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Coxiella burnetii infection in dairy cows and goats: assessment of diagnostic methods, and evaluation of immune response in shedders

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ABSTRACT

Q fever is an infective, contagious and zoonotic disease caused by *Coxiella burnetii,* an obligate intracellular bacterium. The infection in ruminants is frequently subclinical, but late abortions, stillbirths and reproductive disorders can occur as well. Shedding of *C. burnetii* into the environment mainly occurs during parturition or abortion, but the bacterium is shed also in feces, urine, milk, vaginal mucus.

The first goal of this research was to assess the efficacy of a diagnostic strategy based on real-time PCR (r-t PCR) assays on bulk tank milk (BTM) for the detection of infected dairy herds/flocks, with the aim of estimating the prevalence of Q fever infection in dairy herds and in goats flocks. The second goal was to evaluate the dynamics of the antibodies response and the *C. burnetii* excretion in infected animals.

The sensitivity and specificity of a single r-t PCR test on BTM were evaluated using a control-case study in dairy herds and goat flocks. The first step was the identification of infected and negative farms, in which a sample of BTM was taken for a r-t PCR test to detect *C. burnetii* DNA.

In dairy cattle the infected herds were defined as farms with:

- ✓ clinical symptoms of Q fever like abortions or infertility and positive r-t PCR results confirming the presence of *C. burnetii* on specimens from affected animals or abortions,
- ✓ a prevalence at least of 20%.

Negatives farms were defined as farms with the following characteristics:

- ✓ a regular surveillance and diagnosis of abortions,
- ✓ all the r-t PCR test of the previous 2 years negative for *C. burnetii*,
- ✓ a seroprevalence below 20%.

The same features were used for dairy goat flocks but the seroprevalence was set out at 15%.

C burnetii was detected in fetuses or placenta of 17 dairy cattle farms: among these herds only 8 were positive at the BTM r-t PCR (50%). Out of 22 negatives herds, sampled for prevalence assessment, 10 showed a prevalence < 20% and 12 ≥ 20%. All herds defined as uninfected had a r-t PCR negative in BTM sample. The sensitivity and specificity of BTM r-t PCR were respectively equal to 0.5 and 1.

Out of the 29 flocks sampled in the study, 15 had goats shedding *C. burnetii* from vaginal mucus after abortions and also after normal parturition. No abortion storms were detected during the study, but only single cases of abortion. In the flocks where *C. burnetii* was detected, the prevalence was ≥ 15% in 9 farms. Among these flocks, 7 out of 9 had a positive r-t PCR BTM sample.

In the 14 flocks with *C. burnetii* negative vaginal swabs or abortions, none had a prevalence < 15% and a negative r-t PCR in BTM sample. The sensitivity and specificity of BTM r-t PCR was respectively equal to 0.8 and 1. The difference between cattle and goats sensitivity is probably due to the different features of the reproduction cycle. In goats kidding are seasonal and *C. burnetii* is shed in milk more often in the two months following kidding, that was exactly the time of BTM sampling in this study, while in cattle calving they are widespread all over the year.

To study the *C. burnetii* transmission in dairy herds, a longitudinal study was designed as follow:

1. 4 dairy herds were selected, among the infected farms,
2. a sample of blood and milk was taken from every lactating cow and tested, serum with ELISA and CFT for antibodies detection, milk r-t PCR for *C. burnetii* DNA detection,

3. according to test results, 4 groups of nearly 10 cows were established to be sampled 3 times every 2 months. The cows were sorted among the groups as follows:

- ✓ group 1: cows ELISA negative and PCR negative (ELISA-PCR-),
- ✓ group 2: cows ELISA negative and PCR positive (ELISA-PCR+),
- ✓ group 3: cows ELISA positive and PCR negative (ELISA+PCR-),
- ✓ group 4: cows ELISA positive and PCR positive (ELISA+PCR+).

4. From these cows at each sampling a sample of blood, milk and feces was taken and tested: serum with ELISA and CFT for antibodies detection, milk and feces with r-t PCR for *C. burnetii* DNA detection.

The same study was performed in 3 goat flocks, all infected by *C. burnetii.* The goats were sorted among 3 groups and lacked the group ELISA-PCR+ because was found only 1 goat ELISA-PCR+.

