic developmental cycle, a small cell variant (SCV) and a large cell variant (LCV), also called dormant SCV and active LCV. After entering a host cell (alveolar macrophage or a monocyte) due to phagocytosis, SCV transforms into a metabolically active LCV within an acidic (pH ~4.5) lysosome-derived *Coxiella*-containing vacuole (CCV). During the stationary phase of the organism's growth cycle, LCV undergoes sporogenic differentiation to produce resistant, spore-like forms, the small-cell variants (SCV). SCV remais in the environmentt after cell lysis (adapted from http://2015.igem.org/Team:TU_Eindhoven).

Both LPS phenotypes can be distinguished via phase-specific antibodies. Phase I antibodies are directed against the full-length LPS of phase I, whereas phase II antibodies are directed against common surface proteins, that although also being present on the surface of phase I *C. burnetii*, seem to be protected by the long phase I LPS (Roest *et al.*, 2013Beare *et al.*, 2018).

Coxiella burnetii phase variation should not to be confused with phase variation of other Gram-negative bacteria, which is generally a reversible on-off regulatory process. This phase variation is irreversible and seems to be associated with the loss of chromosomal DNA, involving a large group of LPS biosynthetic genes, arranged in an apparent O-antigen cluster (Hoover *et al.*, 2002) and several other genes mutations as well as deletions. One of them is related to the sugars comprising the inner/outer core regions in the O-chain of galactosaminuronyl-a-glucosamide disaccharide and the repeating O-antigen subunits of LPS (Beare *et al.*, 2018). Two unusual O-antigen sugars unique to *C. burnetii* LPS are virenose (6-

deoxy-3-C-methylgulose) (*vir*) and dihydrohydroxystreptose (3-C-(hydroxymethyl)-L-lyxose) (*strep*). Genes have been identified that might be involved in the synthesis of *vir* and *strep* (Beare *et al*., 2018; Mori *et al*., 2018). Unlike other Gram-negative bacteria, where O-antigen generally has a defined repeat size (Aucken & Pitt, 1993), *C. burnetii* O-antigen has populations that differ in size and composition (Vadovic *et al*., 2005); consequently, its carbohydrate structure remains unresolved (Vadovic *et al*., 2005; Beare *et al*., 2018; Mori *et al*., 2018). According to Hotta and colleagues (2002), *C. burnetii* LPS has, at least, four antigenic forms, during phase variation. *C. burnetii* phase I LPS also plays a role in both host cell internalization and survival in the phagosome. Phase I *C. burnetii* bacteria are internalized and survive intracellular killing, whereas phase II bacteria are efficiently phagocytized and then killed (Raoult *et al*.,2005).

At this time the phylogeny of *C. burnetti* agent can be summarized as belonging to the kingdom of Bacteria, phylum of Proteobacteria, class of the Gammaproteobacteria, order of the Legionellales, family of the Coxiellaceae, with the genus *Coxiella* and the only one species (; Roest *et al*., 2013; Garrity *et al*.,2015).

1.2.2. *C. burnetii* genome

In 2003, Seshadri and colleagues, published, for the first time, the complete genome sequence of *C. burnetti* (Seshadri *et al*., 2003). They analyzed the original strain isolated from ticks, by Davies and Cox, in 1938 (called Nine Mile), and revealed a circular genome of 1995 275 base pairs (Seshadri *et al*., 2003; Roest *et al*., 2013). Analysis of the *C. burnetii* genome also revealed the presence of many mobile elements, pseudogenes, and hypothetical proteins, suggesting ongoing genome reduction (Seshadri *et al*., 2003). During reduction, genes accumulate mutations, lose functions, and eventually disappear. It seems probable that these processes are also associated with phase variation at which time a large group of genes is lost. The genome has a G+C content of 43 mol% and 2134 coding sequences are predicted, of which 719 (33.7%) are hypothetical, with no significant similarity to other genes in the database (Seshadri *et al*., 2003; Angelakis & Raoult, 2010).

1.3 Biosafety and biosecurity

Coxiella burnetii is listed by the United States Centers for Disease Control and Prevention (CDC), as a potential bioterrorism agent with the potential to be developed for use in biological warfare, and as a Category B pathogen, by the National Institute of Allergy and Infectious Diseases (NIAID) (Oyston & Davies, 2011; Hanczaruk *et al*., 2012; Heppell *et al*., 2017). *C. burnetti* is an environmentally-persistent ubiquitous organism, considered a containment level 3 organism by the EU, that means an agent that can cause severe human disease and present a serious hazard to workers; an agent that may present a risk of spreading to the community, but there is usually effective prophylaxis or treatment available (directive 2000/54/EC of the European parliament and of the council). Jones *et al*. (2006), working with guinea pigs and humans, concluded that the infectious dose of *C. burnetii* is likely one rickettsia and that the probability of a single organism initiating infection is approximately 0.9. Additionally, *C. burnetii* is able to survive in the environment, even under adverse conditions (Maurin *et al*.,1992; Reimer, 1993; Fournier *et al*.,1998; Palmela *et al*., 2012). To work with containment level 3 organisms, it is mandatory to work in dedicated biosafety level 3 (BSL3) laboratories and animal facilities, and with experienced and trained personnel. All the institutional and personal security measures and emergency procedures designed to prevent the loss, theft, misuse, diversion, or intentional release of pathogens and toxins must be followed (Pastorino *et al*.,2017). Medical monitoring of personnel working with agents such as *C. burnetii* should be implemented. An efficient marker to evaluate the effectiveness of biosafety and to optimize the risk assessment in BSL3 laboratories, is the surveillance of laboratory acquired infection (LAI) (Wurtz *et al*., 2016). *C. burnetti* was one of the agents associated with LAI, in a 1976 study (Pike, 1976) and in another study in 2006, performed by Byers & Harding. However, current practices have minimized worker's pathogen exposition as well as the advance of safety rules and the improvements in containment equipment, engineering controls, and safety training, therefore we have been assisting to a great reduction in LAI (Kozajda *et al*., 2013). Good laboratory practices, regarding biosafety and bioethics, are essential measures to avoid LAIs (Coelho & García Diez, 2015).

1.4 Isolation and cultivation of *C. burnetii*

Coxiella burnetii can only be isolated using embryonated eggs, animal hosts, or mammalian cell culture (Boden *et al.,* 2015). The cell culture system is currently the most widely used in vitro system to isolate and cultivate *C. burnetii*. A number of cell lines can be used for in *vitro* cultures: Vero (African green monkey kidney epithelial) (Heinzen *et al.*,1996), BHK-21 (hamster kidney fibroblast) (Miller *et al.*,2004), L-929 (murine fibroblast) (Baca & Paretsky, 1983), HEL (human embryonic lung fibroblast) (Raoult *et al.*,1990), HeLa (human cervical epithelial) (Berón *et al.*,2002; Mori *et al.*, 2013) and CHO (Chinese hamster ovary fibroblast) (Romano *et al.*,2007) cells.

Guinea pigs were the first laboratory animals to be used in Q fever research. They develop hyperthermia of over 40°C, at five to twelve days post inoculation, and *C. burnetii* can be isolated from the spleen, indicating systemic infection (Vohl & Heinzen, 2007). There are also several mouse strains, with different susceptibilities, that can be used in these studies. In an evaluation of 47 strains of inbred laboratory mice, 33 were found to be resistant to infection with *C. burnetii*, 10 were partially susceptible and 4 were susceptible (Bewley, 2013) The A/J strain of mouse was found to have 100% of morbidity and the highest mortality (70%) but could still be protected by vaccination with formalin-inactivated *C. burnetii* whole cells (Bewley, 2013; Scott *et al.*,1987). The BALB/c strain of mouse was reliably infected and displayed overt signs of illness, including ruffled fur and lethargy in all members of the group, but with no mortality (Bewley, 2013).

In natural infections, *C. burnetti* has a tropism for cells of the mononuclear phagocyte system, such as alveolar macrophages of the lung and Kupffer cells of the liver (Khavkin & Tabibzadeh, 1988; Stein *et al.*, 2005), with organisms also infrequently observed in pneumocytes, fibroblasts and endothelial cells (Khavkin & Tabibzadeh, 1988). Consequently, monocyte/macrophage-like cell lines including J774A.1 (murine macrophage-like) (Howe & Mallavia, 2000; Brennan *et al.*,2004), P388D1 (murine macrophage-like) (Tujulin *et al.*, 1999) and THP-1 cells (human monocyte-like) (Ghigo *et al.*, 2002) have been extensively employed to more accurately mimic the *in vivo* situation, with more recent studies focused on interactions with primary human monocytes/macrophages (Ghigo *et al.*, 2004; Shannon & Heinzen, 2008) and dendritic cells (Shannon *et al.*, 2005).

Embryonated eggs were also used to grow *C. burnetii*. This technique demands time and experience. It involves the inoculation of a tissue suspension containing *C. burnetii*, into 6

to 7 day-old embryonated chicken eggs via the yolk sac, that is harvested after 10 to 15 days (OIE, 2012). On the other hand, culture results are hard to monitor and the results are comparable to cell culture isolation techniques.

A milestone in *C. burnetii* research was reached in 2009 by the development of an axenic culture system for this formerly obligate intracellular bacterium (Boden *et al.*, 2015). This system, acidified citrate cysteine medium (ACCM), was created based on information from acid activation studies, together with analyses using transcription microarray techniques, metabolic pathway reconstruction and metabolite typing (Omsland *et al.*, 2009). The development of this type of host cell-free medium allows the understanding of *C. burnetii* physiology and capacitates the development of advanced genetic tools for biomolecular manipulation (Sandoz *et al.*,2014).

1.5 Quality control in *C. burnetii* cultivation

When working with cell cultures, one of the biggest concerns is to guarantee that there is no contamination and/or cross-contamination. A visual check, on isolate purity and contamination, is hampered by the intracellular nature of *C. burnetii*. Bacterial contamination of cell cultures is easily visible by the change in clarity of the culture medium, especially when culture medium without antibiotics is used. To control these types of contaminations, it is mandatory to implement regular checks for contaminants and monitor cultures by light microscopy at 100× magnification. To detect possible cross-contamination between cultures with different strains, there is the need to regularly identify the strains by genotyping techniques (Vincent *et al.*, 2015).

1.6 Epidemiology

1.6.1 Hosts

Q fever is an ubiquitous zoonosis whose reservoirs include a wide variety of animal species, including arthropods, mainly ticks, wildlife, namely rodents, birds, marine mammals and wild ruminants, as well as domestic animals, like dogs and cats. However, many studies emphasize the importance of wild and domestic ruminants, being the last considered the primary animal reservoir (Million & Raoult, 2013; Meredith *et al.*,2015; Van den Brom *et al.*,2015; Fernández-Aguilar *et al.*,2016).

Domestic ungulates (dairy cattle dairy sheep and goats) are considered the major reservoirs of *C. burnetti* (Kim *et al*.,2005) within these aborting domestic ruminants are the main sources of human infection (Bartelink *et al*., 2000; Woldehiwe, 2004) and environment contamination (Bartelink *et al*., 2000; Woldehiwet, 2004; Million & Raoult, 2013; Van den Brom *et al*., 2015).

1.6.2 Prevalence

Q fever has been described as a disease with worldwide distribution, except in some countries such as New Zealand (Angelakis & Raoult, 2010). However, the estimation of prevalence is depending on the methodology, body fluid or tissue used and region of the globe where the study was performed. In general, is highest in cows, followed by goats and sheep and lower in the other species indicated above. Also, it is generally higher in bulk tank milk tests (BTM). Thus, the prevalence data available in several studies are difficult to compare, being difficult to estimate accurately the real prevalence of infection (Kim *et al*., 2005; Angen *et al*., 2011; Guatteo *et al*., 2011; Astobiza *et al*., 2012; Van den Brom *et al*., 2012). In addition, the results of the BTM samples should be analyzed with caution and carefully correlated with other tests, namely individual tests (Astobiza *et al*., 2012).

1.6.3 Route of transmission

Domestic ungulates (dairy cattle, sheep and goats), specially aborting domestic ruminants, shed widely highly resistant bacteria by all fluids, including feces, urine, milk, placental membranes and birth fluids, which in turn are spread by aerosols (Bartelink *et al*., 2000; Woldehiwet, 2004; Million & Raoult, 2013; Van den Brom *et al*., 2015). Once domestic ruminants undergo subclinical infection they are considered the main sources of human and environment contamination (Bartelink *et al*., 2000; Woldehiwet, 2004).

1.6.4 Occupational hazards

Contaminated aerosols are the main human infection source, by inhalation, affecting mainly occupational groups who deal with placental membranes or birth fluids that contain billions of agents (Colville & Berryhill, 2007; Million & Raoult, 2013; Van den Brom *et al*.,

2015). These groups include veterinarians, veterinary technicians, livestock farmers, dairy workers, slaughterhouse workers and researchers, mainly at facilities where ruminants are housed (Colville & Berryhill, 2007). From the occupational groups, livestock farmers are those with high seroprevalence of antibodies against *C. burnetii*, even in countries where acute Q fever are rare and without registration of outbreaks. Also people who live close to the farms could be contaminated and are potential targets of acute outbreaks (Van den Brom *et al.*, 2015).

1.6.5 Excretion and transmission routes

Awareness of the excretion routes of *C. burnetii* from infected domestic animals is vital to pinpoint transmission routes, and thus, prevent human infection and allow the implementation of effective and reasonable preventive measures. Transmission to humans mainly occurs through inhalation of contaminated aerosols from the environment, such as dust or tick feces (Klemmer *et al.*, 2018), thus making Q fever, essentially, an airborne disease, although some studies refer the evidence that *C. burnetii* may be a food-borne pathogen (Benson *et al.*,1963; Cerf & Condron, 2006; Gale *et al.*, 2015) throughout experiments obtained in which contaminated milk was fed to volunteers, causing seroconversion but any clinical disease (Benson *et al.*, 1963) or through the consumption of unpasteurized milk (Gale *et al.*, 2015). Domestic ruminants' excretion of *C. burnetii* is considered to be the main source of environmental contamination and a key cause of human infection (Roest *et al.*, 2012; Van den Brom *et al.*, 2015), as referred. In the Netherlands, after one of the largest Q fever outbreaks, airborne presence of *Coxiella burnetii* showed to be associated with goat kidding season, and with spatial variation/distance/size of goat farms (Van der Hoek *et al.*, 2010; De Rooij *et al.*, 2016).

Shedding of *C. burnetii* occurs in feces, milk, and mostly, in placental membranes and birth fluids of aborted mammal fetuses, as well as, in stillbirths and healthy infected neonates (Roest *et al.*, 2012; Van den Brom *et al.*, 2015). Subclinically infected animals also shed the organism, but with considerably lower bacterial loads than those observed in animals that underwent abortion (Roest *et al.*, 2012).

During abortion and parturition of infected ruminants, there is a massive excretion of bacteria from birth products. A previous study has found that around 10^9 bacteria/gram of placenta were excreted during abortions in ruminants (Khalili *et al.*, 2012), whereas birthing of healthy neonates, from infected mothers, revealed a lower quantity (Rodolakis, 2009).

Infectious biological excretions are desiccated in the environment, aerosolize, thus becoming airborne and available to be inhaled, allowing for transmission and infection. Environmental surroundings, dust in windy days and fomites (inanimate objects, such as gloves, coveralls, rags, etc.), that have been exposed to contaminated materials, may also result in sources for transmission (The Center for Food Security and Public Health [CFSPH], 2017).

Although *C. burnetii* has been isolated from milk, udder tissue and corresponding lymph nodes, the transmission of Q fever to humans, via ingestion of contaminated unpasteurized dairy products, still remains controversial (Galiero *et al*., 2016; Eldin *et al*., 2017). Nonetheless, shedding of *C. burnetii* in milk should be considered an important vehicle of infection for farmers, veterinarians and for dairy workers in general. Shedding in milk seems to vary in quantity and duration between animal species (Mangili *et al*., 2016; Guidi *et al*., 2017).

When comparing two excretion routes (via vaginal mucus and milk), domestic ruminants (cattle, goats and sheep) exhibit different patterns. Milk shedding seems to be more regular in cattle and goats. Ewes shed more quantity in vaginal mucus and in a more prolonged manner than goats. Both, sheep and goats can shed *C. burnetii* in subsequent pregnancies (Berri *et al*.,2002; Arricau-Bouvery & Rodolakis, 2005; Berri *et al*., 2007).

Rare modes of transmission include tick bites, ingestion of unpasteurized milk or dairy products. Human-to-human transmission is also plausible through exposure to infected human placentas, blood transfusions and sexual contact (CFSPH, 2017; Cleveland, 2017).

1.7 Pathogenesis

The pathogenesis of Q fever still lacks investigation but it is known that the portal of entry is oropharynx, followed by primary multiplication in the regional lymph nodes and then bacteraemia, which lasts for 5–7 days (Woldehiwet, 2004). Once in the bloodstream, the bacteria are directed to the target organs, which are reproductive organs, including mammary glands, placenta of pregnant animals or other organs, like lung, liver, spleen, lymph nodes, intestine and bone marrow (Woldehiwet, 2004).

The virulent factors of *C. burnetii* include the LPS and plasmids, in some strains, triggering, usually, a granulomatous host response, causing variable degrees of pneumonia, granulomatous hepatitis, granulomas in bone marrow and spleen, with splenomegaly, and

chronic endocarditis, affecting aortic and mitral valves, which is most commonly described in humans (Woldehiwet, 2004; Roest *et al*., 2013; Van den Brom *et al*., 2015).

This granulomatous chronic inflammatory response results from the ability of *C. burnetii* to grow and multiply within phagolysosomes, the structure of the phagocytic cells that should destroy it, and its propensity to establish persistent infection (Woldehiwet, 2004). Apparently, infected host cells are detected by the immune system and lysed by antibody dependent cellular cytotoxicity by monocytes and other effector cells, being the released organisms vulnerable to activated macrophages but its intracellular survival ability, probably due to the subversion of some macrophage functions and the impairment of T-cell responses, results in an immune deficient expression, in infections with "chronic" strains (Woldehiwet, 2004).