The results highlighted in dairy cows a great gap between the seroprevalence and the percentage of shedders found in the herds. The seroprevalence was on average 30,6%, while the percentage of shedders was at least 13% and the percentage of strong shedders was only 1,7%. We observed also the presence of 14 cows shedding *C. burnetii* in milk without an appreciable serological response: of these cattle 11 remained seronegative up to six months. The time of shedding in these cows was very short: after 2 months 12 cows were PCR negatives and from the 3rd sampling all cows remained negatives to PCR.

Many ELISA+PCR- cows were found shedding *C. burnetii* in milk in the following samplings. The ELISA+PCR+ cows behaved oppositely : these cattle gradually, during the study, became PCR-, but remained seropositive until the end.

Shedding in feces was sporadic and only 9 cows were found to shed the bacterium occasionally through this route.

In dairy herds we evaluated also the association between the serological response of the

cow to ELISA or CFT test and the occurrence of *C. burnetii* shedding in milk. The results obtained highlighted a high statistically significant association (p value = 0.00) of both ELISA and CFT, especially for the detection of persistent shedders with an odd ratio (OR) equal to 6.8 for CFT and 29.4 for ELISA. By increasing the ELISA cut-off to a value s/p > 2, only cows with a high serological titer were detected and these animals had a greater probability of being persistent shedders. Using this criteria for detection of seropositive cows, a highly significant association (OR = 18.4; p value = 0.00) was found between serological results and r-t PCR in milk.

The goats sampled were 257, coming from 3 flocks very different in size, ranging from 12 to 171 goats. The shedders of *C. burnetii* in milk were 59 (23%), the ELISA seropositive goats 177 (69%), the CFT seropositive goats 11 (4%). The percentage of shedders in milk was very different among the flocks, ranging from 100% of farm 2 to 1% of farm 3. At 2nd sampling in flocks 1 and 2 the numbers of shedders declined sharply, while in flock 3 the 2 shedders founded carried on the excretion until respectively 3rd and 4th sampling. Shedding of *C. burnetii* in stools was detected only occasionally in flocks 2 and 3, while in flock 1 at 2nd sampling 15 positives goats were detected, at the 3rd 13, and at 4th 5. The great majority of ELISA seropositive goats kept this status throughout the study time: only 3 goats out of 38, tested negative for antibodies at 3rd sampling. Among seronegative goats only 3 animals showed seroconversion during the study. The serological response to CFT showed remarkable variations at each sampling: first the percentage of positive goats was low or even 0, in the following samplings the percentage rose to 35% and even to 90% in one flock, then all the goats returned negative.

INTRODUCTION

Q fever is an infective and contagious disease caused by *Coxiella burnetii,* an obligate intracellular bacterium. It’s a zoonotic disease widespread all over the world except for New Zealand and is considered as emerging or re-emerging in many countries. This bacterium can infect a wide range of species, susceptible hosts including farm animals, pets, wild mammals and even non-mammalian such as domestic and wild birds, reptiles and ticks (Raoult et al., 2005).

The most common reservoir of the infection are domestic ruminants and humans are primarily infected by inhaling aerosols contaminated with the bacteria (ECDC, 2012). In domestic ruminants, which represent the major source of human infections, the disease is frequently subclinical, but late abortions, stillbirths and reproductive disorders can occur as well (Arricau-Bouvery and Rodolakis, 2005). Shedding of *C. burnetii* into the environment mainly occurs during delivery or abortion, but the bacterium is shed also in feces, urine, milk, vaginal mucus (Angelakis E., Raoult D., 2010).

In cattle, as in small ruminants, in addition to the peripartum excretion other periods of shedding can be observed: infected cows can persistently shed bacteria in milk for months without symptoms, while sporadic or intermittent shedding can occur in feces or vaginal mucus (Guatteo et al., 2007).

Factors affecting the maintenance of *C. burnetii* infection in animal populations can be grouped according to:

a) Agent factors, related to the characteristics of *C. burnetii,* and in particular infectivity, virulence and resistance to environmental conditions;

b) Host factors, including animal species, susceptibility, infectiousness, age and sex;

c) Environment factors , related to animal management, as well as manure management and farm characteristics.

Diagnosis, particularly the determination of the shedder status, is a critical and expensive process that is not yet completely standardized. Several methods are available for diagnosis of *C. burnetii* infection in animals, including both direct identification of the agent and serological testing. Isolation of *C. burnetii* can be done by cell or embryonated chicken egg culture. Such methods are complex and require level 3 containment (OIE) so they are not usually adopted in routine diagnostics laboratories (EFSA, 2010). Polymerase chain reaction (PCR) is one of the most analytically sensitive and rapid method for both the direct detection of *C. burnetii* and the identification of shedders, and the ability to detect and quantify *C. burnetii* DNA by real-time PCR has dramatically enhanced diagnostic and research approaches. Real-time PCR can be used on a wide range of samples: vaginal discharge, abortion material, feces and milk, bulk or individual (Kim et al., 2005). However, also with real-time PCR the detection of shedders is still complex because the shedding dynamics are not well known (Guatteo et al., 2007; Sidi-Boumedine et al., 2009;).

For the serological diagnosis of Q fever there is no officially (OIE) prescribed test, but among the various techniques that can be employed, the 3 most often used are the indirect immunofluorescence assay (IFA), enzyme linked immunoassay test, (ELISA) and the complement fixation test (CFT). Serological assays are suitable for the screening of herds or flocks, but interpretation at the individual animal level is not possible. The CFT was considered the reference test for historical reasons but its diagnostic sensitivity was highly variable . The analytical sensitivity of the ELISA was found to be 8-16 times higher than that of the best CFTs (EFSA, 2010). At present, ELISA is the recommended choice for seroprevalences studies (Natale et al., 2012).

During the last decade our knowledge on Q fever has increased, however the persistent considerable uncertainty about this infection calls for more research. Recently the European food safety authority (EFSA) scientific opinion on Q fever (2010) pointed out several items to be clarified by new investigations, concerning diagnostic methods, factors influencing the maintenance of infection and characteristics of the bacterium, like host specificity and virulence factors.

Several methods are available for diagnosis of *C. burnetii* infection in animals, including both direct identification of the agent and serological testing, but none of these is an officially designated test. The diagnosis of the infection should involve the use of multiple techniques and can be effectively interpreted only at herd or flock level.

The first goal of this research was to assess the efficacy of a diagnostic strategy based on real-time PCR assays on bulk tank milk (BTM) for the detection of infected dairy herds/flocks, with the aim of estimating the prevalence of Q fever infection in dairy herds and in goats flocks. The second goal was to evaluate in infected animals the dynamics of the antibodies response and the *C. burnetii* excretion.

CHAPTER 1: *Coxiella burnetii* in animals and humans

1.1 HISTORICAL BACKGROUND

Q fever was described in 1935 as an outbreak of febrile illness in slaughterhouse workers in Brisbane, Australia (Derrick EH, 1973). Derrick examined all those who were affected and could not produce a diagnosis, as a result, he named the illness ‘‘Q’’ for query fever.

Later, some workers suggested that the Q stood for Queensland, the state in which the disease was first described.

Burnet and Freeman (1937) reproduced the disease in guinea pigs and mice with an emulsion of infectious guinea pig liver received from Derrick and demonstrated Rickettsial organisms in spleen sections from infected mice (Burnet and Freeman 1983).

In the same period, Davis and Cox (1938),working on the possible vectors of Rocky Mountain spotted fever at the Rocky Mountain Laboratory in Hamilton,Montana, USA, allowed *Dermacentor andersoni* ticks collected near Nine Mile Creek, Montana, to feed on guinea pigs and found that some animals developed a febrile illness with enlarged spleens. They further characterized the “Nine Mile” agent. The organism was observed intravacuolarly in infected tissue cultures and was found to cause an infection in people (Cox et al., 1947).

Both groups in Brisbane and Montana demonstrated that the etiological agent displayed properties of both viruses and Rickettsiae in 1938, *Rickettsia diaporica*, the proposed name for the organism, which incorporated both Rickettsial features and the ability of the organism to pass through a bacteriological filter, was propagated in tissue cultures and in developing chicken embryos (Cox, 1941).