Relatively to the cellular response, in vitro studies revealed that gamma interferon (IFN-γ) limits the multiplication of *C. burnetii* and enhances its killing through apoptotic mechanisms, mediated by tumour necrosis factor (TNF-α), which induces apoptosis in infected monocytes. Also, IFN-γ mediate the killing of *C. burnetii*, through the alteration of conditions within the phagosomes and nitric oxide, up-regulated by IFN-γ and TNF-α, inhibits the multiplication of the organism in vitro (Woldehiwet, 2004; Roest *et al*., 2013).

As referred above, animals are infected from the environment and undergo a primary infection, with discrete clinical expression. However, the organism can persist after initial acute or subclinical disease, being shed in large numbers when persistently infected female animal becomes pregnant (Woldehiwet, 2004). At pregnancy the level of antibody, probably IgG, climbs and in subsequent pregnancies the animal does not excrete again or excrete at a low titre. Whereas the *C. burnetii* may continue to be shed in the milk, particularly in dairy cattle, for long periods of time (Woldehiwet, 2004).

The bacteria have tropism and multiply in the trophoblast cells of the inter-cotyledonary allantochorion and base of the cotyledonary villi, spreading through the placenta, causing placentitis and not affecting the trophoblasts that cover the cotyledonary villi. Once these trophoblasts are involved in the exchange of gasses and nutrients, the foetal development continues until inflammation spreads and causes abortion, so aborted fetuses appear normal, occasionally autolytic, or could have only granulomatous hepatitis (Woldehiwet, 2004; Van den Brom *et al*., 2015).

Macroscopically, infected placenta exhibits thickening, intercotyledonary leathery and a discoloured or yellow-brownish purulent exudate of variable consistency which corresponds,

microscopically, to an acute, diffuse inflammatory infiltrate in the chorion without vasculitis. The severity of inflammation varies from mild mononuclear infiltration to severe supurative exsudate or necrosis (Woldehiwet, 2004; Van den Brom *et al*., 2015). Intact infected chorionic epithelial cells, especially at the base of the cotyledonary villi, show cytoplasmic vacuoles, containing large numbers of basophilic granulation, highly suggestive of *C. burnetii* organisms (Woldehiwet, 2004; Van den Brom *et al*., 2015).

1.8 Clinical Manifestation in animals

1.8.1 Clinical findings

Coxiella burnetii is capable of infecting several animal species, such as ruminants, companion animals, birds and reptiles, as well as, humans, thus being considered a widespread zoonosis (Roest *et al*., 2012; van den Brom *et al*., 2015; Cleveland, 2017). Q fever is reputed to be non-pathogenic for domestic livestock (Blood & Radostitis, 1989) and the truth is that there is very little information about the clinical signs of Q fever in domestic animals. Despite the earlier reports of respiratory disease due to *C. burnetii* in sheep, goats and cattle, anorexia was the only consistent clinical finding (Blood & Radostitis, 1989; Woldehiwet, 2004). However, it is currently recognized that *C. burnetii* causing placentitis and abortion in dairy goats and sheep. Recent studies performed in dairy cattle, reveal the possibility of a relationship between *C. burnetii* infection and an increased number of endometritis, subfertility, and retained foetal membranes (De Biase *et al*., 2018).

Most *C. burnetii* infections in mammals result in asymptomatic seroconversions, but when infection is clinically expressed, it displays a variety of clinical forms depending on the infected species, virulence of the strain and inoculation route. In guinea pigs and mice, pneumonia and hepatitis can be observed, as well as, occurrence of splenomegaly. In contrast, reproductive expression constitutes the highest concern in livestock, although rare cases of pneumonia can ensue after infection. Clinically infected humans may exhibit atypical pneumonia, hepatitis, flu-like symptoms, or even life-threatening endocarditis, in the presence of persistent infection (Marrie *et al*.,1996; Arricau-Bouvery & Rodolakis, 2005; Russell-Lodrigue *et al*., 2006; Russel-Lodrigue *et al*. 2009; Roest *et al*., 2012; van den Brom *et al*., 2015; Cleveland, 2017).

Ruminants, particularly goats and sheep, constitute the most relevant zoonotic sources since they are the main animal reservoirs of *C. burnetii*, as referred previously The most frequent

clinical signs in these species are abortion, premature delivery, stillbirth, and weak offspring (de Biase *et al*., 2018). Abortions occur without previous warning signals and mainly during the late to end stages of pregnancy (Berri *et al*., 2001; Berri *et al*., 2002; Hatchette *et al*., 2003; Arricau-Bouvery & Rodolakis, 2005; Rousset *et al*., 2009) (Figure 1.4).

In dairy goat herds where Q fever abortions occurred, metritis can be present, as well as neonate lambs can be equally affected, showing general weakness with low body weight and high mortality in the neonatal phase. Those that appear to be healthy may later exhibit respiratory and digestive tract disorders displaying very low viability (Arricau-Bouvery & Rodolakis, 2005; Wouda & Dercksen, 2007; Roest *et al*., 2013).

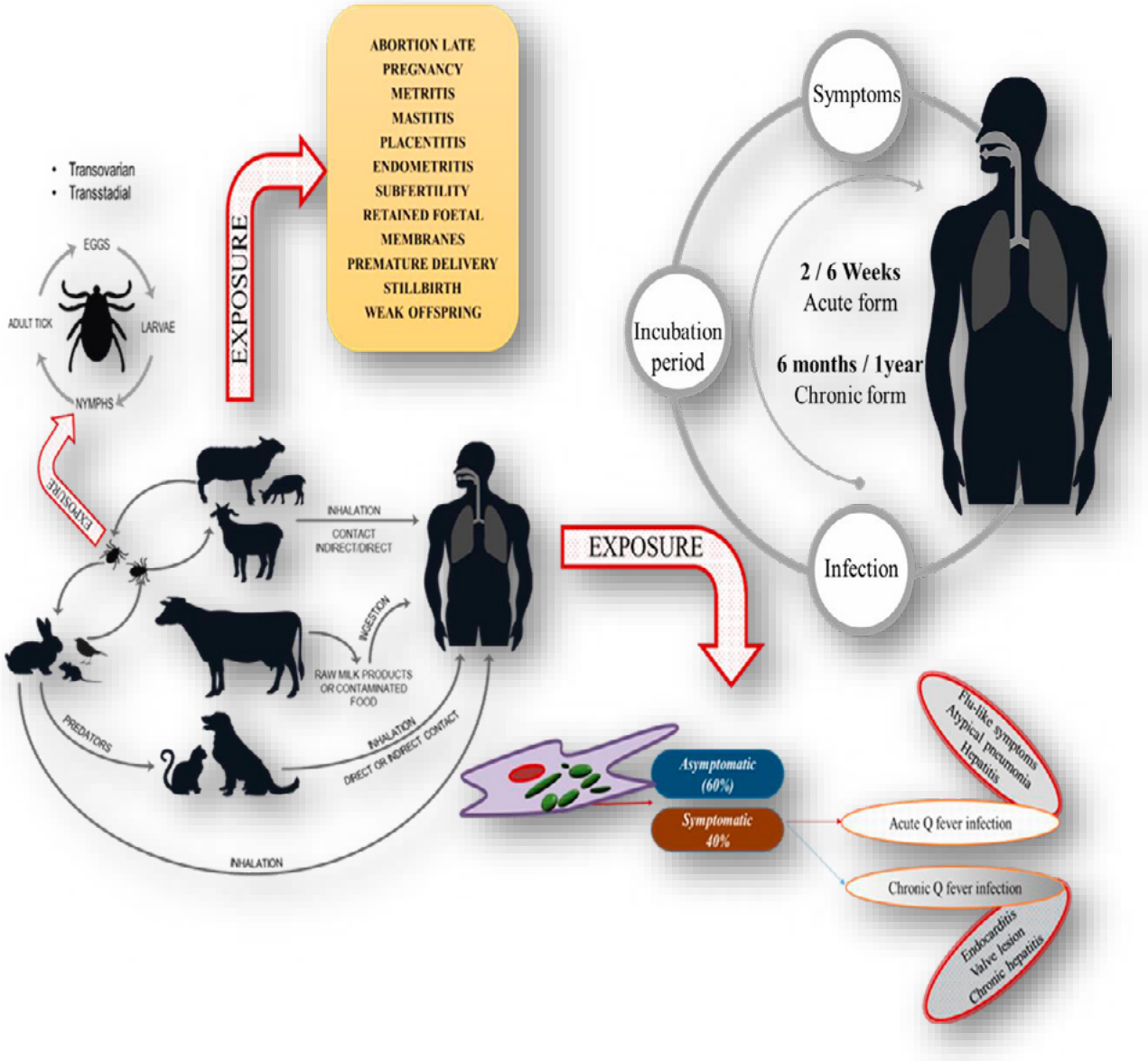

Figure 1.4. Schematic representation of the main symptoms of Q fever in animals and humans, routes of transmission. The image represents the most significant zoonotic pathways of *C. burnetii*.

In dairy cattle, endemic infection is considered to reduce fertility (Lang, 1990; de Biase *et al*. 2018) (Figure 1.5.a) and may predispose to sub-clinical mastitis, resulting in reduction of milk production and final break down of the quarter (Klemmer *et al*., 2018).

Although aborted fetuses appear grossly normal, infected placentas display unspecific alterations of intercotyledonary placentitis, showing opacity and thickening due to exudative inflammation, necrosis and intercotyledonary fibrosis (Figure 1.5.c), with the cotyledons exhibiting areas of grey discoloration, as mentioned previously (McGavin & Zachary, 2007). Owing to the unspecific nature of lesions, establishing *C. burnetii* as the causal agent requires mandatory confirmation by alternative laboratory methods (Agerholm, 2013).

Figure 1.5. Images representing the main symptoms of animal Q fever in ruminants: a) endometritis, b) abortion in late pregnacy and c). In same cases the lesions in the reproductive tract, include placentitis with unspecific lesions such as cotyledonary thickening and necrosis as well as and intercotyledonary fibrosis.

It is still unclear if infection always leads to abortion in ruminants (Figure 1.5.b). Some studies refer that in cattle and in other species, it seems that *C. burnetii* is an infrequent cause of abortion, while high abortion rates are found in goats (Muskens *et al*., 2012; Agerholm, 2013). The cause of these differences in pregnancy outcomes after infection is unknown because extensive assessments, of the effects of *C. burnetii* in each species reproductive profile, is lacking. However, there are biological indications of species differences in relation to the

impact on reproduction. Recent molecular studies have already shown the existence of different strains of *C. burnetii*, these strains may be associated with different ruminant hosts despite the occurrence of cross infection (Agerholm, 2013).

1.9 Q fever in humans: pathogenesis of Q fever and immune response

In humans' Q fever is a complex and polymorphic disease that can manifest itself in the acute form with self-limiting, mild to moderate course and therefore a benign prognosis, as well as in the persistent or chronic form of the disease (Fournier *et al.*,1998).

The acute form normally appears after a period of incubation that varies between 2 and 6 weeks and may be asymptomatic or cause a "simple" flu-like illness (Bartelink *et al.*, 2000; Kovácová & Kazár, 2002) or could be more severe and cause a pneumonia or hepatitis (Kovácová & Kazár, 2002) (Figure 1.6).

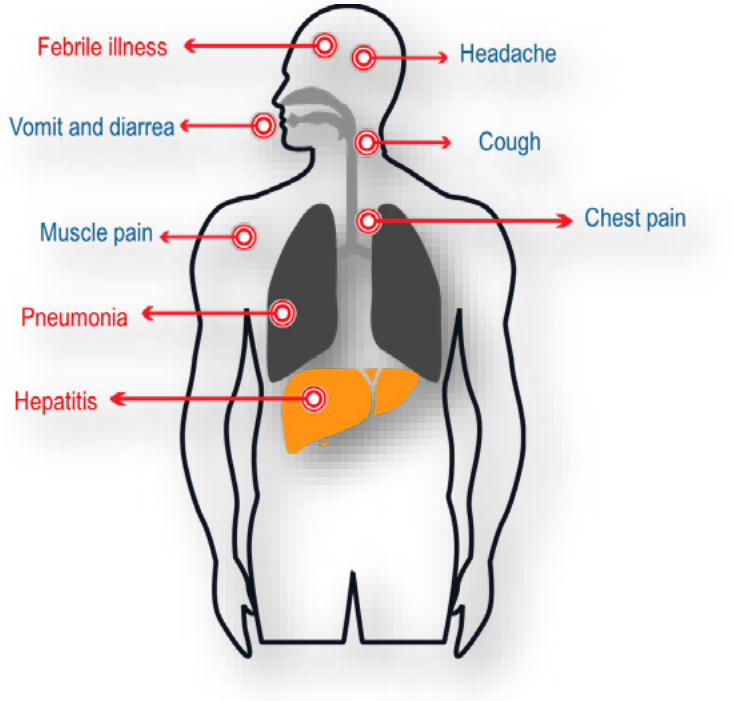

Figure 1.6. Human Q fever: the most comon symptoms of acute Q fever in humans.

The chronic form (Figure 1.7) may be mainly responsible for endocarditis, but also hepatitis and chronic fatigue syndrome (Bartelink *et al.*, 2000; Kovácová & Kazár, 2002; Kazar, 2005). In some cases, *C. burnetti* can be responsible for isolated cases and in outbreaks, associated

with a severe or with a potentially fatal course, with important implications in public health (Santos *et al*., 2007b; Siciliano *et al*. 2008).

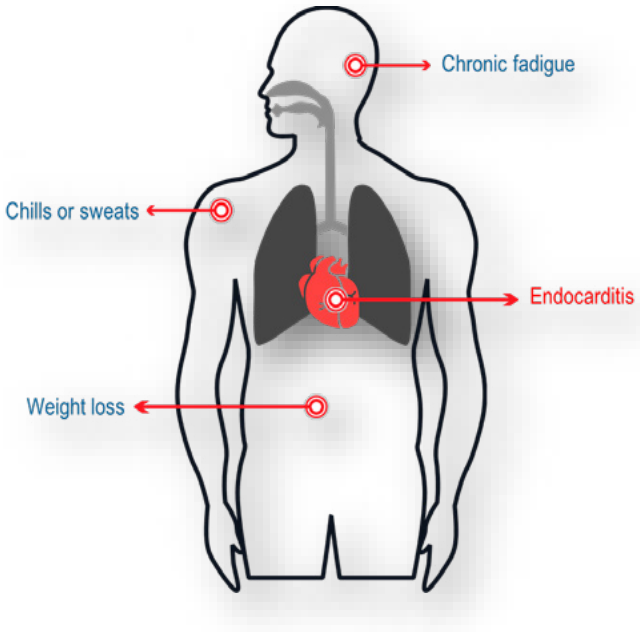

Figure 1.7. Human Q fever: the most comon symptoms of chronic Q fever in humans.

The primary route of infection for humans is airborne, via inhalation of aerosolized particles from animal birthing products, urine and faeces, or possibly via ingestion of raw milk (Maurin & Raoult, 1999; Angelakis & Raoult, 2010). After aerossol transmission, *C. burnetii* targets alveolar macrophages and other mononuclear phagocytes (Baca *et al*.,1993; Ghigo *et al*., 2012). *C. burnetii* passively enters macrophages cells by actin-dependent phagocytosis (van Schaik *et al*.,2013).

As an obligate intracellular pathogen, *C. burnetii* presents some challenging features to immunologists. The organism takes up residence in a lysosome-like compartment in a phagocyte (Shannon & Heinzen, 2009) (Figure 1.8). The survival of *C. burnetii* in human macrophages is based on the control of phagocytosis and the prevention of ultimate phagosome lysosome fusion (Ghigo *et al*., 2004). The bacteria are retained in this phagosomal compartment and the moderately acidic pH (<5) of this compartment is actually needed for its metabolism and subsequent replication (Hackstadt & Williams, 1981; Shannon & Heinzen, 2009).

The host defence relies on systemic cell-mediated immunity, involving innate and adaptive partners of the immune response. One of the main features of the immune response is the formation of a granuloma under the control of IFN-γ. Granulomas show an accumulation of immune cells around a central open space and limited by a fibrin ring, which led to the

definition of a doughnut granuloma. They are rich in macrophages with different levels of maturation, including epithelioid cells and multinucleated giant cells (Pellegrin *et al*., 1980). *C. burnetii*, via its LPS, interacts with macrophages through αvβ3 integrins, and avoids internalization by inhibiting the interaction between αvβ3 integrins and CR3, which is essential for bacterial uptake (Capo *et al*., 1999; Capo *et al*., 2003).

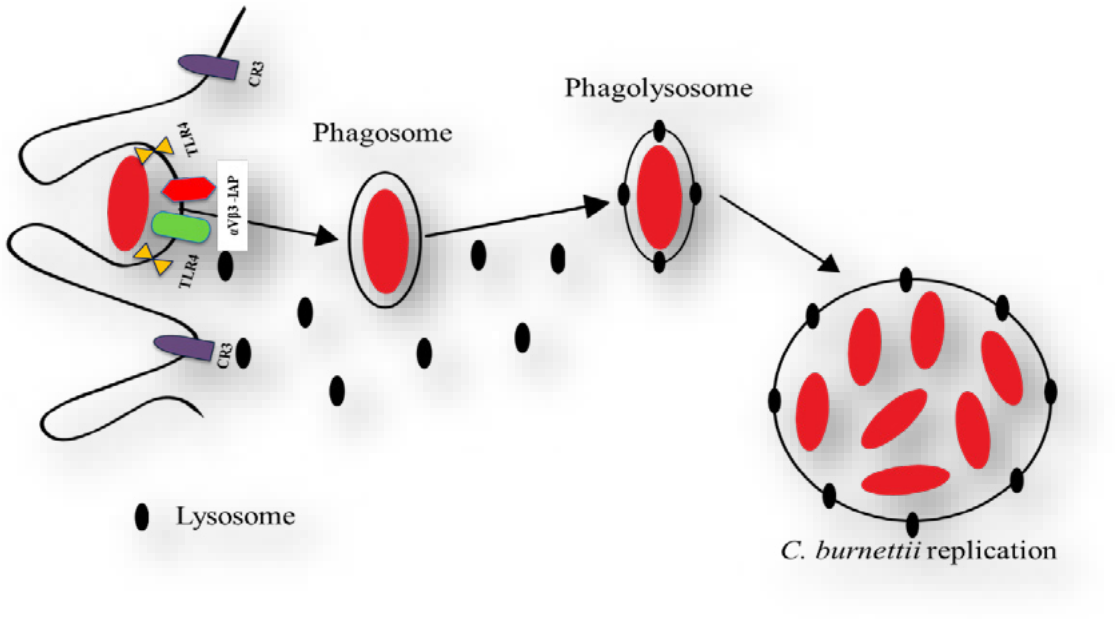

Figure 1.8. Shematic representation of the formation of phagosomes inside a host cell in a phase I (virulent) *C. burnetii* infection. Formation of "rufles" (pseudopodal formation) after the interaction between αvβ3 integrins and protein integrin-associated protein (IAP). Thus leading to cytosqueleton reorganization and consequent survival of *C. burnetii*. In this process TLR4 (Toll-like receptor 4) is envolved in the recognition of lipopolysaccharide of phase I. In this phase intracellular bacteria its not killed because there is a inhibition of the catepsin fusion (Mori *et al*., 2018).