American and Australian groups started exchanging information and infected materials

after a laboratory-acquired Q fever infection occurred in the Rocky Mountain Laboratory in 1938 (Dyer, 1949). They demonstrated that the Australian Q fever agent, the zoonotic agent, and the Nine Mile agent were in fact isolates of the same microorganism, *Rickettsia burnetii* (Maurin and Raoult, 1999), later renamed as *Coxiella burnetii* (Philip, 1952), a name which honours both Cox and Burnet as pioneers in this field.

1.2 ETIOLOGY

1.2.1 Bacteriology

C. burnetii is a small pleomorphic rod (0.2–0.4 mm wide, 0.4–1.0mm long) obligate intracellular bacterium, with a membrane similar to that of a Gram-negative bacterium (Maurin and Raoult, 1999). Historically the bacterium was classified in the order of Ricketsiales, but now, based on 16S rRNA sequence analysis, had been placed in the *Coxiellaceae* family in the order *Legionellaes* of the gamma subdivision of *Proteobacteria* (Raoult et al., 2005).

C. burnetii replicates, with an estimated doubling time of 20–45 hours, within a parasitophorous vacuole of eukaryotic host cells (Mertens and Samuel, 2007). This microorganism has a intracellular development cycle that involves 3 distinct cells type: large cell variant (LCV), small-cell variant (SCV) or small dense cell (SDC) (Angelakis and Raoult, 2010).

The 3 forms can be distinguished by morphologic, antigenic, metabolic differences, and physical and chemical resistance (Heinzen and Hackstadt, 1999). The LCV, which share features common with Gram-negative bacteria, have diffuse chromatin and possess clearly distinguishable outer and cytoplasmic membranes with exposed LPS on the surface. They are the metabolically active intracellular form of *C. burnetii*, and are larger, more pleomorphic and less electron dense than the other two forms (Arricau-Bouvery and Rodolakis, 2005). The LCV form undergoes sporogenic differentiation to produce resistant, spore-like forms, the SDC and SCV, that are able to survive extracellularly as infectious particles (Angelakis and Raoult, 2010). The SCV is a compact small rod with a center of condensed nucleoid filaments. The SDC resemble the SCV in morphology but are distinct from this form as a result of a higher physical stability (McCaul et. al., 1991), that

is highlighted from the resistance of these cells to a pressure treatment of 50,000 psi (corresponding to 350,000 KPa) (Samuel et al., 2003). The SDC have been visualized in LCV as endospores and may be liberated upon the lysis of LCV or binary transverse fission with unequal cell division (McCaul et. al., 1981).

The formation of the different forms is linked to the lifecycle of *C. burnetii*, a strategy developed to survive in and out of the parasitophorous vacuole. The SCV and SDC forms enter into the eukaryotic cells by microfilament dependent endocytosis and after the acidification of the phagosome (about pH 5.5) the SCV and SDC cells multiply by transverse binary fission and differentiate to LCV (Arricau-Bouvery and Rodolakis, 2005). The fusion of the phagosome with the lysosome leads to the formation of the phagolysosom, and to a further acidification (about pH 4.5) that is an absolute requirement for the replication of the LCV cells. At this pH, an exponential growth of the LCV cells occurs for 4-6 days, then a stationary phase starts and coincides with the reappearance of SCV cells (Rodolakis, 2011).

These 3 bacterial forms express different proteins, specific for each form, in the developmental life cycle (Heinzen and Hackstadt, 1996; Heinzen et al., 1999; Seshadri et. al, 1999) and recognized by antibodies produced during a *C. burnetii* infection. These differentially expressed antigens could allow the bacteria to escape the immune response. In particular the major outer protein P1, which functions as porin, is absent in SDC cells and (McCaul et al., 1991) and is responsible for the great resistance of *C. burnetii* in the environment.

C. burnetii displays antigenic variation similar to the smooth–rough variation of other Gram-negative bacteria and this variation is related to changes in the lipopolysaccharides (LPS) layer (Woldehiwet, 2004). After several passages in embryonated eggs or cell culture, the bacterial population shifts from a virulent (phase I) to an avirulent phase (phase

II), which corresponds to the rough variants of other Gram negative bacteria. *C. burnetii* phase I bacteria expresses a smooth full-length LPS and are highly infective and naturally found in infected animals, humans and ticks, while phase II bacteria are avirulent and characterized by a truncated LPS that lacks completely the O-antigenic polysaccharide chain (Mertens and Samuel, 2007), and also some protein cell surface determinants (Amano and Williams, 1984). Early activation of immune cells depends primarily on LPS, and the full-length LPS of phase I bacteria seems to have a masking effect protecting the bacteria from Toll-like receptors type 2 (TLR2) recognition (Mertens and Samuel, 2007).