Although the virulence of *C. burnetii* does not only depend on LPS, the particular composition of *C. burnetii* LPS allows several axes of the immune response to be modulated, ranging from phagocytosis to vesicular trafficking (Abnave *et al*., 2017).

Specific immunoglobulins are secreted following infection. IgG is mainly directed against phase II antigen, whereas IgM is directed against both phase I and II cells (Maurin & Raoult, 1999). Once established, chronic Q fever presents defective cell-mediated immunity, highlighting the major role of cell-mediated immunity in the protection against *C. burnetii* (Koster *et al*., 1985).

2. Diagnostic methods

Coxiella burnetii can be detected in numerous ways, depending on the type of sample and the purpose of the analysis. The organism, or its nucleic acids, may be researched in vaginal discharge, placentas, other tissues, birth fluids, aborted foetuses, milk, colostrum, blood, urine and feces (Guatteo *et al*., 2006; Centers for Disease Control and Prevention [CDC], 2017).

Histopathology of the placenta, with modified Ziehl– Neelsen, Gimenez, Stamp, Giemsa or modified Koster stain, permit the observation of *C. burnetii* in inflammatory exudates (Guatteo *et al*., 2006; Sanchez *et al*., 2006). Due to similarities, *C. burnetii* can be confused with *Chlamydophila abortus* or *Brucella* spp., and thus, immunohistochemistry (IHC), using *C. burnetii* specific antibodies, or other more conclusive methods, should be used for definitive identification. Abortion induced by *C. burnetii* usually yields high numbers of bacteria for observation in trophoblasts of the allantochorion (Sanchez *et al*., 2006).

Histopathology, combined with serological testing, has been the diagnostic mainstay for Q fever for some years, as well as the key methodology to differentiate between other causes of abortion in ruminants. Currently, direct detection and quantification by polymerase chain reaction (PCR) and serological ELISA (enzyme-linked immunosorbent assay) should be considered the methods of choice for clinical diagnosis, as PCR is still the most reliable tool for the diagnosis of infectious abortions and ELISA is the most sensitive method in detecting *C. burnetii* specific antibodies (Sidi-Boumedine *et al*., 2010; Niemczuk *et al*.,2014).

PCR detects *C. burnetii* DNA in a wide range of samples, such as placenta tissues, feces, vaginal mucus and milk. Primers and probes targeting the IS*1111* element, which has multiple copies in the genome of *C. burnetii*, are more sensitive than primers and probes that target single copy genes, resulting in increased detection. Capability to detect and quantify *C. burnetii* DNA by real-time PCR has heightened diagnostic and study approaches. Individual vaginal discharge, milk and colostrum samples, or collective milk from a tank, can be used for analysis of bacterial shedding. Therefore, active monitoring systems may be implemented in regions that need to evaluate the prevalence of Q fever in their animal population, through PCR testing of bulk tank milk (BTM). Although vaginal swabs and birth products are indicated for abortion diagnosis, BTM samples are more appropriate to scrutinize the sanitary condition of dairy cattle and goat herds. This collective diagnostic approach to screen *C. burnetii* is deemed as more

significant in relation to public health risk (Klee *et al*., 2006; EFSA Panel on Animal Health and Welfare (AHAW), 2010; Sidi-Boumedine *et al*., 2010).

A serological survey is the correct approach to evaluate prevalence. Serological assays are suitable for screening herds or flocks, but interpretation at the individual animal level is not possible. Indeed, a significant proportion of animals shedding *C. burnetii* bacteria, and even some aborted animals, are found to be seronegative, but the presence of specific anti-*C. burnetii* antibodies may provide evidence of a recent infection, as well as, of a past exposure (Guatteo *et al*., 2007; Rousset *et al*., 2007; Rousset *et al*., 2009; de Cremoux *et al*., 2012b). Sampling should target a representative number of animals. Serological analyses may be carried out using enzyme-linked immunosorbent assay (ELISA), indirect immunofluorescence assay (IFA) or complement fixation test (CFT). Numerous studies have shown that relative sensitivity is lowest for the CFT, but on the contrary, it has high specificity for high levels of anti-*C. burnetii* antibodies, generated in aborted herds or flocks (Rousset *et al*., 2007; Rousset *et al*., 2009; Horigan *et al*., 2011; Emery *et al*., 2014; Niemczuk *et al*., 2014).

The CFT is the most widely used test in veterinary laboratories, although commercially available ELISA tests for *C. burnetii* antibodies, in ruminants, are increasingly used. Compared to ELISA, the CFT lacks the sensitivity to detect *C. burnetii* specific antibodies in ruminant serum. IFA is the less reproducible test between operators and therefore between laboratories and research institutions. Although ELISA methods are not yet fully validated and harmonised, they are consistent and can be automated. Consequently, ELISA testing is recommended for routine serological screening of animals for Q fever and to date, is considered the most sensitive technique to detect *C. burnetii* specific antibodies (Rousset *et al*., 2009; Horigan *et al*., 2011).

Genotyping methods are very useful for epidemiological research, particularly to clarify links to the source of infection, understanding of epidemiological emerging factors, elucidating human outbreaks, and to a lesser extent, for evaluating control measures. Several typing methods have been used for the characterisation of *C. burnetii* strains, such as restriction endonuclease of genomic DNA, pulsed-field gel electrophoresis (PFGE), and sequence and/or restriction fragment length polymorphism (PCR-RFLP) analysis of *icd*, *com1* and *mucZ* genes. More recently, two PCR-based typing methods have been described, multi-*locus* variable number of tandem repeats analysis (MLVA) and multispacer sequence typing (MST) that permit the typing of *C. burnetii* without the need for isolation of the organism. To date, MLVA and MST are considered to be the most selective methods for *C. burnetii*, allowing the identification of up to 36 distinct genotypes. Additionally, databases have been established and

their availability allows inter-laboratory comparisons to be made easily. This enables a better understanding of the propagation of *C. burnetii* isolates (linking outbreaks to their source) or to identify new emerging strains. Standardisation of MLVA is underway and should be available in the near future (World Organisation for Animal Health [OIE], 2015).

For specific laboratory research, it may be necessary to isolate the agent. For low contaminated inoculates of *C. burnetii*, direct isolation by inoculation of embryonated chicken eggs or cell culture is possible, being the latter a superior method (Samuel & Hendrix, 2009).

Various other methods are or have been evaluated for analysis of *C. burnetii* in different samples, like *in-situ* hybridisation, DNA amplification, fluorescent *in-situ* hybridisation (FISH) and loop mediated isothermal amplification (LAMP) assays (Jensen *et al.*, 2007; Samuel & Hendrix, 2009).

3. Disease control

In common with all zoonotic diseases, control of the disease in animals will influence the level of disease seen in man (Angelakis & Raoult, 2010). In the case of a Q fever outbreak, sanitary and prophylactic measures should be applied at herd and human level, in order to limit disease transmission. Human and animal infections must be diagnosed early and treated immediately to prevent the development of chronic infections and secondary complications (Porter *et al.*,2011). Balancing economic livestock interests and human health is particularly demanding when controlling zoonotic diseases outbreaks, including Q fever (van Asseldonk *et al.*,2015).

According to the EFSA Panel on AHAW (2010), the choice of a Q fever control strategy will depend on the main objective of the control effort, that can be limited to avoiding the spreading of the disease or attempt the complete eradication of *C. burnetii* infection.

Therapeutic and prophylactic measures in small ruminants are intended to diminish abortion rates and bacterial shedding, thereby aiming to reduce environmental contamination (Van den Brom *et al.*, 2015).

In spite of the difficulty to determine susceptibility of *C. burnetii* to antibiotics, antibiotic treatment may be performed to reduce the number of abortions and the quantity of *C. burnetii* shed at parturition (Angelakis & Raoult, 2010). In ruminants, antibiotic treatment generally consists in administering two injections of oxytetracycline (20 mg per kg body weight), during

the last month of gestation, although this treatment does not totally suppress the abortions and the shedding of *C. burnetii* at lambing (Berri *et al.*,2007; Angelakis & Raoult, 2010). Abortion in animals can be prevented with vaccination, and it is undoubted that a phase I vaccine must be used to control the disease and to reduce environmental contamination and therefore, the risk of transmission to humans, and might be considered a long-term control policy, especially in heavily infected herds (Angelakis & Raoult, 2010; EFSA Panel on AHAW, 2010).

In goats, vaccination induces an overall decrease in shedding levels and the highest reduction is found in nulliparous animals (de Cremoux *et al.*, 2012a). Thereby, before the very first pregnancy, the susceptible animals should be vaccinated (de Cremoux *et al.*, 2012a; Bontje *et al.*,2016).

The efficacy of the phase I vaccine was also studied in naturally infected cows (Guatteo *et al.*,2008). When vaccinated while not pregnant, an initially non infected animal had a five times lower probability of becoming a shedder than an animal receiving a placebo (EFSA Panel on AHAW, 2010).

Besides antibiotic treatment and vaccination, other preventive measures can be adopted at the farm level, including the removal of risk material (placentas, aborted foetuses), manure management, culling of pregnant animals, identifying and culling of shedders, temporary breeding ban, control of animal movement between farms of differing infection status, appropriate tick control strategies, removing infected animals from herds or providing separate containment facilities in which to give birth, among others (Angelakis & Raoult, 2010; EFSA Panel on AHAW, 2010).

In humans, regarding the clinical polymorphism of *C. burnetii* infection, there is no single management strategy. Recent studies have revealed that each situation requires specific treatment and follow-up. In case of primary infection, the main issues after diagnosis are screening for potential risk factors for complications and choice of a recommended regimen with doxycycline (200 mg/day) and hydroxychloroquine (600 mg/day), to prevent progression to persistent focalized infection (Eldin *et al.*, 2017). Human Q fever may be prevented with a vaccine developed and commercialized in Australia, where it was used to immunize slaughterhouse personnel. This vaccine must not be administered to individuals having been in contact with *C. burnetii*, because this may lead to serious adverse reactions, such as sterile abscesses or systemic inflammatory syndrome (Parker *et al.*,2006; Schneeberger *et al.*,2014).

4. Q fever in Portugal

Q fever was identified for the first time in Portugal in 1948 (Fonseca *et al*.,1949a; Fonseca *et al*.,1949b). For many years it had been thought that acute Q fever merely expressed itself in Portugal as a pulmonary disease (Fonseca *et al*., 1949b), but in 1974, the first case with hepatic involvement was identified in the Infectious Diseases Department at Santa Maria's University Hospital (Mendes *et al*.,1989b). Several seroepidemiological studies have been conducted in Portugal confirming that the disease is endemic in the country (Mendes *et al*., 1989a; Filipe *et al*.,1990; Parreira, 2008). Q fever is a notifiable disease in Portugal since January 1, 1999 and an average of 0.1 cases per 10^5 inhabitants per year have been reported (Direção-Geral da Saúde [DGS], 2011).

Unlike in other countries within Europe, where there is a clear trend towards a decrease in the number of human Q fever cases, in Portugal the numbers have been stable which has been reason for alert within Public Health stakeholders (Direção-Geral da Saúde [DGS], 2015; European Centre for Disease Prevention and Control [ECDC], 2014). Although no epidemics have been reported, it is believed that the disease is underdiagnosed and underreported (Santos *et al*.,2007a; Palmela *et al*.,2012). For instance, a study performed by the Centre for Vectors and Infectious Diseases of the Ricardo Jorge National Health Institute (CEVDI/INSA) found serological confirmation of 32 cases between 2004 and 2005, as opposed to the 12 cases reported to the General Directorate of Health (DGS) countrywide for the same period (Santos *et al*., 2007a; DGS, 2011).

Sheep and goat traditional farming and has been widely present in Portugal and is part of the cultural and gastronomical background of the country. This close proximity to small ruminants may contribute to the endemicity of Q fever in Portugal (Alves *et al*., 2016). In a recent case series describing 7 cases of acute Q fever, admitted in a Portuguese University Hospital, between 2014 and 2015, Alves *et al*. (2016) have been able to retrieve detailed epidemiological data of all cases, mostly regarding contact or close proximity to sheep, cattle or goats, suggesting aerosol inhalation as the main route of transmission and a strong relationship between disease and occupational exposure (Alves *et al*., 2016). Risk for environmental exposure to Q fever has been previously pointed in a *Coxiella burnetii* prosthetic valve endocarditis of a 53-year-old patient with recurrent mechanical valve dehiscence on mitral position (Ferraz *et al*., 2016). This man had lived in a rural area with sheep and goats on the surroundings. Also recently, a study reported 2 rare manifestations of Q fever, namely

splenic and hepatic abscesses and cerebral venous thrombosis, in a 47 and a 55-year-old man, both living on the country side and in contact with chicken and other farm animals (Gomes *et al*., 2014).

Nonetheless, a few studies also report disease in those exposed to companion animals. Q fever was detected in a 16-year-old female that presented to the emergency unit of a major Portuguese Hospital, complaining of severe retro-orbital headaches for the previous 6 days, with photophobia and vomiting for 2 days (Figueiredo *et al*.,2016). The patient used to visit her grandparents in a rural area, where they had 2 dogs. Interestingly, another very recent case report described a 60-year-old man who underwent kidney transplantation with a 34-year-old living donor renal allograft and underwent immunosuppressive therapy, and was only exposed to his cat (no other animal) (Godinho *et al*., 2015).

More recent attention has been given to Q fever in animals. In two studies, evidence for Q fever in aborted foetuses and in Zoo animals, using PCR technique with primers targeting *C. burnetii* genome (Clemente *et al*., 2008; Clemente *et al*.,2009). In a later study, Cumbassá *et al*. (2015) sought evidence for *C. burnetii* infection in domestic, captive and wild animals, having found *C. burnetii* DNA in tissue of domestic, wild and zoo animals (Cumbassá *et al*., 2015). Simultaneously, a few serosurveys with wider sampling approaches, both in large and small ruminants, have shown *C. burnetii* circulation in Portugal. A 2013 cross-sectional study evaluated the exposure to *C. burnetii* in sheep and goats in the Centre of Portugal (Anastácio *et al*., 2013). Results showed a global herd prevalence of 32.6%, higher in mixed herds (38.5%) and in sheep herds (37.5%) than in goat herds (28.8%). Global individual prevalence was estimated at 9.6% being higher in goats (10.4%) than in sheep (8.6%). Interestingly, authors have associated seropositivity to goats, older animals and larger herds. Another study from central Portugal studied bulk tank milk samples from cattle (n = 45) and small ruminant (n = 64) herds by both ELISA and PCR, showing an apparent seroprevalence of 45.9%, being higher in small ruminants (51.6%) than in cattle (37.8%). PCR analysis showed shedding of *C. burnetii* in 11.9% of bulk milk tank samples, being higher in cattle (20%) than in sheep and mixed herds (6.3%) (Anastácio *et al*., 2013). A study from the same year confirmed results of substantial endemicity of *C. burnetii* (Pimenta *et al*., 2015). Authors have studied bulk tank milk samples from 90 dairy farms and assayed using an ELISA kit, showing an apparent prevalence of 61.1% (Pimenta *et al*., 2015). Taken together, these serosurveys show a high level of exposure to *C. burnetii* in Portuguese small and large ruminant herds, proving the need to provide clearer

understanding of Q fever epidemiology in Portugal, ideally by implementing monitoring programs on sentinel herds.

5. Serra da Estrela region

Serra da Estrela is a small region classified as NUTS III statistical subregion of Portugal integrated in the NUTS II Centre region and has its name due to the largest mountain in Portugal. Serra da Estrela is bordered to the north by Dão-Lafões, to the west by Beira Interior Norte, to south by Cova da Beira and to the east by Pinhal Interior Norte. The subregion contains the Serra da Estrela, as referred, the largest highest mountain in Portugal and much of the 1000 km² Natural Park of Serra da Estrela. The subregion itself is only 867.8 km² (Figure 1.9).

The Serra da Estrela sheep autochtonous breed is one of the most important autochthonous sheep breeds in Portugal, with approximately 70,000 animals (Dinis, 2013). According to the rules of the Genealogical Book, the Serra da Estrela sheep may be white or black (Dinis, 2013) (Figure 1.10). This breed has as its natural territory covering the municipalities of Seia; Gouveia; Celorico da Beira; Guarda; Fornos de Algodres; Oliveira do Hospital; Tábua; Arganil; Mangualde; Carregal do Sal; Penalva do Castelo; Tondela and Viseu, still being distributed throughout Baixo Mondego, downstream of Coimbra.

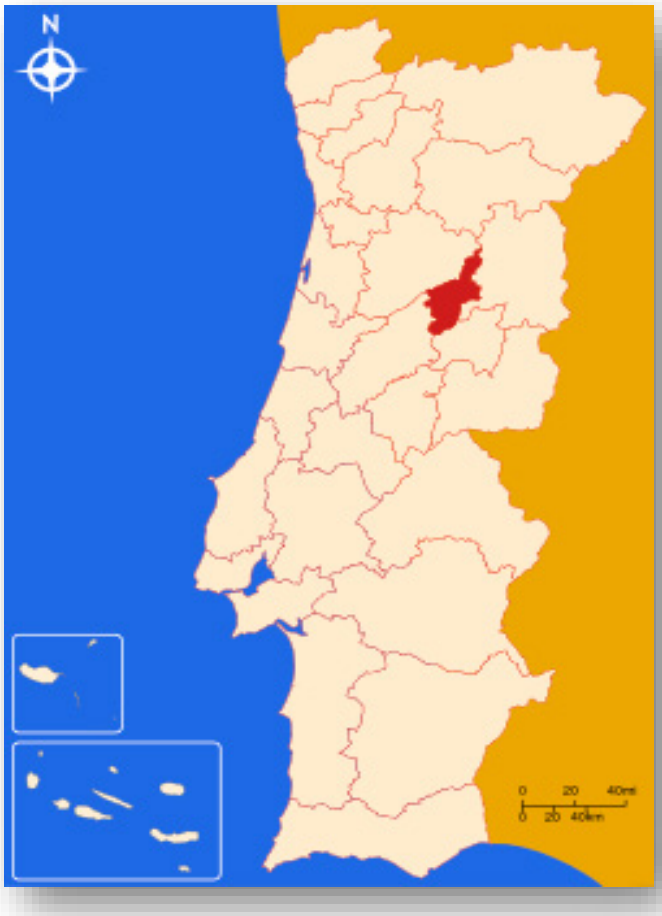

Figure 1.9. Serra da Estrela region, according to The Nomenclature of Territorial Units for Statistics level III (NUTS III) (adapted from https://pt.wikipedia.org/wiki/Serra_da_Estrela).

It is a predominantly a dairy breed and its milk is used for the production of Serra da Estrela cheese (Protected Denominated of Origin) and creamy cheese Serra da Estrela (Protected Denominated of Origin). These are characteristic products, of excellent quality, with worldwide recognition, being this production one of the socioeconomic cornerstone of this whole area.

Milk production is strongly influenced by both the environmental context of the farm and the climatic effects over throughout the years (Dinis, 2013). There are great differences between farmers with regards to milk production, such as the farming characteristics, the management practices and the sheep genetics.

a) b)

Figure 1.10. Serra da Estrela authoctonous sheep. The photos show the black a) and white b) varieties.

Milk production tipically increases from the first lactation to the intermediate ones, reaching a maximum, beginning to decrease thereafter (Carolino *et al*., 1994, 1998 and 2003, Dinis, 2013).

Besides milk production in Serra da Estrela sheep, this breed has also been linked to meat production through Serra da Estrela (DOP) milk lambs (Dinis, 2013), due to its with good reproductive characteristics either, in terms of fertility and prolificacy. Their sexual activity is extended throughout the year, however, there are two breeding seasons: the main in spring and the secondary in autumn for young females and older non-pregnant female sheep. The prolificity of the Serra da Estrela sheep has remained approximately constant in recent years, with an average value of 1.42 lambs/calving (Dinis, 2013).

This area is characterized by small farms in which the average number of animals *per* farm is about 50-60 animals. In most cases these small farms may be predominantly run by owner-operators wich are the the responsible for all phases of production of Serra da Estrela sheep. This official breed is highly rustic and extremely well adapted to the surrounding agricultural areas, revealing a great adaptability, even in less fertile areas, contributing to the conservation of landscapes and ecosystems. Although many of the farms are based on the continuation of the work of previous generations, there has been an adaptation in production, in which cheese producers undergone specialization by adapting their farm to milk production, in compliance with the rules of required by oficial certification.

The farming practices of this region is a traditional and familiar method of production based on a closed proximity. In the region focused, one characteristic activity in this type of farming is transhumance, which, although practically in disuse, still occurs in particular areas such as Seia and Gouveia, especially during times of greater resource scarcity, namely between June and August (Pinto, 1982; Dinis, 2013). Currently transhumance is no longer performed, due to several factors, such as the higher production intensity originating an increased sowing and grazing areas in the lowlands of the Serra, as well as emigration, with consequent abandonment agricultural activity and by sanitary factors.

6. Conclusion

Coxiella burnetii infection can result in huge economic loss due to epizootic abortions, which are often associated with bacteria shedding in birth fluids and placentas, increasing the risk of infection to humans and animals. Important work has been made to prevent the spread of *C. burnetii* from small ruminants to humans, using either nonspecific and specific measures. However, these efforts have been hindered by the absence of effective veterinary interventions.

The knowledge of the epidemiology and the implementation of measures it seems to be urgent to an effective control, in animals which will have an important impact in the maintenance of human health. Consequently, it is imperative to determine the prevalence of *C. burnetii* infection in portuguese sheeps, evaluating simultaneously the prevalence in occupational portuguese workers, to determine and estimate the real circulation of *C. burnetii* among the risk population, trying, therefore, to determine the major types of specific economical loss and the main sources of infection that occur in Portuguese professional workers.

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CHAPTER 2 - OBJECTIVES

The main objective of the present thesis was to evaluate the circulation of *Coxiella burnetii* in portuguese sheep, as well to study in detail the effective presence and circulation of this agent in Serra da Estrela sheep (an autochthonous portuguese sheep breed) in the central region of Portugal.

To accomplish this general objective, several specific goals were achieved, particularly:

1. Estimate the prevalence of *Coxiella burnetii* infection in continental Portugal. This goal was acomplished by performing a seroepidemiologic survey on portuguese sheep from all regions of continental Portugal.

2. Evaluate the epidemiology of *Coxiella burnetii* in a selected portuguese breed of sheep. This specific aim was performed through a prospective serosurvey in a population of Serra da Estrela sheep in the central region of Portugal.

3. Perform surveillance of *Coxiella burnetii* in herds in Central Portugal, in order to study the epidemiological features of *Coxiella burnetii* outbreaks and the emergence of this agent in the central region of Portugal. This objective was achieved by the molecular diagnosis of *Coxiella burnetii* in small ruminant flocks that presented abortion outbreaks and by the findings of a herd-level study on IgG anti- *Coxiella burnetii* in bulk-tank milk samples of sheep from Central Portugal.

CHAPTER 3 - A NATIONWIDE SEROEPIDEMIOLOGICAL STUDY ON Q FEVER ANTIBODIES IN SHEEP OF PORTUGAL

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Introduction

Q fever is an almost global zoonotic disease caused by *Coxiella burnetii*, which is able to infect several animal species and of which cattle, sheep and goats are the primary animal reservoirs (Van der Brom *et al*., 2015; Khor *et al*., 2018). Q fever in humans can produce acute disease that usually leads to pneumonia, hepatitis and self-limited illness (Guatteo *et al*., 2011). Chronicity is associated to endocarditis in immuno-compromised individuals or abortions and stillbirths in pregnant women (Angelakis & Raoult, 2010; Dabaja *et al*., 2018). Human infection occurs by *C. burnetii*-contaminated aerosols of animal origin that are inhaled (Guatteo *et al*., 2011). Although rare, oral transmission by consumption of contaminated dairy products is also possible as is also sexual and vertical transmission (Kruszewska & Tylewska-Wierzbanowska, 1997; Milazzo *et al*., 2001). Though almost neglected in the past, recent outbreaks have alerted the public and the scientific community (van der Hoek *et al*., 2010). In the Netherlands, a large outbreak affecting 2,357 human cases that lasted several years was notified in 2009 and linked to abortion waves on dairy goat flocks (van der Hoek *et al*., 2010). In fact, Q fever in small ruminants (sheep and goats) is associated with vast shedding of bacteria in placentas, faeces, milk, and birth fluids, and usually manifest as abortions, which greatly increases the risk of disease spread (O´Neill *et al*., 2014; Filioussis *et al*., 2017).

In the Iberic Peninsula, very few regional studies on Q fever in ruminants have been made (Anastácio *et al*., 2013; Cumbassá *et al*., 2015), and no serologic survey in the full continental territory Portugal has ever been done. Hence, an epidemiologic survey was set up to estimate the seroprevalence of Q fever antibodies in sheep of all regions of Portugal.

Materials and methods

Sample size

This study used samples collected in 2014 for a previous study (Esteves *et al*., 2016). Sample size was calculated considering the following a priori assumptions: population size of 2,092,175 sheep (IFAP, 2018), an expected *C. burnetii* seroprevalence of 50% (allowing for the largest sample possible), an absolute error of 3%, and a 95% confidence level (DESCRIBE package, WINPEPI updated, version 11.43). A calculated sample size of 1,068 sheep was obtained and a stratified random sampling design was obtained by categorizing according to the Nomenclature of Territorial Units for Statistics level II (NUTS II) regions (North, Centre, Lisboa and Vale do Tejo, Alentejo and Algarve), to reduce possible confounders associated to the heterogeneous geographical distribution of sheep in Portugal. The 2014 official animal census data of Portugal reported the following distribution on sheep head according to region: 315,506 sheep are located in the North (15.1%), 481,017 in the centre (23%), 42,861 in Lisboa and Vale do Tejo (2%), 1,206,876 in Alentejo (57.7%) and 45,915 in Algarve (2.2%) (IFAP, 2018). As no herd level statistics are available, to better represent the distribution in the 5 regions of Continental Portugal, samples from 4 farms spread within each region (at the North, South, East and West) were selected for screening.

ELISA screening

Blood samples had been collected aseptically by jugular vein puncture into sterile labelled Vacutainer tubes without additives (BD Vacutainer Systems, Plymouth, UK). Samples were kept cold during transport to the laboratory. Sera was removed after centrifugation and stored at −20 °C until analysis. All sera belonged from healthy female sheep with 6 months to 10 years of age (average age of 5 years) born in Portugal, randomly selected upon the moment of the official brucellosis control. In total, 161 samples from the North, 246 from the Centre, 21 from Lisboa and Vale do Tejo, 616 from Alentejo and 24 from Algarve were selected. Sera were tested for the presence of anti-*C. burnetii* IgG antibodies using a commercial indirect ELISA, ID Screen Q Fever Indirect Multi-species Kit (IDvet™, Montpellier, France), following the manufacturer's instructions. Sensitivity and specificity of this assay has shown to be 100% (IDvet™, according to the manufacturer internal validation report). Briefly, sample-to-positive control (S/P) ratio in each serum was calculated according to the formula provided: S/P =

(OD_{450} sample – OD_{450} NC)/(OD_{450} PC – OD_{450} NC); where OD_{450} sample = optical density of the sample, OD450 NC = optical density of the negative control and OD_{450} PC = optical density of the positive control. Results were expressed as an index (S/P x 100). Indices were stratified as 4 different rising categories. Samples with S/P indices <40% were considered negative, samples with S/P indices between 40 and 50% were considered doubtful, samples with S/P indices between 50 and 80% were considered low positive, and samples with S/P indices >80% were considered strong positive. Doubtful samples were retested and if resulting doubtful, considered as negative. Obtained data were used to calculate NUTS II-specific seroprevalence values. Exact binomial 95% confidence intervals (CI) were established for proportions.

Results and discussion

The presence of anti-*C. burnetii* antibodies was found in 122 sheep, which represents a 11.4% (95% CI 9.6–13.5%) seroprevalence of IgG anti-*C. burnetii* in sheep of Portugal. Of these 122 positive sheep, 53 (43.4%, 95% CI 34.5–52.7%) were considered low positive and 69 (56.6%, 95% CI 47.3-65.5%) considered strong positive. Regarding the distributions according to regions, anti-*C. burnetii* were found in 18 of 161 sheep of the North of Portugal (11.2%, 95% CI 6.8-17.1%), of which 7 (38.9%; 95% CI 17.3-64.3%) were low positive and 11 (61.1%, 95% CI 35.7-82.7%) were strong positive; in 44 of 246 sheep of the Centre of Portugal (17.9%, 95% CI 13.3–23.3%), of which 13 (29.5%, 95% CI 16.8-45.2%) were low positive and 31 (70.5%, 95% CI 54.8-83.2%) were strong positive; in none of the 21 sheep from Lisboa and Vale do Tejo (0%, 95% CI 0.0-0.0%); in 59 of the 616 sheep of Alentejo (9.6%, 95% CI 7.4-11.9%), of which 32 (54.2% , 95% CI 40.8-67.3%) were low positive and 27 (45.8%, 95% CI 32.7-59.2%) were strong positive; and in only 1 of the 24 sheep of Algarve (4.2%, 95% CI 0.1–21.1%), considered a low positive (100%, 95% CI 2.5-100%) (Table 3.1).

The seroprevalence found in Portugal, even considering individual regions, seems to be within the low ranges when comparing with the seroprevalence observed among sheep in the neighboring country of Spain, reported to be from 9.8% in the Basque country (northern country) to approximately 30% in the islands of Canarias, considered a highly endemic region (Gárcia-Pérez *et al*., 2009; Rodriguez *et al*., 2010; Ruiz-Fons *et al*., 2010; Fernandez-Aguillar *et al*., 2016; Bolaños-Rivero *et al*., 2017). In fact, seroprevalence in sheep is estimated to be around 15–20% in many countries of the world (Guatteo *et al*., 2011), which confirms that sheep of Portugal showed relatively low Q fever seroprevalence.

Table 3.1. Screening for anti-*C. burnetii* IgG antibodies in 1,068 sheep of all 5 regions of continental Portugal.

	Anti-*C. burnetii* low pos/region total pos: no. (%; CI)	**Anti-*C. burnetii* strong pos/region total pos: no. (%; CI)**	**Anti-*C. burnetii* pos/total: no. (%; CI)**
Region			
North	7/18 (38.9; 17.3-64.3)	11/18 (61.1; 35.7-82.7)	18/161 (11.2; 6.8-17.1)
Centre	13/44 (29.6; 16.8-45.2)	31/44 (70.4; 54.8-83.2)	44/246 (17.9; 13.3-23.3)
Lisboa and Vale do Tejo	0/0 (0.0; 0.0-0.0)	0/0 (0.0; 0.0-0.0)	0/21 (0.0; 0.0-0.0)
Alentejo	32/59 (54.2; 40.8-67.3)	27/59 (45.8; 32.7-59.2)	59/616 (9.6; 7.4-11.9)
Algarve	1/1 (100; 2.5-100)	0/1 (0; 0-97.5)	1/24 (4.2; 0.1-21.1)
Total	53/122 (43.4; 34.5-52.7)	69/122 (56.6; 47.3-65.5)	122/1068 (11.4; 9.6-13.5)

*CI: 95% confidence interval.

When comparing anti-*C. burnetii* presence in sheep according to NUTS II distribution, a higher seroprevalence in the Centre region can be observed when comparing to the other regions of Portugal (Figure 3.1). Unlike the regions southern to the Centre region (Alentejo, Lisboa and Vale do Tejo and Algarve) that have vast flat plains and low density extensive husbandry systems, the North region has a highly irregular mountainous terrain (favoring intensive husbandry) and the Centre has a mixture of hills and mountains that transition to plains to the south (Tibério & Dinis, 2014). This terrain profile shifts from mountainous to plains in the Centre region favor semi-extensive husbandry systems, where sheep are housed during the night and are allowed movement during the day (Fraga *et al*., 2014; Tibério & Dinis, 2014). The semi-extensive husbandry that allow use of common pasture by sheep during the day can favour infection by spore like forms that are deposited in the soil and have a long survival time, helping explain the increased anti-*C. burnetii* seroprevalence in this region. Also from a climatic standpoint the country presents a relatively large set of mesoclimates, spanning from dryer in the Southern regions, to more humid and windy in the north and Centre regions (Santos *et al*., 2012). This windy climate in the Centre region may also favour *C. burnetii* airborne dispersion and transmission in the outdoor environment and help explain the increased seroprevalence in this region, when compared to the remaining regions of Portugal. To this moment only one sheep seroprevalence study has been performed in Portugal, collecting blood from 2011 (Anastácio *et al*., 2013). In this study, the global individual seroprevalence was 8.6% and animals belonged from the Centre region of Portugal which is lower that the seroprevalence detected in this study for that region and may indicate an increasing circulation of *C. burnetii*.

However, caution must be taken in this comparison since different Enzyme Immunoassays were used as well as different sampling designs.

Figure 3.1. Seroprevalence of IgG Anti-*C. burnetii* in sheep of Portugal. Anti-*C. burnetii* presence in sheep according to NUTS II distribution. Centre region of Portugal shows a higher in comparision with the other portuguese regions (North, Lisboa and Vale do Tejo, Alentejo and Algarve).

In conclusion, this is the first study nationwide seroepidemiologic survey Q fever in sheep in Portugal also profiling the distribution of seropositive animals according to regions. Although seroprevalence seems low, the agent appears to be distributed across the country hence alerts for the possibility of zoonotic transmission have to be made. Moreover, sheep traditional farming is widely present in Portugal and is part of the cultural and gastronomical background of the country. This close proximity to small ruminants may contribute to the zoonotic transfer to humans. Although preliminary results from this study show relatively low Q fever seroprevalence in Portugal, there is the need to provide a clearer understanding of *C. burnetii* epidemiology in Portugal. Implementing monitoring programs on sentinel herds may help prevent or mitigate the effects of potential epidemics.

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CHAPTER 4 - PROSPECTIVE SEROSURVEY OF *COXIELLA BURNETII* IN SELECTED SHEEP OF PORTUGAL

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Introduction

Q fever is a zoonotic disease, prevalent in most places in the world (Angelakis & Raoult, 2010). It is a zoonosis caused by *Coxiella burnetii*, a small, Gram-negative, nonmotile, obligate intracellular bacterium with a high infectivity capacity (Mori *et al*., 2018). Transmission in humans is mainly accomplished through inhalation of contaminated aerosols (Angelakis & Raoult, 2010) but, occasionally, infection can occur through the digestive tract, percutaneous exposure, transfusion or sexual intercourse (Angelakis & Raoult, 2010; Georgiev *et al*., 2013; Mori *et al*., 2018;). This illness is associated with a wide clinical spectrum, and infection may lead to asymptomatic seroconversion, acute disease (ranging from a flu-like syndrome to severe pneumonia requiring intensive care), chronic infection (manifesting mainly as endocarditis) and even a fatal outcome (Mori *et al*., 2018).

The most commonly identified sources of human infection are farm animals, such as cattle, goats, and sheep, and infected animals shed *C. burnetii* in urine, feces, milk, and birth products, in particular. In sheep, the most important clinical presentations of Q fever are abortion and stillbirth (Van den Brom *et al*., 2015). Abortion occurs most frequently with no preceding clinical symptoms, at the end of pregnancy (Arricau-Bouvery & Rodolakis, 2005). In this study, a prospective serosurvey was performed to study *C. burnetii* circulation in a population of sheep in the central region of Portugal, where the economic and social impact have a strong dependency on local produce, namely sheep cheese of the brand "Serra da Estrela".