1.2.2 Genomic

Strains of *C. burnetii* have been shown to belong to six (I–VI) genomic groups on the basis of restriction fragment length polymorphism (RFLP) (Hendrix et al., 1991). It remains to be clarified whether the phase variation of *C. burnetii* is related to genetic variation (Maurin and Raoult, 1999) similar to that observed in *Pseudomonas aeruginosa* (Zielinski et al., 1991).

C. burnetii possesses a small circular chromosome of approximately 2 Mbp (Mertens and Samuel, 2007). Most isolates harbor additionally one out of four plasmids of 32 to 51 kb in size, defined as: QpH1, QpRS, QpDG, QpDV (Jäger, 1998). Strains without a resident plasmid carry instead a 16 kb plasmid-like sequence integrated in the chromosome (Mertens and Samuel, 2007). The plasmids seem to be of major importance for virulence because their common sequences are conserved among all isolates of *C. burnetii*, but their biological significance is still unclear (Arricau-Bouvery and Rodolakis, 2005).

The genome is predicted to encode 2,134 coding sequences larger than 30 aa, of which 719 (33.7%) are hypothetical with no significant similarity to other genes in the database (Arricau-Bouvery and Rodolakis, 2005). In the genome of *C. burnetii* 83 pseudogenes have been identified: many of these genes contains single frameshifts, points mutations,

truncations, which suggest that the genome reduction is a relatively early, and still ongoing process. Unlike other intracellular bacteria, *C. burnetii* genome has a great variety of mobile elements: in particular 29 insertion sequence (IS) elements are present. There are 21 copies of a unique IS110-related isotype, named IS1111, 5 IS30 and 3 ISAs1 family elements, and 3 degenerate transposase genes of unknown lineage (Hoover et al., 1992). The IS1111 sequence analysis highlighted a less than 2% divergence among the strains tested (Mertens and Samuel, 2007).

Several attempts were made to discriminate among the different *C. burnetii* strains based on phenotypic and genomic characteristics, especially for the individuation of specific pathological features. Initially, the plasmid profile was associated with the so-called acute or chronic *C. burnetii* isolates, originating respectively from acute or chronic Q fever patients. However, recent findings by PCR analysis of *C. burnetii* strains from patients exhibiting chronic Q fever have revealed that there is no correlation between the plasmid type and the acute or chronic human infection (Arricau-Bouvery and Rodolakis, 2005). Restriction fragment length polymorphism (RFLP) analysis of genomic DNA demonstrates a considerable heterogenic banding pattern for the classification of genomic groups (Mertens and Samuel, 2007). Six distinct genomic groups were identified that can be correlated with specific plasmid types and clinical symptoms (Hendrix, 1991). Multispacer sequence typing (MST) was performed on 173 isolates to characterize different isolates of *C. burnetii* at the molecular level (Glazunova et al., 2005). This method is based on the comparison of the nucleotide sequences of internal regions between different genes, because they are considered highly variable since they are subject to lower selection pressure than the adjacent genes (Mertens and Samuel, 2007). Three monophyletic groups were identified based on MST: a correlation between the geographical distribution and the sequence type was found. Moreover this study demonstrated a correlation between

plasmide type and the disease outcome (acute or chronic infection) and an association between some genotypes and disease type (Glazunova et al., 2005).

Recently, researchers from different countries have applied the multiple loci variable number of tandem repeat analysis (MLVA) method to *C. burnetii* strains typing because of its quite high discriminatory power and its applicability either to isolated bacterial strains or directly to DNA extracted from clinical samples (Arricau-Bouvery et al., 2006; Svraka et al., 2006). French researchers identified within the C. burnetii genome a total of 17 different minisatellite and microsatellite markers to be used in MLVA genotyping in 2 successive panels (Arricau-Bouvery, 2006). A third panel was implemented during the Dutch Coxiella burnetii outbreak, involving 6 microsatellite markers (Klaassen et al., 2009; Tilburg et al., 2012).