Materials and methods

In the present study, the geographical focus was the Serra da Estrela Mountain, central region of Portugal, under the influence of the National Association of Serra da Estrela Sheep breed - ANCOSE (40º 26´8.84´´ North, 51º 59.94´´ West), where sheep from the autochthonous breed (breed Serra da Estrela) are located. Only Serra da Estrela autochthonous breed were selected given their geographical restriction to the region and mobility restraints (sheep are housed during the night and are allowed movement uniquely on the farm premises). This provides a clear picture on *C. burnetii* circulation in sheep of the central part of Portugal.

A sample size of 168 was calculated assuming an a priori 10% anti-*C. burnetii* seroprevalence (Anastácio *et al*., 2013) a confidence in the estimate of 95%, a maximum allowable error in the prevalence of 5%, a sheep Serra da Estrela breed size of 70,000 (based on the latest animal census data; www.ancose.com), and an oversampling of 20% to account for possible losses (deaths, sales and trades) (WINPEPI updated, version 11.35). Blood from 168 sheep was collected in January/February 2015 and again in January/February 2016, in order to study both the dissemination/circulation and also the emergence of *C. burnetii* in the region. The sampling frame consisted of sheep (N=4) aging more than 6 months, randomly selected from each of the 42 Serra da Estrela breed official farms, located in 9 municipalities of the geographical coverage of this breed (Seia, Gouveia, Celorico da Beira, Fornos de Algodres, Mangualde, Carregal do Sal, Arganil, Oliveira do Hospital and Tábua) (Figure 4.1).

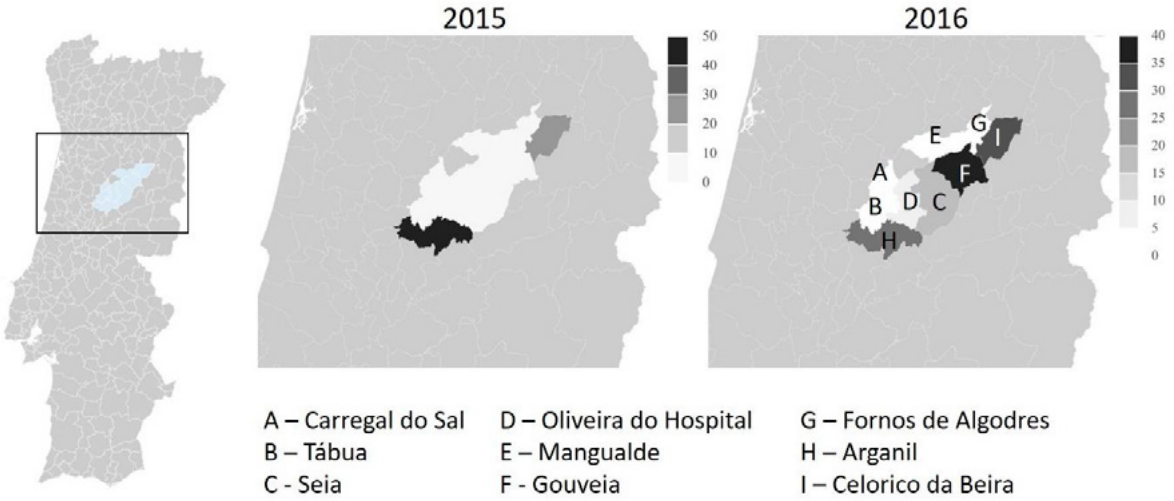

Figure 4.1. Location of the 9 municipialities in the central region of Portugal, from where the 42 samples of Serra da Estrela official breed were randomly selected.

Blood samples were collected by venipuncture of the jugular vein. Blood tubes were kept in ice and transferred immediately to the laboratory at the Department of Zootechnics, Rural Engineering and Veterinary, Agrarian Superior School, Polytechnic Institute of Viseu. The collected blood samples were centrifuged at 1800×*g* for 10 min and the sera were separated and frozen at − 20 °C until analysis.

Individual blood serum samples were used to detect *C. burnetii* IgG antibodies, using a commercial indirect ELISA, ID Screen Q Fever Indirect Multi-Species Kit (IDvet™, Montpellier, France), according to the manufacturer's instructions. Both the sensitivity and specificity of this ELISA have been shown to be 100% (IDvet™, internal validation report).

The sample-to-positive control (S/P) ratio in each serum was calculated according to the formula provided: S/P = (OD_{450} sample – OD_{450} NC)/ (OD_{450} PC – OD_{450} NC); where OD_{450} sample = optical density of the sample, OD_{450} NC = optical density of the negative control and OD_{450} PC = optical density of the positive control. Results were expressed as an index (S/P x 100). In accordance with the manufacturer, indices were categorized into 4 different rising categories. Samples with S/P indices < 40% were considered negative, samples with S/P indices between 40 and 50% were considered doubtful, samples with S/P indices between 50 and 80% were considered positive, and samples with S/P indices > 80% were considered strong positive. Doubtful samples were retested and if resulting doubtful, considered as negative (Seo *et al*., 2017). Data obtained from the analysis of sera by ELISA were used to calculate population and geographic (municipality)-specific seroprevalence values. Chi-square test was used to study differences between groups (GraphPad Prism version 5.04; GraphPad Software Inc., La Jolla, CA, USA). A p value < 0.05 was considered statistically significant.

Results and discussion

All selected Serra da Estrela breed official farms (N=42), of the 9 municipalities of the geographical coverage of this breed, were visited and agreed to participate in the study. The total of 168 animals were sampled both in 2015 and 2016 and sera were tested for IgG anti-*C. burnetii*. Of the 168 sera from the 2015 sample collection, 13 animals tested positive for IgG anti-*C. burnetii* (of which 4 were positive and 9 were strongly positive), showing a 2015 population seroprevalence of 7.7 %). From the total of 168 sera of the 2016 sample collection, 29 tested positive for IgG anti-*C. burnetii* (of which 16 were positive and 13 were strongly positive), showing a 2016 population seroprevalence of 17.3%. Anti-*C. burnetii* seroprevalence

differences in the 2015 and 2016 samplings showed to be statistically significant (p = 0.008) (Table 4.1).

Table 4.1. Screening for anti-*C. burnetii* IgG antibodies in selected sheep, in 2015 and 2016.

	2015	2016	
	C. burnetii IgG positives Total/% (95% CI)	*C. burnetii* IgG positives no. / % (95% CI)	**P value**
Total	168/7.74% (4.2-12.9)	168/17.26% (11.9-23.8)	0.008*
Region (Municipality)			
Carregal do Sal	12/0% (0.0-0.0)	12/0% (0.0-0.0)	-
Tábua	4/0% (0.0-0.0)	4/0% (0.0-0.0)	-
Seia	36/5.55% (0.7-18.7)	36/19.44% (8.2-36.0)	0.074
Oliveira do Hospital	32/3.12% (0.1-16.2)	32/9.37% (2.0-25.0)	0.301
Mangualde	8/0% (0.0-0.0)	8/0% (0.0-0.0)	-
Gouveia	20/5.0% (0.1-24.9)	20/40.0% (19.1-63.9)	0.008*
Fornos de Algodres	20/0% (0.0-0.0)	20/0% (0.0-0.0)	-
Arganil	4/50.0% (6.8-93.2)	4/25.0%% (0.6-80.6)	0.465
Celorico da Beira	32/21.87% (9.3-40.0)	32/31.25% (16.1-50.0)	0.395

* p value < 0.05;

Within the 2015 cohort, of the 155 initially anti-*C. burnetii* seronegative animals, 17 (10.9%) have seroconverted by 2016. Of the 13 initially anti-*C. burnetii* seropositive animals, 12 (92.3%) have maintained their immunological status regarding *C. burnetii* and 1 (7.7%) became seronegative.

Taking into account geographic location, a distinction between endemic and non-endemic municipalities can be observed. Sheep from 4 municipalities (Carregal do Sal, Tábua, Mangualde and Fornos de Algodres) were all negative in both 2015 and 2016. On the other hand, all but one of the remaining municipalities had farms where sheep increased anti-*C. burnetii* seropositivity. In fact, in Gouveia, a statistically significant increase (p = 0.008) was observed between anti-*C. burnetii* seropositivity in 2015 (5.0%; 95% confidence interval: 0.1-24.9%) and 2016 (40.0%; 95% confidence interval: 19.1-63.9%).

The present research studied the circulation of Q fever in sentinel sheep during 2 consecutive years, 2015 and 2016. On the first screening (2015), 13 (7.7 %) of the 168 animals tested positive for IgG anti-*C. burnetii*. On the second screening (2016), 29 (17.3%) of the same 168 animals tested positive for IgG anti-*C. burnetii*, showing a statistically significant increase in anti-*C. burnetii* seroprevalence in sheep herds of Central Portugal. Interestingly, in a recent

study in Portugal, exposure to *C. burnetii* in sheep and goats were evaluated showing a global individual seroprevalence of 9.6%, being higher in goats (10.4%) than in sheep (8.6%) (Anastácio *et al*., 2013). Our results show higher seroprevalences than those, supporting the notion that Q fever is increasing its occurrence in Central Portugal (Figure 4.1). Results also confirm a high level of exposure to *C. burnetii* in small ruminant herds of Central Portugal.

In general, little is known regarding Q fever status in animals in Europe, however European countries have reported, in general, decreasing number of human Q fever cases, whereas in Portugal the numbers have remained unchanged which has given rise to alert within Public Health stakeholders and decision makers (DGS, 2015; ECDC, 2014). Sheep traditional backyard farming has been widely present in Portugal and is part of the cultural and gastronomical background of the country. This close proximity to small ruminants may contribute to the endemicity of Q fever in Portugal (Alves *et al*., 2016). Additionally, an increase in *C. burnetii* seroprevalence in ruminant herds is considered to be a useful index for studying their occurrence in the human population (Mori *et al*, 2018). Interestingly, during the same time span of the present study, a recent case series described 7 cases of acute Q fever admitted in a Portuguese University Hospital, where authors have been able to retrieve detailed epidemiological data of all cases, mostly regarding contact or close proximity to sheep, cattle or goats, suggesting aerosol inhalation as the main route of transmission and a strong relationship between disease and occupational exposure (Alves *et al*., 2016). The present study is the first in Portugal to provide prospective data on the serological status of a sheep cohort of Portugal and showing an increase in the occurrence of Q fever. There is the need to provide clearer understanding of Q fever epidemiology in Portugal, ideally by implementing monitoring programs on sentinel herds.

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CHAPTER 5 - OUTBREAKS OF ABORTIONS BY *COXIELLA BURNETII* IN SMALL RUMINANT FLOCKS AND A LONGITUDINAL SEROLOGICAL APPROACH ON ARCHIVED BULK TANK MILK SUGGEST Q FEVER EMERGENCE IN CENTRAL PORTUGAL

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Introduction

Q fever is a worldwide zoonotic infectious disease caused by *Coxiella burnetii* and ruminants, namely, cattle, sheep and goats are known to be the main reservoir for human infection, however ticks are also considered a common reservoir (Arricau-Bouvery & Rodolakis, 2005; Van den Brom *et al*., 2015; Angelakis & Raoult, 2010; Djerbib *et al*., 2018). *C. burnetii* infection in ruminants can result in epizootic abortions, which are often associated with vast bacteria shedding in birth fluids and placentas, significantly increasing the risk of disease spread (O´Neill *et al*., 2014; Filioussis *et al*., 2017). Human infections mainly occur in persons handling infected animals and their products but until recently, the zoonotic transfer of *C. burnetii* to the human population did not generate important alerts in both Veterinary and Human Public Health (Tselentis *et al*., 1995; Ergas *et al*., 2006).

In the last decade, a strong paradigm shift has occurred in the scientific community due to a major epidemic that has occurred in the general population in the Netherlands, resulting in 3 525 notified cases in humans and the subsequent national cull of carrying goat herds (van der Hoek *et al*., 2010). Abortion clusters in goat herds that started a few years earlier, as a consequence of the intensification of dairy goat production systems in the region, were initially suggested as the source of this large human epidemic (van der Hoek *et al*., 2010). This was later supported by the results of geospatial studies indicating an association between the human cases and the dairy goat farms (Schimmer *et al*., 2010). Since then, important work has been made to prevent the spread of *C. burnetii* from sheep or goats to humans, however these efforts have been hindered by the limitation of effective veterinary interventions in these small ruminants (Angelakis & Raoult, 2010).

Much is known on the role of sentinel animals in detecting risks to humans by providing early warning of an emerging infectious disease, being the particular case of sheep regarding *C. burnetii* (Mori *et al*., 2018). In particular, the important tradition behind primary production

of sheep in the centre of Portugal, mainly done at a small scale and in intimate contact with humans, can provide seroepidemiological studies with sentinel flocks and thus potentially mitigate *C. burnetii* shedding to the human population. The present study describes two epizooties of *C. burnetii* affecting sheep and goat flocks, and also provides the results of a 2-year prospective serosurvey in bulk-tank milk samples to assess *C. burnetii* circulation in a population of sheep living in close contact to the human population in Central Portugal.

Materials and methods

Outbreaks investigation

The first outbreak of abortions started on 15 november 2017 and lasted for 2 months in a sheep farm in the municipality of Mangualde (40.58633 North; -7.760661 West), district of Viseu, Central Portugal. The flock was not vaccinated to Q fever and had sheep from "Serra da Estrela" breed, the autochthonous breed of this region that produces the best sheep milk in Portugal. This milk is used for the highly valued and recognized worldwide, cheese "Serra da Estrela". There were 155 sheep in the flock, of which 100 were pregnant and 20 aborted. The first abortion occurred in 15 November 2017 and the last in 18 January 2018. Aborted foetuses (N=2) were taken and refrigerated until arriving at the Laboratory (within 24 h). The second outbreak of abortions started on 10 January 2018 and lasted for 3 weeks in a goat farm in the municipality of Aguiar da Beira (40.81443 North; -7.54440 West), also in the district of Viseu, Central Portugal, approximately 50 km distant from the first outbreak. The flock had goats from "Murciana" breed and was also not vaccinated to Q fever. There were 60 goats in the flock, all pregnant, of which 25 aborted. The first abortion occurred in 10 January 2018 and the last in 30 January 2018. Placenta (N=1) was taken and refrigerated until arriving at the Laboratory (within 24 h). Tissues from both outbreaks were tested for a panel of abortion pathogens, namely *Toxoplasma gondii*, Chlamydiaceae and *Coxiella burnetii*. DNA was extracted using NucleoSpin® Tissue kit, (Macherey Nagel, Duren, Germany), according to the manufacturers instructions. For pathogen genomic detection, 3 commercial real-time PCR probe assay kits were used, according to the manufacturers instructions (EXOone *Toxoplasma gondii* oneMIX Kit; EXOone Chlamydiaceae one MIX Kit; EXOone *Coxiella burnetii* oneMIX Kit, Zaragoça, Spain). All reactions were performed using a positive, a negative and an endogenous control (β-actin target).

A questionnaire was applied to the farm owners and families, as well as to the workers for symptoms fitting acute Q fever case definition (acute fever and one or more of the following: rigors, severe retrobulbar headache, acute hepatitis, pneumonia,) (CDC, 2009), during the period of the outbreaks and the following months, with the intervention and help of a human health team (a medical doctor and a nurse) and none fit the case definition.

To address *C. burnetii* spread the delivered questionnaires included queries on similar epizootic abortions in nearby farms during the period of the outbreaks and the following months. The farm owner from the first outbreak reported that a neighbouring sheep farm had experienced 5 to 7 abortions during the same period. Despite efforts we were not able to retrieve epidemiological data and samples from that sheep farm for analysis.

Bulk tank milk collection

The study geographical location was Estrela Mountain ("Serra da Estrela"), located in Central Portugal, where the National Association of Serra da Estrela Sheep breed - ANCOSE (Associação Nacional de Criadores de Ovinos da Serra da Estrela; http://www.ancose.com) is responsible for the administration of one of Portugal's autochthonous sheep breeds, the "Serra da Estrela". This breed provides for several European Union´s Protected Designation of Origin (PDO) products, being exclusively bred in this region and thus having residual animal movement due to its confined production to the farm premises. For the serological analysis to study *C. burnetii* emergence, samples from a previous study on Schmallenberg virus (data not published) were used. All registered sheep flocks (ANCOSE official, N=180) were invited to participate in this study, which required a bulk tank milk collection (one in January/February 2015, the other in January/February 2016). A total of 78 sheep milk farms from 46 parishes of 5 municipalities the centre region of Portugal (Celorico da Beira, Fornos de Algodres, Gouveia, Seia and Tábua) answered and accepted to participate (response rate= 43.3%). The farm where the 2017 abortion outbreak occurred did not participate. All farms provided a 2 ml bulk-milk sample both in January 2015 and in January 2016, which was swiftly transported to the laboratory at 4 °C. Samples were processed according to the manufacturers instructions with slight modifications (Chaintoutis *et al*., 2014). Briefly, bulk-milk samples were centrifuged at 1,000×*g* at 4 °C for 10 min. After centrifugation, the fat fraction was removed using a sterile spatula, and the remaining fraction was transferred to a 1.5 ml microcentrifuge tube and immediately frozen (-20 °C) until analysis.

Enzyme linked immunosorbent assay

Samples were tested for the presence of anti-*C. burnetii* IgG antibodies, using a commercial indirect ELISA, ID Screen Q Fever Indirect Multi-species Kit (IDvet™, Montpellier, France), following the manufacturer's instructions. The positive control of this assay is a pool of positive bovine sera (field infected, from France) and the assay has a sensitivity and a specificity of 100% (according to the manufacturer). For test interpretation, sample-to-positive control (S/P) ratio in each serum was calculated, according to the formula provided: S/P = (OD450 sample – OD450 NC)/ (OD450 PC – OD450 NC); where OD450 sample = optical density of the sample, OD450 NC = optical density of the negative control and OD450 PC = optical density of the positive control. Results were expressed as an index (S/P x 100). Indices stratified as 3 different rising categories. Samples with S/P indices <30% were considered negative, samples with S/P indices between 30 and 40% were considered doubtful, samples with S/P indices >40% were considered positive. Doubtful samples were retested and if resulting doubtful, considered as negative. Obtained data were used to calculate NUTS II-specific seroprevalence values and differences between NUTS II-specific seroprevalence, in 2015 and 2016, were evaluated by Fisher's exact test, using GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA) and considered statistically significant if $p < 0.05$. Exact binomial 95% confidence intervals (CI) were established for proportions.