1.2.3 Resistance features

C. burnetii is able to survive in the outdoor environment for long time and persist in contaminated food, due to its physical characteristics that include stability against acids (up to pH 4.5), temperature (62° C for 30 minutes), UV light and pressure (up to 300.000 kPa) (EFSA, 2010). *C. burnetii* can survive for up to 42 months at 4-6°C in milk, 12 to 16 months in wool, 120 days in dust, 49 days in dried urine, 30 days in dried sputum (EFSA, 2010). Further, the organism can survive for more than 6 months in 10% saline (Williams 1991). *C. burnetii* is killed following exposure to 5% chloroform or formaldehyde gas (in an 80% humidified environment) with less than 30 minutes exposure, to 5% H2O2, 0.5% hypochlorite and 70% ethanol (all with 30 minute exposure), and following pasteurization (at least 72°C for 40 seconds) (Angelakis and Raoult, 2010; EFSA 2010).

1.3 EPIDEMIOLOGY

1.3.1 Transmission

C. burnetii is characterized by a remarkable resistance to adverse environmental conditions. The organism is readily excreted in milk, urine, feces and uterine discharge of affected cattle, sheep, goats and other ungulates (Rodolakis, 2009). *C. burnetii* is present in very high numbers in the amniotic fluid, the placenta and foetal membranes of parturient ewes, goats, cattle and infected aerosols are generated from desiccation of infected placenta, body fluids, manure or contaminated dust (Arricau-Bovery and Rodolakis, 2005). Animals may continue to shed infectious particles for time long after abortion (Berri et al., 2001). Parturition and abortions in animals associated with specific climatic conditions, especially dry, windy weather, has been reported as the main risk factors associated with spillover of infection from domestic ruminants to humans in different European Union (EU) members state (EFSA, 2010). Infection of domestic ruminants is usually seasonal and it occurs mainly in relation to lambing or calving seasons. In sheep, some experimental evidence suggests that pregnant animals are more susceptible to infection than non-pregnant ones (Woldehiwet, 2004).

C. burnetii is unique among the members of the family *Rickettsiaceae* in its non-dependence on arthropod transmission, but ticks and other arthropods may be a source of infection for domestic animals. Marrie (1988) hypothesized that there may be an epidemiological circuit involving ticks, wild rodents, cats and man. Infected ticks are important for the maintenance of the whole cycle of the organism in nature but not essential vectors for animal or human infection (Woldehiwet, 2004; Marrie, 2007).

The factors affecting the maintenance of *C. burnetii* infection in animal populations can be grouped according to:

a) agent, relating to the characteristics of *C. burnetii*, and in particular infectivity, virulence and resistance to environmental conditions;

b) host, including animal species, susceptibility, infectiousness, age and sex;

c) environment, related to animal management, as well as manure management and farm characteristics.

Agent factors

The importance of strain as a risk factor for both maintenance of infection and disease progression is unknown. The relationship between genotype/isolates and virulence is at the moment unclear, but the presence of a more virulent strain or a genetic shift to a more virulent strain has been suggested as an important factor in the occurrence of the outbreaks (Roest, 2011). Using MLVA Roest and others found that, in the outbreaks occurred in The Netherland during 2007-2010, 1 genotype of *C. burnetii* predominated on all dairy goat farms. This finding strongly suggests a clonal spread of *C. burnetii* with this predominant genotype over the dairy goat farms in the southeastern part of the Netherlands. The clonal spread of this single genotype could have been facilitated by the emergence of a genotype of *C. burnetii* causing abortion in dairy goats, that could then spread successfully over the dense goat population in the southeastern part of the country (Roest et al., 2012).

C. burnetii is highly resistant to environmental conditions, surviving for many months under a range of conditions (EFSA, 2010; Rodolakis 2011). This feature can explain the ubiquitous nature of *C. burnetii* and the occurrence of outbreaks in humans far away from their animal source as happened in the Marseille area and in a town in the French Alps (Marrie, 2007). In the first outbreak the presence of contaminated waste from sheep and of strong wind were the key factors for the widespread of infection, while in the second outbreak the presence of a helicopter landing area near a slaughterhouse created winds that facilitated the airborne spread of *C. burnetii* (Marrie, 2007).

Host factors

Host factors play a key role in the natural history of *C. burnetii* infection in human and animals. Animal proximity and contact with infected animals and/or their contaminated products (e.g. birth products) have been identified as important risk factors for humans. In most outbreaks, there are reports of spill-over of infection to humans from infected domestic small ruminants like goats or sheep. In contrast, there is no evidence of any major contribution of cattle in the history of Q fever in humans (Georgiev et al., 2013). Further, the evolution of disease, including clinical signs, is not the same in cattle, as in sheep or goats. The duration of excretion of the agent, husbandry conditions and other factors may each play an important role in the differences observed in animal seroprevalence and the persistence of infection. *C. burnetii* excretion can last up to several months in ruminants, with some differences among the species. The longest duration of excretion found during the follow up of naturally or experimentally infected animals were:

- ✓ 14 days in feces, 13 months in milk in cows,
- ✓ 14 days in vaginal mucus, 20 days in feces, 52 days in milk in goats,
- ✓ 71 days in vaginal mucus, 8 days after lambing in feces, 8 days in milk in sheep (Arricau-Bouvery and Rodolakis, 2005).

Environment factors

A range of environmental factors have been suggested to influence the maintenance of infection in farmed animal population, including increased herd/flock size, animal density, and herd/flock density (EFSA, 2010). Pathogen pressure is likely to increase in association with farm factors that increase the concentration of *C. burnetii* in the environment.

C. burnetii contaminated manure has been identified as a source for Q fever in human outbreaks ((Arricau-Bouvery and Rodolakis, 2005; EFSA, 2010; Roest et al, 2011a). It is likely that it also plays a role on the maintenance of infection in livestock.

1.3.2.Host distribution

Q fever is a zoonosis affecting a wide range of hosts. Horses, pigs, dogs, cats, camels, buffaloes and also wild and domestic birds such as chickens, pigeons, ducks, geese and turkeys can be infected without showing any clinical signs. *C. burnetii* has also been isolated from rabbits, cats squirrels, mice, deer and other free-living animals (Woldehiwet, 2004). Farm animals such as cattle, sheep and goats are the most important reservoirs for human infections (Rodolakis, 2006; Marrie, 2007). Evidence of infection has also been shown in various species of ticks, fleas, mites, flies and other arthropods (Dyer, 1949; Babudieri and Moscovici, 1952). Over 40 tick species can be naturally infected with *C. burnetii:* the organism multiplies in the gut cells of ticks and large numbers of *C. burnetii* are shed in tick feces. Nevertheless infected ticks seems not to be important in the maintenance of infections in livestock or humans, they are probably most important in maintaining the whole cycle of *Coxiella burnetii* (Marrie, 2007).

C. burnetii infection is widespread all over the word with the exception of New Zealand, occurring in diverse geographic regions and climatic zones (Hilbink et al., 1993; Woldehiwet, 2004). The real incidence of the infection both in humans and animals is underestimated in several countries due to the preponderance of asymptomatic cases and the lack of efficient diagnostic tools (Porter 2001; EFSA, 2010). In Africa studies on the seroprevalence of the infection have been carried out mainly in Egypt, Sudan, Morocco, Tunisia, Chad, South Africa, and in all the states of the Western part of the continent. A seroprevalence ranging from 4% up to 55% was found in cattle, while in small ruminants the seroprevalence ranged from 13% to 33%. In camels a study carried out in Chad found a seroprevalence of 80% (Vanderburg et al., 2014).

In the United States of America (USA) the prevalence of the disease in dairy cattle was estimated greater than 90% analyzing bulk tank milk in one study (Kim et al., 2005), and

in a recent study involving both dairy and goats herds the presence of *C. burnetii* DNA was found in the 96% of the farms tested (Pearson et al., 2014). Several seroprevalence studies have been performed in USA to assess the diffusion of *C. burnetii* in animals. The greater prevalence was found in goats (41.6%) and in sheep (16.5%): in dairy cattle, despite the widespread of the etiological agent in dairy farms, the seroprevalence founded was lower than 5% (McQuiston and Childs, 2002).