Results and discussion

Results from the qPCR screening in the aborted foetuses and placenta showed that samples were negative for *T. gondii* and *Chlamydia*, and positive for *C. burnetii*, strongly suggesting that the abortion outbreaks that affected both farms were due to Q fever. All farm owners, family members and workers replied to the questionnaire however none fitted acute Q fever case definition. Nonetheless, the genotypes involved in the outbreaks could have been associated to low virulence in humans, and asymptomatic infections could have occurred (Van Schaik *et al*., 2013)

As for the serological survey, anti-*C. burnetii* antibodies were found in both years. From the 2015 sampling, eight (10.2%; 95%CI: 4.5-19.2%) of the 78 bulk tank milk samples presented IgG antibodies against *C. burnetii*, while from the 2016 sampling, 20 (25.6%; 95%CI: 16.4-36.8%) of the total 78 bulk tank milk samples were positive. Of the eight initially (2015)

positive farms, five (62.5%) maintained their seropositive status regarding Q fever and three (37.5%) became seronegative. Of the anti-*C. burnetii* seronegative farms from 2015, 15 (25.8%) had seroconverted by 2016. This steep increase in the number of anti-*C. burnetii* farms between the 2015 and 2016 collections showed to be statistically significant (p = 0.020).

Regarding the distribution of IgG anti-*C. burnetii* positive bulk tank milk samples, according to geographical location (Table 5.1), in 2015 and 2016, an increase was observed in all but one municipality, namely in Seia (8.7% [CI: 1.1-28.0%] versus 21.7% [CI: 7.5-43.7%]), Gouveia (18.8% [CI: 4.0-45.6%] versus 31.3% [CI: 11.0-58.7%]), Celorico da Beira (0% [CI: 0.0-0.0%] versus 26.7% [CI: 7.8-55.1%]) and Tábua (12.5% [CI: 1.6-38.3%] *versus* 31.3% [CI: 11.1-58.7%]). Only Fornos de Algodres showed no change in the number of IgG anti-*C. burnetii* positive bulk tank milk samples (12.5% [CI: 0.3-52.7%]). The steep increase of IgG anti-*C. burnetii* bulk tank milk samples across the central region of Portugal is strongly suggestive of Q fever emergence in Central Portugal.

Table 5.1. Distribution of IgG anti-*C. burnetii* positive bulk tank milk samples, according to geographical location. Generaly there was an increase in Ig G in two milking seasons in a row (2015/2016), namely in Seia municipiality.

Municipality	**2015**	**2016**	**P value**
	C.burnetii IgG positive/Total: no. (%; CI)	*C. burneti* IgG positives/Total: no. (%; CI)	
Seia	2/23 (8.7; 1.1-28.0)	5/23 (21.7;7.5-43.7)	0.414
Gouveia	3/16 (18.8;4.0-45.6)	5/16 (31.3;11.0-58.7)	0.685
Fornos de Algodres	1/8 (12.5;0.3-52.7)	1/8 (12.5;0.3-52.7)	†
Celorico da Beira	0/15 (0.0;0.0-0.0)	4/15 (26.7;7.8-55.1)	0.099
Tábua	2/16 (12.5; 1.6-38.3)	5/16 (31.3; 11.1-58.7)	0.394
Total	8/78 (10.2; 4.5-19.2)	20/78 (25.8; 16.4-36.8)	0.021 *

CI: 95% confidence interval; †: not determined; * p value < 0.05

ELISA on bulk tank milk samples have shown in the past to be a valuable matrix for the screening of *C. burnetii* infection within animals in lactation by providing information about the exposure to *C. burnetii* (Guatteo *et al.*, 2007, van den Brom *et al.*, 2012) and producing comparable results to those obtained in serum samples due to the immunoglobulin transfer from blood to milk (Nielsen *et al.*, 2011). Interestingly another study in Portugal has detected IgG anti-*C. burnetii* in milk (Anastácio *et al.*, 2016). Authors have collected 39 bulk milk samples from sheep in the same region, from 2009 to 2013, and showed that 51.3% flocks had positive samples (Anastácio *et al.*, 2016). Although authors have obtained a much higher prevalence of positive bulk tank milk samples, they have tested samples for the presence of specific anti-*C.*

burnetii antibodies using a different commercial ELISA (LSIVET Ruminant Milk/Serum Q Fever; Laboratoire Service International, Lissieu, France). Thus, we find difficult to compare values as a different assay with distinct sensitivity/specificity has been used in the present study.

While considered to remain unaltered, Q fever prevalence in the human population of Portugal is conflicting with European data that shows a clear increase of cases (DGS, 2015; ECDC, 2014). Although little is known regarding Q fever in Portugal, a few recent case reports in humans have been linked to animals, highlighting the concern for zoonotic transfer from ruminants (Alves *et al*., 2016). It is therefore of the upmost importance to provide results on the circulation of *C. burnetii* in sheep, so as to implement measures on animal health and control the disease spread to the human population.

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CHAPTER 6 - CONCLUSIONS AND FUTURE WORK

During the attainment of this doctoral study, valuable data has been obtained that point to, not only endemicity, but also to the recent emergence of Q fever in small ruminants. This work was based on the fact that during the last years of the authors' intensive practical clinical work in small ruminants' health, the number of cases of animal Q fever has grown, which has ultimately lead to considerable impacts to public health as well as at a socioeconomic level of the farm itself. In particular, during the last phase of these studies, 2 cases of human Q fever (livestock farmers) have occurred with serious impact on their health (personnal communication).

The main objective of this work was to evaluate the circulation of Q fever in sheep from continental Portugal, subsequently limiting the geographic focus to the central region of Portugal, more specifically the region of Serra da Estrela, characterized by a very traditional production system, based on intimate contact between farmer and animals. This allowed to provide a detailed analysis of the effective presence and circulation of this agent in Serra da Estrela sheep.

Thus, the major conclusions of the present study are:

1. *Coxiella burnetii* is widespread across Portuguese territory, as shown by this first nationwide seroepidemiologic study on Q fever in Portugal - Anti-*C. burnetii* IgG seroprevalence in sheep of Portugal was of 11.4%;

2. A higher anti-*C. burnetii* IgG seroprevalence was found in the centre region, when compared to the other NUTS II regions of continental Portugal;

3. A statistically significant increase in anti-*C. burnetii* IgG seroprevalence was found in sheep of central Portugal (7.7%, in 2015 *versus* 17.3%, in 2016).

4. *Coxiella burnetii* was found to be responsible for 2 abortion outbreaks, in late 2017 and in early 2018.

5. A statistically significant increase in anti-*C. burnetii* IgG seroprevalence in bulk tank milk samples was found in sheep farms of central Portugal (10.2%, in 2015 *versus* 25.6%, in 2016).

6. Combined data from this study, that included evidence for the increase in *C. burnetii* exposure in animals and herds of central Portugal, and also showed that *C. burnetii* is responsible for epizootics, provides a strong support for the notion of Q fever emergence in Central Portugal. As sheep traditional farming is widely present in Portugal with a strong impact in the cultural and gastronomical history of the country, and given the close proximity to small ruminants that may contribute to the zoonotic transfer to humans, public health warnings should be addressed

Future Work

Based on the present work, future research is needed to:

1. Clarify the understanding of *C. burnetii* epidemiology in Portugal, having in mind the close relationship between the animal-human interface, characteristic of the traditional central Portugal milk production system. This is currently being performed by the author of this thesis in a case-control serological survey in sheep Shepherds (occupationaly exposed) *versus* the general population (controls). Risk factors for seropositivity in Shepherds will be assessed;

2. Estimate Human Q fever seroprevalence in this region, establishing the main occupational hazards defining the main sources of infections and therefore implementing preventive and control measures (work in progress);

3. Given the current results that point to low Q fever seroprevalence in sheep, further studies should be focused on the possibility of natural predisposition in Serra da Estrela breed;

4. Evaluate if the specific and typical climatic conditions of Serra da Estrela contributes to the dissemination of the agent;

5. Determine and quantify the diret effect on production (milk, meet and cheese) and, consequently, the impact at socioeconomical level;

6. Determine the possibility of shepherd dogs acting as reservoirs in the transmission and dissemination of *C. burnetii* to portuguese sheep.

In short, it is important to point out that this doctoral work, represents just the begining of the study of *C. burnetii* infection in Portugal and much is yet still to know. Farmers continue to face heavy losses due to abortion outbreaks often attributed to unknown causes. Continuing education of farmers may pose as a protective measure for Q fever infection, as increased knowledge can provide tools for disease control. As such, efforts should be addressed by the national authorities to implement educational workshops in this target group.

ANNEXES

ANNEX A - Cruz, R., Esteves, F., Vasconcelos-Nóbrega, C., Santos, C., Ferreira, A.S., Mega, C., Coelho, A.C., Vala, H., Mesquita, J.R. A nationwide seroepidemiologic study on Q fever antibodies in sheep of Portugal. Accepted in to *Vector-Borne and Zoonotic Diseases*

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A nationwide seroepidemiologic study on Q fever antibodies in sheep of Portugal

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Keyword:	Coxiella, Zoonosis
Manuscript Keywords (Search Terms):	q fever, seroprevalence, sheep, Portugal
Abstract:	Introduction. Q fever is an almost global zoonotic disease caused by Coxiella burnetii. Human infections can produce acute and chronic disease that can lead to abortions and stillbirths in pregnant women, usually infected by the inhalation of C. burnetii-contaminated aerosols or through consumption of contaminated products. Sheep are one of the primary animal reservoirs with disease being associated with vast shedding of bacteria in placentas, faeces, milk, and birth fluids. Although almost neglected in the past, recent outbreaks of sheep origin have alerted the public and the scientific community. Materials and Methods. An epidemiologic survey to estimate the seroprevalence of Q fever antibodies was performed in a representative number of sheep of all regions of continental Portugal (n=1,068), using a commercial ELISA (ID Screen Q Fever Indirect Multi-species Kit; IDvet™, Montpellier, France). Results and Discussion. An anti-C. burnetii seroprevalence of 11.4% (95% CI 11.0%–15.0%) was found, with a clear distinction between high (north) and low (south) density sheep farming regions in the territory. Sheep

	traditional farming is widely present in Portugal and is part of the cultural and gastronomical background of the country. This close proximity to small ruminants may contribute to the zoonotic transfer to humans.

A nationwide seroepidemiologic study on Q fever antibodies in sheep of Portugal

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Introduction

Q fever is an almost global zoonotic disease caused by *Coxiella burnetii*, which is able to infect several animal species and of which cattle, sheep and goats are the primary animal reservoirs (Van der Brom et al., 2015; Khor et al., 2018). Q fever in humans can produce acute disease that usually leads to pneumonia, hepatitis and self-limited illness (Guatteo et al., 2011). Chronicity is associated to endocarditis in immuno-compromised individuals or abortions and stillbirths in pregnant women (Angelakis and Raoult, 2010; Dabaja et al., 2018). Human infection occurs by *C. burnetii*-contaminated aerosols of animal origin that are inhaled (Guatteo et al., 2011). Although rare, oral transmission by consumption of contaminated dairy products is also possible as is also sexual and vertical transmission (Kruszewska and Tylewska-Wierzbanowska, 1997; Milazzo et al., 2001). Though almost neglected in the past, recent outbreaks have alerted the public and the scientific community (van der Hoek et al., 2010). In the Netherlands, a large outbreak affecting 2,357 human cases that lasted several years was notified in 2009 and linked to abortion waves on dairy goat flocks (van der Hoek et al., 2010). In fact, Q fever in small ruminants (sheep and goats) is associated with vast shedding of bacteria in placentas, faeces, milk, and birth fluids, and usually manifest as abortions, which greatly increases the risk of disease spread (O´Neill et al., 2014;: Filioussis et al., 2017).

In the Iberic Peninsula, very few regional studies on Q fever in ruminants have been made (Anastácio et al., 2013; Cumbassá et al., 2015), and no serologic survey in the full continental territory Portugal has ever been done. Hence, an epidemiologic survey was set up to estimate the seroprevalence of Q fever antibodies in sheep of all regions of Portugal.

Materials and methods

Sample size

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This study used samples collected in 2014 for a previous study (Esteves et al., 2016). Sample size was calculated considering the following a priori assumptions: population size of 2,092,175 sheep (IFAP, 2018), an expected *C. burnetii* seroprevalence of 50% (allowing for the largest sample possible), an absolute error of 3%, and a 95% confidence level (DESCRIBE package, WINPEPI updated, version 11.43). A calculated sample size of 1,068 sheep was obtained and a stratified random sampling design was obtained by categorizing according to the Nomenclature of Territorial Units for Statistics level II (NUTS II) regions (North, Centre, Lisboa and Vale do Tejo, Alentejo and Algarve), to reduce possible confounders associated to the heterogeneous geographical distribution of sheep in Portugal. The 2014 official animal census data of Portugal reported the following distribution on sheep head according to region: 315,506 sheep are located in the North (15.1%), 481,017 in the Centre (23%), 42,861 in Lisboa and Vale do Tejo (2%), 1,206,876 in Alentejo (57.7%) and 45,915 in Algarve (2.2%) (IFAP, 2018). As no herd level statistics are available, to better represent the distribution in the 5 regions of Continental Portugal, samples from 4 farms spread within each region (at the North, South, East and West) were selected for screening.

ELISA screening

Blood samples had been collected aseptically by jugular vein puncture into sterile labelled Vacutainer tubes without additives (BD Vacutainer Systems, Plymouth, UK). Samples were kept cold during transport to the laboratory. Sera was removed after centrifugation and stored at −20 °C until analysis. All sera belonged from healthy female sheep with 6 months to 10 years of age (average age of 5 years) born in Portugal, randomly selected upon the moment of the official brucellosis control. In total, 161 samples from the North, 246 from the Centre, 21 from Lisboa and Vale do Tejo, 616 from Alentejo and 24 from Algarve were selected. Sera were tested for the presence of anti-*C. burnetii* IgG antibodies using a commercial indirect ELISA, ID Screen Q Fever Indirect Multi-species Kit (IDvet™, Montpellier, France), following the manufacturer's instructions. Sensitivity and specificity of this assay has shown to be 100%

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(IDvet™, according to the manufacturer internal validation report). Briefly, sample-to-positive control (S/P) ratio in each serum was calculated according to the formula provided: S/P = (OD_{450} sample – OD_{450} NC)/(OD_{450} PC – OD_{450} NC); where OD_{450} *sample* = optical density of the sample, OD_{450} *NC* = optical density of the negative control and OD_{450} *PC* = optical density of the positive control. Results were expressed as an index (S/P x 100). Indices stratified as 4 different rising categories. Samples with S/P indices <40% were considered negative, samples with S/P indices between 40 and 50% were considered doubtful, samples with S/P indices between 50 and 80% were considered low positive, and samples with S/P indices >80% were considered strong positive. Doubtful samples were retested and if resulting doubtful, considered as negative. Obtained data were used to calculate NUTS II-specific seroprevalence values. Exact binomial 95% confidence intervals (CI) were established for proportions.

Results and discussion

The presence of anti-*C. burnetii* antibodies was found in 122 sheep, which represents a 11.4% (95% CI 9.6–13.5%) seroprevalence of IgG anti-*C. burnetii* in sheep of Portugal. Of these 122 positive sheep, 53 (43.4%, 95% CI 34.5–52.7%) were considered low positive and 69 (56.6%, 95% CI 47.3-65.5%) considered strong positive. Regarding the distributions according to regions, anti-*C. burnetii* were found in 18 of 161 sheep of the North of Portugal (11.2%, 95% CI 6.8-17.1%), of which 7 (38.9%; 95% CI 17.3-64.3%) were low positive and 11 (61.1%, 95% CI 35.7-82.7%) were strong positive; in 44 of 246 sheep of the Centre of Portugal (17.9%, 95% CI 13.3–23.3%), of which 13 (29.5%, 95% CI 16.8-45.2%) were low positive and 31 (70.5%, 95% CI 54.8-83.2%) were strong positive; in none of the 21 sheep from Lisboa and Vale do Tejo (0%, 95% CI 0.0-0.0%); in 59 of the 616 sheep of Alentejo (9.6%, 95% CI 7.4-11.9%), of which 32 (54.2% , 95% CI 40.8-67.3%) were low positive and 27 (45.8%, 95% CI 32.7-59.2%) were strong positive; and in only 1 of the 24 sheep of Algarve (4.2%, 95% CI 0.1–21.1%), considered a low positive (100%, 95% CI 2.5-100%) (Table 1; Figure 1). The seroprevalence found in Portugal,

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even considering individual regions, seems to be within the low ranges when comparing with the seroprevalence observed among sheep in the neighboring country of Spain, reported to be from 9.8% in the Basque country (northern country) to approximately 30% in the islands of Canarias, considered a highly endemic region (Gárcia-Pérez et al., 2009; Rodriguez et al., 2010; Ruiz-Fons et al., 2010; Fernandez-Aguillar et al., 2016; Bolaños-Rivero et al., 2017). In fact seroprevalence in sheep is estimated to be around 15–20% in many countries of the world (Guatteo et al., 2011), which confirms that sheep of Portugal showed relatively low Q fever seroprevalence.

When comparing anti-*C. burnetii* presence in sheep according to NUTS II distribution, a generally higher seroprevalence in the Southern (Alentejo and Algarve) versus the Northern (North and Centre) regions of Portugal can be observed. Interestingly, a clear difference in the types of husbandry systems between Southern/Northern Portugal is also present, with typical low density farming in the Southern part of Portugal, and high density farming in the Northern (Tibério and Dinis, 2014). Also from a climatic standpoint the country presents a relatively large set of mesoclimates, spanning from dryer in the southern regions, to more humid in the northern regions (Santos et al., 2012). Topography and soils are also different, ranging from extended flatland areas in the southern regions to steep mountainous in the northern regions, each with different crop selections (Fraga et al., 2014). Both the fewer animal density (less likelihood for horizontal transmission) and the high temperatures and low air humidity (reduced environmental persistency of *C. burnetii*) in the Southern regions compared to the Northern can help explain the observed differences in anti-*C. burnetii*.

To this moment only one sheep seroprevalence study has been performed in Portugal, collecting blood from 2011 (Anastácio et al., 2013). In this study, the global individual seroprevalence was 8.6% and animals belonged from the Centre region of Portugal which is lower that the seroprevalence detected in this study for that region and may indicate an

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increasing circulation of *C. burnetii*. However, caution must be taken in this comparison since different Enzyme Immunoassays were used as well as different sampling designs.

In conclusion, this is the first study nationwide seroepidemiologic survey Q fever in sheep in Portugal also profiling the distribution of seropositive animals according to regions. Although seroprevalence seems low, the agent seems to be distributed across the country hence alerts for the possibility of zoonotic transmission have to be made. Moreover, sheep traditional farming is widely present in Portugal and is part of the cultural and gastronomical background of the country. This close proximity to small ruminants may contribute to the zoonotic transfer to humans. Although preliminary results from this study show relatively low Q fever seroprevalence in Portugal, there is the need to provide a clearer understanding of *C. burnetii* epidemiology in Portugal. Implementing monitoring programs on sentinel herds may help prevent or mitigate the effects of potential epidemics.

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Author Disclosure Statement

Authors have no conflict of interest

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228 Figure 1. Percentages of anti-*C. burnetii* seropositive sheep in the 5 NUTS II regions of
229 Continental Portugal.

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Table 1. Screening for anti-*C. burnetii* IgG antibodies in 1,068 sheep of all 5 regions of continental Portugal

	Anti-*C. burnetti* low pos/region total pos: no. (%; CI)	Anti-*C. burnetti* strong pos/region total pos: no. (%; CI)	Anti-*C. burnetti* pos/total: no. (%; CI)
Region			
North	7/18 (38.9; 17.3-64.3)	11/18 (61.1; 35.7-82.7)	18/161 (11.2; 6.8-17.1)
Centre	13/44 (29.6; 16.8-45.2)	31/44 (70.4; 54.8-83.2)	44/246 (17.9; 13.3-23.3)
Lisboa and Vale do Tejo	0/0 (0.0; 0.0-0.0)	0/0 (0.0; 0.0-0.0)	0/21 (0.0; 0.0-0.0)
Alentejo	32/59 (54.2; 40.8-67.3)	27/59 (45.8; 32.7-59.2)	59/616 (9.6; 7.4-11.9)
Algarve	1/1 (100; 2.5-100)	0/1 (0; 0-97.5)	1/24 (4.2; 0.1-21.1)
Total	53/122 (43.4; 34.5-52.7)	69/122 (56.6; 47.3-65.5)	122/1068 (11.4; 9.6-13.5)

CI: 95% confidence interval

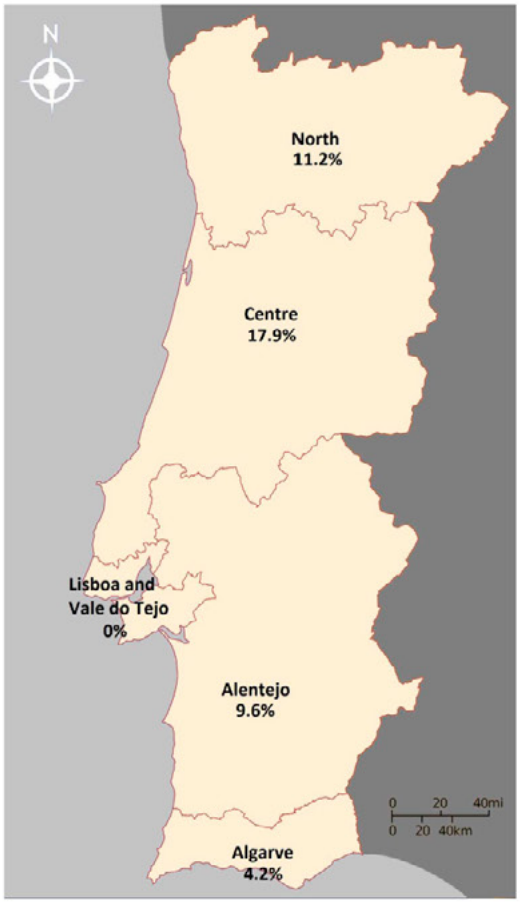

Figure 1

122x213mm (300 x 300 DPI)

ANNEX B - Cruz R, Esteves F, Vasconcelos-Nóbrega C, Santos C, Ferreira AS, Mega C, Coelho AC, Vala H, Mesquita JR. Prospective serosurvey of *Coxiella burnetii* antibodies in selected sheep of Portugal. Submitted to Ecohealth on 14/02/2018 (Ref: ECH-18-0026)

EcoHealth

Prospective serosurvey of Coxiella burnetii antibodies in selected sheep of Portugal

Journal:	*EcoHealth*
Manuscript ID	ECH-18-0026
Manuscript Type:	Short Communication
Keywords:	Q fever, emergence, Portugal

Springer

Abstract

Q fever is a zoonotic disease caused by *Coxiella burnetii* that is highly prevalent across the. In this study, a prospective serosurvey was performed to study *C. burnetii* circulation in a population of sheep in the central region of Portugal. Blood from a representative sample of 168 animals were drawn both in 2015 and 2016, and sera were tested for IgG anti-*C. burnetii* by EIA. Of the 2015 sample collection, 7.7% (13/168) animals tested positive for IgG anti-*C. burnetii* while of the 2016 collection 17.3% (29/168) tested positive, showing a statistically significant (p = 0.008) increase in anti-*C. burnetii* seroprevalence. Results support the notion that Q fever is emerging in central Portugal.

Key words

Q fever, *Coxiella burnetii*, Portugal, emergence

Wordcount: 1460

Text

Q fever is a zoonotic disease, prevalent in most places in the world (Angelakis & Raoult, 2010). It is a zoonosis caused by *Coxiella burnetii*, a small, Gram-negative, nonmotile, obligate intracellular bacterium with a high infectivity capacity (Mori et al, 2018). Transmission in humans is mainly accomplished through inhalation of contaminated aerosols (Angelakis & Raoult, 2010) but, occasionally, infection can occur through the digestive tract, percutaneous exposure, transfusion or sexual intercourse (Angelakis & Raoult, 2010; Mori et al, 2018; Georgiev et al, 2013). This illness is associated with a wide clinical spectrum, and infection may lead to asymptomatic seroconversion, acute disease (ranging from a flu-like syndrome to severe pneumonia requiring intensive care), chronic infection (manifesting mainly as endocarditis) and even a fatal outcome (Mori et al, 2018).

The most commonly identified sources of human infection are farm animals, such as cattle, goats, and sheep, and infected animals shed *C. burnetii* in urine, feces, milk, and birth products, in particular. In sheep, the most important clinical presentations of Q fever are abortion and stillbirth (Van den Brom et al, 2015). Abortion occurs most frequently with no preceding clinical symptoms, at the end of pregnancy (Arricau-Bouvery & Rodolakis, 2005). In this study, a prospective serosurvey was performed to study *C. burnetii* circulation in a population of sheep in the central region of Portugal, where the economic and social impact have a strong dependency on local produce, namely sheep cheese of the brand "Serra da Estrela".

In the present study, the geographical focus was the Serra da Estrela Mountain, central region of Portugal, under the influence of the National Association of Serra da Estrela Sheep breed - ANCOSE (40º 26'8.84'' North, 51º 59.94'' West), where sheep from the autochthonous breed (breed Serra da Estrela) are located. Only Serra da Estrela autochthonous breed were selected

given their geographical restriction to the region and mobility restraints (sheep are housed during the night and are allowed movement uniquely on the farm premises). This provides a clear picture on *C. burnetii* circulation in sheep of the central part of Portugal.

A sample size of 168 was calculated assuming an a priori 10% anti-*C. burnetii* seroprevalence (Anastácio et al, 2013) a confidence in the estimate of 95%, a maximum allowable error in the prevalence of 5%, a sheep Serra da Estrela breed size of 70,000 (based on the latest animal census data; www.ancose.com), and an oversampling of 20% to account for possible losses (deaths, sales and trades) (WINPEPI updated, version 11.35). Blood from 168 sheep was collected in January/February 2015 and again in January/February 2016, in order to study both the dissemination/circulation and also the emergence of *C. burnetii* in the region. The sampling frame consisted of sheep (N=4) aging more than 6 months, randomly selected from each of the 42 Serra da Estrela breed official farms, located in 9 municipalities of the geographical coverage of this breed (Seia, Gouveia, Celorico da Beira, Fornos de Algodres, Mangualde, Carregal do Sal, Arganil, Oliveira do Hospital and Tábua).

Blood samples were collected by venipuncture of the jugular vein. Blood tubes were kept in ice and transferred immediately to the laboratory at the Department of Zootechnics, Rural Engineering and Veterinary, Agrarian Superior School, Polytechnic Institute of Viseu. The collected blood samples were centrifuged at 1800×g for 10 min and the sera were separated and frozen at − 20 °C until analysis.

Individual blood serum samples were used to detect *C. burnetii* IgG antibodies, using a commercial indirect ELISA, ID Screen Q Fever Indirect Multi-species Kit (IDvet™, Montpellier, France), according to the manufacturer's instructions. Both the sensitivity and specificity of this ELISA have been shown to be 100% (IDvet™, internal validation report).

The sample-to-positive control (S/P) ratio in each serum was calculated according to the formula provided: S/P = (OD_{450} sample – OD_{450} NC)/(OD_{450} PC – OD_{450} NC); where *OD_{450} sample* = optical density of the sample, *OD_{450} NC* = optical density of the negative control and *OD_{450} PC* = optical density of the positive control. Results were expressed as an index (S/P x 100). In accordance with the manufacturer, indices were categorized into 4 different rising categories. Samples with S/P indices < 40% were considered negative, samples with S/P indices between 40 and 50% were considered doubtful, samples with S/P indices between 50 and 80% were considered positive, and samples with S/P indices > 80% were considered strong positive. Doubtful samples were retested and if resulting doubtful, considered as negative (Seo et al, 2017). Data obtained from the analysis of sera by ELISA were used to calculate population and geographic (municipality)-specific seroprevalence values. Chi-square test was used to study differences between groups (GraphPad Prism version 5.04; GraphPad Software Inc., La Jolla, CA, USA). A p value < 0.05 was considered statistically significant.

All selected Serra da Estrela breed official farms (N=42), of the 9 municipalities of the geographical coverage of this breed, were visited and agreed to participate in the study. The total of 168 animals were sampled both in 2015 and 2016 and sera were tested for IgG anti-*C. burnetii*. Of the 168 sera from the 2015 sample collection, 13 animals tested positive for IgG anti-*C. burnetii* (of which 4 were positive and 9 were strongly positive), showing a 2015 population seroprevalence of 7.7 % (Table 1). From the total of 168 sera of the 2016 sample collection, 29 tested positive for IgG anti-*C. burnetii* (of which 16 were positive and 13 were strongly positive), showing a 2016 population seroprevalence of 17.3%. Anti-*C. burnetii* seroprevalence differences in the 2015 and 2016 samplings showed to be statistically significant (p = 0.008). Within the 2015 cohort, of the 155 initially anti-*C. burnetii* seronegative animals, 17 (10.9%) have seroconverted by 2016. Of the 13 initially anti-*C. burnetii* seropositive

animals, 12 (92.3%) have maintained their immunological status regarding *C. burnetii* and 1 (7.7%) became seronegative.

Taking into account geographic location, a distinction between endemic and non-endemic municipalities can be observed. Sheep from 4 municipalities (Carregal do Sal, Tábua, Mangualde and Fornos de Algodres) were all negative in both 2015 and 2016. On the other hand, all but one of the remaining municipalities had farms where sheep increased anti-*C. burnetii* seropositivity (Figure 1). In fact, in Gouveia, a statistically significant increase ($p = 0.008$) was observed between anti-*C. burnetii* seropositivity in 2015 (5.0%; 95% confidence interval: 0.1-24.9) and 2016 (40.0%; 95% confidence interval: 19.1-63.9).

The present research studied the circulation of Q fever in sentinel sheep during 2 consecutive years, 2015 and 2016. On the first screening (2015), 13 (7.7 %) of the 168 animals tested positive for IgG anti-*C. burnetii.* On the second screening (2016), 29 (17.3%) of the same 168 animals tested positive for IgG anti-*C. burnetii*, showing a statistically significant increase in anti-*C. burnetii* seroprevalence in sheep herds of central Portugal. Interestingly, in a recent study in Portugal, exposure to *C. burnetii* in sheep and goats were evaluated showing a global individual seroprevalence of 9.6%, being higher in goats (10.4%) than in sheep (8.6%) (Anastácio et al, 2013). Our results show higher seroprevalences than those, supporting the notion that Q fever is increasing its occurrence in central Portugal (Figure 1). Results also confirm a high level of exposure to *C. burnetii* in small ruminant herds of central Portugal.

In general, little is known regarding Q fever status in animals in Europe, however European countries have reported, in general, decreasing number of human Q fever cases, whereas in Portugal the numbers have remained unchanged which has given rise to alert within Public Health stakeholders and decision makers (DGS, 2015; ECDC, 2014). Sheep traditional backyard farming has been widely present in Portugal and is part of the cultural and gastronomical

Figure 1. Anti-HEV IgG antibodies by municipality (%), in sheep sampled in 2015 and in 2016

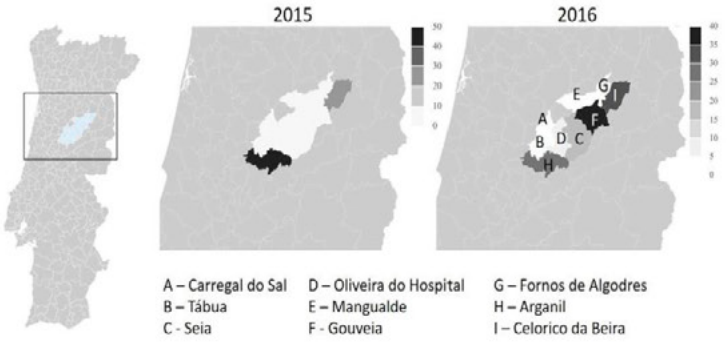

Figure 1. Anti-HEV IgG antibodies by municipality (%), in sheep sampled in 2015 and in 2016

320x139mm (150 x 150 DPI)

ANNEX C - Cruz R, Esteves F, Vasconcelos-Nóbrega C, Santos C, Ferreira AS, Mega C, Coelho AC, Vala H, Mesquita JR. Outbreaks of abortions by *Coxiella burnetii* in small ruminant flocks and a longitudinal serological approach on archived bulk tank milk suggest Q fever emergence in Central Portugal. Transboundary and Emerging Diseases

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RAPID COMMUNICATION

WILEY Transboundary and Emerging Diseases

Outbreaks of abortions by *Coxiella burnetii* in small ruminant flocks and a longitudinal serological approach on archived bulk tank milk suggest Q fever emergence in Central Portugal

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Abstract

Q fever is a worldwide zoonotic infectious disease caused by *Coxiella burnetii* and sheep and goats are known to be the main reservoir for human infection. This study describes the epidemiological and laboratory findings of *C. burnetii* outbreaks affecting sheep and goat flocks and also provides the results of a prospective serosurvey in bulk tank milk samples to assess *C. burnetii* circulation in a population of sheep living in close contact to the human population in Central Portugal. In the epizooties, *C. burnetii* was identified in tissues of the resulting abortions by qPCR. As for the serological survey, 10.2% (95%CI: 4.5-19.2) of the 78 bulk tank milk samples collected in 2015 presented IgG antibodies against *C. burnetii*. The same farms were visited and sampled in 2016 and 25.6% (95%CI: 16.4-36.8) were positive. This steep increase in the number of anti-*C. burnetii* farms between the 2015 and 2016 collections showed to be statistically significant ($p = 0.020$) and is strongly suggestive of Q fever emergence in Central Portugal. Measures on animal health and on disease spread control to the human population should be considered.

KEYWORDS
Coxiella burnetii, epidemiology, outbreaks, Q fever, small ruminants

1 | INTRODUCTION

Q fever is a worldwide zoonotic infectious disease caused by *Coxiella burnetii* and ruminants, namely cattle, sheep and goats are known to be the main reservoir for human infection, however, ticks are also considered a common reservoir (Angelakis & Raoult, 2010; Arricau-Bouvery & Rodolakis, 2005; van den Brom et al., 2012; Djerbib et al., 2018). *C. burnetii* infection in ruminants can result in epizootic abortions, which are often associated with vast bacteria shedding in birth fluids and placentas, significantly increasing the risk of disease spread (Filioussis et al., 2017; O'Neill, Sargeant, & Poljak, 2014). Human infections mainly occur in persons handling infected animals and their products but until recently, the zoonotic transfer of *C. burnetii* to the human population did not generate important alerts in both Veterinary and Human Public Health (Ergas, Keysari, Edelstein, & Sthoeger, 2006; Tselentis et al., 1995).

In the last decade, a strong paradigm shift has occurred in the scientific community due to a major epidemic that has occurred in the general population in the Netherlands, resulting in three 525 notified cases in humans and the subsequent national cull of carrying goat herds (van der Hoek et al., 2010). Abortion clusters in goat herds that started a few years earlier, as a consequence of the intensification of dairy goat production systems in the region, were initially suggested as the source of this large human epidemic (van der Hoek et al., 2010). This was later supported by the results of geospatial studies indicating an association between the human cases and the dairy goat farms (Schimmer et al., 2010). Since then, important work has been made to prevent the spread of *C. burnetii*

from sheep or goats to humans, however, these efforts have been hindered by the limitation of effective veterinary interventions in these small ruminants (Angelakis & Raoult, 2010).

Much is known on the role of sentinel animals in detecting risks to humans by providing early warning of an emerging infectious disease, being the particular case of sheep regarding *C. burnetii* (Mori & Roest, 2018). In particular, the important tradition behind primary production of sheep in the Centre of Portugal, mainly done at a small scale and in intimate contact with humans, can provide seroepidemiological studies with sentinel flocks and thus potentially mitigate *C. burnetii* shedding to the human population. This study describes two epizooties of *C. burnetii* affecting sheep and goat flocks, and also provides the results of a 2-year prospective serosurvey in bulk tank milk samples to assess *C. burnetii* circulation in a population of sheep living in close contact to the human population in Central Portugal.

Impacts

- We report two outbreaks of Q fever in sheep and goat flocks
- We describe a steep increase in Q fever antibodies in milk from sheep farms
- There is the possibility for Q fever emergence in central Portugal

2 | MATERIALS AND METHODS

2.1 | Outbreaks investigation

The first outbreak of abortions started on November 15, 2017, and lasted for 2 months in a sheep farm in the municipality of Mangualde (40.58633 North; -7.760661 West), district of Viseu, Central Portugal. The flock was not vaccinated to Q fever and had sheep from "Serra da Estrela" breed, the autochthonous breed of this region that produces the best sheep milk in Portugal. This milk is used for the highly valued and recognized worldwide, cheese "Serra da Estrela." There were 155 sheep in the flock, of which 100 were pregnant and 20 aborted. The first abortion occurred in November 15, 2017, and the last in January 18, 2018. Aborted foetuses (n = 2) were taken and refrigerated until arriving at the laboratory (within 24 hr). The second outbreak of abortions started on January 10, 2018, and lasted for 3 weeks in a goat farm in the municipality of Aguiar da Beira (40.81443 North; -7.54440 West), also in the district of Viseu, Central Portugal, approximately 50 km distant from the first outbreak. The flock had goats from "Murciana" breed and was also not vaccinated to Q fever. There were 60 goats in the flock, all pregnant, of which 25 aborted. The first abortion occurred in January 10, 2018, and the last in January 30, 2018. Placenta (n = 1) was taken and refrigerated until arriving at the laboratory (within 24 hr). Tissues from both outbreaks were tested for a panel of abortion pathogens, namely *Toxoplasma gondii*, *Chlamydiaceae* and *Coxiella burnetii*. DNA was extracted using NucleoSpin® Tissue kit, (Macherey Nagel, Duren, Germany), according to the manufacturer's instructions. For pathogen genomic detection, three commercial real-time PCR probe assay kits were used, according to the manufacturer's instructions (EXOone *T. gondii* oneMIX Kit; EXOone Chlamydiaceae one MIX Kit; EXOone *Coxiella burnetii* oneMIX Kit, Zaragoça, Spain). All reactions were performed using a positive, a negative and an endogenous control (β-actin target).

A questionnaire was applied to the farm owners and families, as well as to the workers for symptoms fitting acute Q fever case definition (acute fever and one or more of the following: rigours, severe retrobulbar headache, acute hepatitis, pneumonia) (CDC, 2009), during the period of the outbreaks and the following months, with the intervention and help of a human health team (a medical doctor and a nurse) and none fit the case definition.

To address *C. burnetti* spread, the delivered questionnaires included queries on similar epizootic abortions in nearby farms during the period of the outbreaks and the following months. The farm owner from the first outbreak reported that a neighbouring sheep farm had experienced 5–7 abortions during the same period. Despite efforts, we were not able to retrieve epidemiological data and samples from that sheep farm for analysis.

2.2 | Bulk tank milk collection

The study geographical location was Estrela Mountain ("Serra da Estrela"), located in central Portugal, where the National Association of Serra da Estrela Sheep breed—ANCOSE (Associação Nacional de Criadores de Ovinos da Serra da Estrela; http://www.ancose.com) is responsible for the administration of one of Portugal's autochthonous sheep breeds, the "Serra da Estrela." This breed provides for several European Union's Protected Designation of Origin (PDO) products, being exclusively bred in this region and thus having residual animal movement due to its confined production to the farm premises. For the serological analysis to study *C. burnetii* emergence, samples from a previous study on Schmallenberg virus (data not published) were used. All registered sheep flocks (ANCOSE official, N = 180) were invited to participate in this study, which required a bulk tank milk collection (one in January/February 2015, the other in January/February 2016). A total of 78 sheep milk farms from 46 parishes of five municipalities the Centre region of Portugal (Celorico da Beira, Fornos de Algodres, Gouveia, Seia and Tábua) answered and accepted to participate (response rate = 43.3%). The farm where the 2017 abortion outbreak occurred did not participate. All farms provided a 2 ml bulk milk sample both in January 2015 and in January 2016, which was swiftly transported to the laboratory at 4°C. Samples were processed according to the manufacturer's instructions with slight modifications (Chaintoutis et al., 2014). In brief, bulk milk samples were centrifuged at 1,000 g at 4°C for 10 min. After centrifugation, the fat fraction was removed using a sterile spatula, and the remaining fraction was transferred to a 1.5 ml microcentrifuge tube and immediately frozen (−20°C) until analysis.

TABLE 1 Screening for anti- *Coxiella burnetii* IgG antibodies in bulk milk tank samples from Serra da Estrela sheep, years 2015 and 2016

Municipality	2015 SBV IgG positive/Total: no. (%; CI)	2016 SBV IgG positives/Total: no. (%; CI)	*p* value
Seia	2/23 (8.7; 1.1-28.0)	5/23 (21.7;7.5-43.7)	0.414
Gouveia	3/16 (18.8;4.0-45.6)	5/16 (31.3;11.0-58.7)	0.685
Fornos de Algodres	1/8 (12.5;0.3-52.7)	1/8 (12.5;0.3-52.7)	[a]
Celorico da Beira	0/15 (0.0;0.0-0.0)	4/15 (26.7;7.8-55.1)	0.099
Tábua	2/16 (12.5; 1.6-38.3)	5/16 (31.3; 11.1-58.7)	0.394
Total	8/78 (10.2; 4.5-19.2)	20/78 (25.8; 16.4-36.8)	0.021*

CI, 95% confidence interval.
[a]Not determined.
*p value <0.05.

2.3 | Enzyme-linked immunosorbent assay

Samples were tested for the presence of anti-*C. burnetii* IgG antibodies, using a commercial indirect ELISA, ID Screen Q Fever Indirect Multi-species Kit (IDvet™, Montpellier, France), following the manufacturer's instructions. The positive control of this assay is a pool of positive bovine sera (field infected, from France) and the assay has a sensitivity and a specificity of 100% (according to the manufacturer). For test interpretation, sample-to-positive control (S/P) ratio in each serum was calculated, according to the formula provided: $S/P = (OD_{450}\ sample - OD_{450}\ NC)/(OD_{450}\ PC - OD_{450}\ NC)$; where OD_{450} *sample* = optical density of the sample, OD_{450} *NC* = optical density of the negative control and OD_{450} *PC* = optical density of the positive control. Results were expressed as an index (S/P × 100). Indices stratified as three different rising categories. Samples with S/P indices <30% were considered negative, samples with S/P indices between 30% and 40% were considered doubtful, samples with S/P indices >40% were considered positive. Doubtful samples were retested and if resulting doubtful, considered as negative. Obtained data were used to calculate NUTS II-specific seroprevalence values and differences between NUTS II-specific seroprevalence, in 2015 and 2016, were evaluated by Fisher's exact test, using GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA) and considered statistically significant if $p < 0.05$. Exact binomial 95% confidence intervals (CI) were established for proportions.

3 | RESULTS AND DISCUSSION

Results from the qPCR screening in the aborted foetuses and placenta showed that samples were negative for *T. gondii* and Chlamydia, and positive for *C. burnetii*, strongly suggesting that the abortion outbreaks that affected both farms were due to Q fever. All farm owners, family members and workers replied to the questionnaire, however, none fitted acute Q fever case definition. Nonetheless, the genotypes involved in the outbreaks could have been associated with low virulence in humans, and asymptomatic infections could have occurred (Van Schaik, Chen, Mertens, Weber, & Samuel, 2013).

As for the serological survey, anti-*C. burnetii* antibodies were found in both years. From the 2015 sampling, eight (10.2%; 95%CI: 4.5-19.2) of the 78 bulk tank milk samples presented IgG antibodies against *C. burnetii*, while from the 2016 sampling, 20 (25.6%; 95%CI: 16.4-36.8) of the total 78 bulk tank milk samples were positive. Of the eight initially (2015) positive farms, five (62.5%) maintained their seropositive status regarding Q fever and three (37.5%) became seronegative. Of the anti-*C. burnetii* seronegative farms from 2015, 15 (25.8%) had seroconverted by 2016. This steep increase in the number of anti-*C. burnetii* farms between the 2015 and 2016 collections showed to be statistically significant ($p = 0.020$).

Regarding the distribution of IgG anti-*C. burnetii*-positive bulk tank milk samples, according to geographical location (Table 1), in 2015 and 2016, an increase was observed in all but one municipality, namely in Seia (8.7% [CI: 1.1%-28.0%] versus 21.7% [CI: 7.5%-43.7%]), Gouveia (18.8% [CI: 4.0%-45.6%] versus 31.3% [CI: 11.0%-58.7%]), Celorico da Beira (0% [CI: 0.0%-0.0%] versus 26.7% [CI: 7.8%-55.1%]) and Tábua (12.5% [CI: 1.6%-38.3%] versus 31.3% [CI: 11.1%-58.7%]). Only Fornos de Algodres showed no change in the number of IgG anti-*C. burnetii*-positive bulk tank milk samples (12.5% [CI: 0.3%-52.7%]). The steep increase in IgG anti-*C. burnetii* bulk tank milk samples across the Central region of Portugal is strongly suggestive of Q fever emergence in Central Portugal.

ELISA on bulk tank milk samples has shown in the past to be a valuable matrix for the screening of *C. burnetii* infection within animals in lactation by providing information about the exposure to *C. burnetii* (van den Brom et al., 2012; Guatteo, Beaudeau, Joly, & Seegers, 2007) and producing comparable results to those obtained in serum samples due to the immunoglobulin transfer from blood to milk (Nielsen, Nielsen, Agger, Christoffersen, & Agerholm, 2011). In an interesting manner, another study in Portugal has detected IgG anti-*C. burnetii* in milk (Anastácio, Carolino, Sidi-Boumedine, & da Silva, 2016). Authors have collected 39 bulk milk samples from sheep in the same region, from 2009 to 2013, and showed that 51.3% flocks had positive samples (Anastácio et al., 2016). Although authors have obtained a much higher prevalence of positive bulk tank milk samples, they have tested samples for the presence of specific anti-C. burnetii antibodies using a different commercial ELISA (LSIVET Ruminant Milk/Serum Q Fever; Laboratoire Service

International, Lissieu, France). Thus, we find difficult to compare values as a different assay with distinct sensitivity/specificity has been used in the present study.

While considered to remain unaltered, Q fever prevalence in the human population of Portugal is conflicting with European data that shows a clear increase in cases (DGS, 2015; ECDC, 2014). Although little is known regarding Q fever in Portugal, a few recent case reports in humans have been linked to animals, highlighting the concern for zoonotic transfer from ruminants (Alves et al., 2016). It is therefore of the upmost importance to provide results on the circulation of *C. burnetii* in sheep, so as to implement measures on animal health and control the disease spread to the human population.

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CONFLICT OF INTEREST

Authors have no conflict of interest.

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ANNEX D – Q Fever epidemiological survey performed in centre Portugal

Inquérito sobre Febre Q

Nome _____ Sexo: ____ Marca de exploração: _____

Concelho _____

A. IDENTIFICAÇÃO

1. Idade: _____
2. Habilitações literárias:
   - ○ Sem habilitações
   - ○ Escola primária concluída
   - ○ Ensino obrigatório
   - ○ Curso profissional
   - ○ Licenciatura ou superior
3. Formação na área de produção animal:
   - ○ Sem formação específica
   - ○ Formação jovem agricultor
   - ○ Formação superior na área da produção animal
   - ○ Formação superior noutra área, não relacionada com a produção animal
4. Qual a atividade profissional?
   - ○ Pastor
   - ○ Técnico superior (mv/ev/eap)
   - ○ Ordenhador
   - ○ Queijeira
5. Qual a sua função na exploração?
   - ○ Pastor e ordenhador
   - ○ Só pastor
   - ○ Só ordenhador
   - ○ Ordenhador
6. Há quanto tempo trabalha com ovelhas e cabras _____
7. Alguma vez saiu de Portugal Continental?
   - ○ Sim
   - ○ Não
8. No caso de a resposta anterior ser SIM, indique qual o País para o qual viajou.

_____

1

Inquérito sobre Febre Q

9. Já alguma vez foi ao Algarve e/ou Alentejo?
   - ○ Sim
   - ○ Não

10. Já alguma vez saíram da região centro?
   - ○ Sim
   - ○ Não

11. Alguma vez teve doente com o seguinte (tremores, dores musculares e dores de cabeça? - https://www.cdc.gov/ncezid/conditions/q-fever/case-definition/2009/-) _____

11.1. Quando?
   - ○ Durante o ultimo ano
   - ○ 1-3 anos
   - ○ 3-5 anos
   - ○ Resposta livre _____

12. Alguma vez teve doente com o seguinte (tremores, dores musculares e dores de cabeça e com um ou mais dos seguintes: fadiga, suores noturnos, dificuldade em respirar, náusea, dor barriga, vomito, tosse e dor de peito) _____

12.1. Quando?
   - ○ Durante o ultimo ano
   - ○ 1-3 anos
   - ○ 3-5 anos
   - ○ Resposta livre _____

13. Alguma vez foi ao médico com estes sintomas ou similares e o médico diagnosticou febre Q (ou coxiellose)? _____

B. CARACTERIZAÇÃO EXPLORAÇÃO

14. Qual o tipo de exploração?
   - ○ Ovinos SC
   - ○ Ovinos raça exótica
   - ○ Caprinos
   - ○ Misto

15. Qual o encabeçamento da exploração? _____

16. Qual o objetivo da produção?
   - ○ Produção leite

Inquérito sobre Febre Q

- ○ Produção carne
- ○ Misto
- ○ Produção leite para transformação em queijaria própria

17. Qual o sistema de produção?
    - ○ Extensivo
    - ○ Intensivo
    - ○ Semi-extensivo

18. Tipo de ordenha
    - ○ Manual
    - ○ Mecânica
    - ○ Manual e mecânica
    - ○ Não ordenha

19. Tipo de pastagem
    - ○ Pastoreio em pastos semeados
    - ○ Pastoreio em pastos naturais sem sementeira
    - ○ Pastoreio em mata
    - ○ Sem pastoreio

C. CARACTERIZAÇÃO FATORES EPIDEMIOLÓGICOS

20. Faz controlo de ectoparasitas nomeadamente carraças em ovinos e caprinos?
    - ○ Sim
    - ○ Não
    - ○ Só no caso de superinfestação
    - ○ Só no período de seca

21. Isola as fêmeas quando estão para parir?
    - ○ Sim
    - ○ Nao

22. Já alguma vez ocorreram alguns abortos no seu efectivo de animais?
    - ○ Sim
    - ○ Não

(Nota: se a resposta a questão anterior for negativa passe para a questão 24)

23. O que faz aos produtos co aborto?

Inquérito sobre Febre Q

- ○ Enterra
- ○ Incinera
- ○ Deita no lixo
- ○ Deixa ao ar

24. Isola os animais que abortaram do restante efectivo?
    - ○ Sim
    - ○ Não

25. Faz algum tratamento médico (antibiático) aos animais depois do aborto?
    - ○ Sim
    - ○ Não

26. Usa luvas ao manipular os produtos do parto?
    - ○ Sim
    - ○ Não

27. Usa mascara ao manipular os produtos do parto?
    - ○ Sim
    - ○ Não

28. No caso da ordenha utiliza luvas na ordenha?
    - ○ Sim
    - ○ Não

29. Depois da ordenha, toma banho?
    - ○ Sim
    - ○ Não

30. No caso do processamento do leite para produção de queijo, pasteuriza o leite?
    - ○ Sim
    - ○ Não

31. No processo de fabrico utiliza luvas e/ou mascara no fabrico de queijo?
    - ○ Sim para os 2
    - ○ Não para os 2
    - ○ Só luvas
    - ○ Só máscara

32. Já alguma vez bebeu leite cru de ovelha ou cabra?

Inquérito sobre Febre Q

- ○ Sim
- ○ Não

33. Já ouviu falar em febre Q ou coxeirose em ovinos e caprinos?
    - ○ Sim
    - ○ Não

34. Sabia que o aborto pode ser um sintoma de febre Q em ovinos e caprinos?
    - ○ Sim
    - ○ Não

35. Sabia que a febre Q pode ser transmitida dos PR para os humanos?
    - ○ Sim
    - ○ Não

36. Sabia que esta bactéria pode ser eliminada através do leite e disseminada através de aerossóis?
    - ○ Sim
    - ○ Não

37. Sabia que esta doença pode ser diagnosticada através de uma análise de sangue, quer em humanos quer em PR?
    - ○ Sim
    - ○ Não

38. Conhece alguém (amigo ou familiar) que tenha tido febre Q?
    - ○ Sim
    - ○ Não

39. Alguma vez foi picado por carraças?
    - ○ Sim
    - ○ Não

40. Os cães de pastoreio são controlados para as carraças?
    - ○ Sim
    - ○ Não

41. De que forma?
    - ○ Coleira
    - ○ Injeção
    - ○ Pipeta

Inquérito sobre Febre Q

- ○ Comprimidos para as pulgas e carraças

42. Quando identifica carraças tem o cuidado de usar luvas na sua remoção?
    - ○ Sim
    - ○ Não

43. Quando faz a aplicação destes produtos?
    - ○ Quando desparasito as ovelhas
    - ○ Mensalmente
    - ○ Trimestralmente ou de 6 em 6 meses
    - ○ Quando vejo as carraças

44. Já teve algum cão diagnosticado com febre do carraça?
    - ○ Sim
    - ○ Não

ANNEX E – Approval of the ethical committee of UTAD

UNIVERSIDADE DE TRÁS-OS-MONTES E ALTO DOURO
Comissão de Ética da UTAD

Parecer da Comissão de Ética N:	19/2017
Data:	08.06.2017
Assunto:	Doc 15/CE/2017 Projeto de investigação "Coxiella burnetii surveillance in small ruminants of Portugal and the zoonotic impact to humans occupationally exposed"
Requerente:	Rita Marisa Paiva/**Coord:** Ana Cláudia Coelho; João Rodrigo Mesquita; Helena Vala

Dado o interesse para a ciência na área da Zootecnia, Medicina Veterinária e Medicina Humana, tendo em consideração que estão garantidos os direitos das pessoas envolvidas, nomeadamente consentimento informado e confidencialidade, a CE não tem objeções a colocar ao estudo submetido para apreciação.

Pela Comissão de Ética

O Presidente da Comissão

Pedro Miguel Mestre Alves da Silva