In Humans the 3.1 % of the population was found seropositive (Anderson et al., 2009) but among veterinarians a seroprevalence almost equal to 22% was found in a different study (Whitney et al., 2009).

In Europe *C. burnetii* infection is prevalent in domestic ruminants (cattle, sheep, and goats) in a wide range of countries, based on the results of serological testing over the last decades, listed in table 1, 2 and 3 (EFSA, 2010). In recent studies herd prevalence for cattle was estimated to be up to 73.0%, in France, and up to 37.0 % in the Netherlands. For goats it was 40.0% in France and 17.8% in the Netherlands, while for sheep values of 89.0% in France, and 14.5% in the Netherlands were respectively found (Georgiev et al., 2013). Within-herd prevalence estimates for cattle were up to 20.8% in Bulgaria, 15.0% in France, 19.3% in Germany,, 21.0% in the Netherlands, for goats up to 40.0% in Bulgaria, 88.1% in France, 2.5% in Germany, 7.8% in the Netherlands, and for sheep up to 56.9% in Bulgaria, 20.0% in France, 8.7% in Germany, 3.5% in the Netherlands (Georgiev et al., 2013).

In humans Q fever has been endemic in large parts of Europe for several decades. Estimates of prevalence of *C. burnetii* infection in human, based on serological studies conducted in France, The Netherlands, Bulgaria and Germany since 1982 to 2010, are listed in Table 4 (Georgiev et al., 2013). Seroprevalence studies from the period 1970–2009 show that 10–30% of rural populations in different parts of Europe have antibodies

against *C. burnetii*. The seroprevalence is higher in farmers working with cattle or sheep, and highest in persons who are in contact with the products of animal births or abortions. Other high-risk groups for infection are veterinarians and personnel in research laboratories working with animals (Lahuerta et al., 2009; Frode Forland et al., 2010).

There is significant variation in the levels of seroconversion throughout the EU, but that at the general population level, there are typically a small percentage of individuals (often between 2-10%) that have evidence of having been infected with *C. burnetii*. This evidence inevitably rises in areas with outbreaks of human Q fever, or where outbreaks are commonly reported and there is some endemicity of *C. burnetii* (Eurosurvilliance, 2010).

In 2007, the broad epidemiological situation from reported data was that 22 EU and European Free Trade Association (EFTA) countries reported a total of 669 cases of Q fever (8 countries reported zero cases), 637 of which were confirmed. In 2008, 24 EU/EFTA countries reported 1,599 Q fever cases (1,594 confirmed), representing an increase of over 170% from the previous year. This increase was mainly attributed to the increase in the Netherlands. The number of cases per year reported usually in the Netherlands from 1980 to 2007 was on average 20 a year (van der Hoek et al., 2012). In 2007 a total of 168 human cases was notified in a specific area, the Noord-Brabant. Dairy goats were identified as the source of the human Q fever due to a considerable number of Q-fever abortions in goats: the unusually hot and dry weather in the spring 2007 seems to have caused airborne transmission of contaminated dust particles (van der Hoek et al., 2012). The number of Q fever cases increased dramatically in 2008 and it became evident that the 2007 outbreak was not an isolate incident. A total of 3,489 patients affected from Q fever were reported between 2007 and 2009 and looking at the epidemic curve of the disease (figure 1) during this time, a seasonal pattern could be observed with the most cases occurring in spring and early summer (van der Hoek et al., 2012). The exponential spread of Q fever stopped since

2010 and the notification of cases ended by 2011. Drastic veterinary intervention such as culling of pregnant goats from infected farms, massive use of vaccination and enforced hygiene measures were able to stop the widespread of the disease in humans and animals.
A posteriori, it would be difficult to establish if this epidemic in The Netherlands, with 3523 human cases within 3 consecutive years, represents a unique phenomenon (Roest et al., 2011). The epidemiological situation of Q fever infection in The Netherlands till the year 2006 was very similar to the most of European countries, with high seroprevalence in animal population but few human cases (EFSA, 2010).
It is not clear why Q fever became a major problem in The Netherlands but not elsewhere. Several factors might have facilitated a change in epidemiology in goats : first, an increase in goat density in specific areas of The Netherlands and second, the extension of farms over the years. The increase in goat density took place in the highly populated province of Noord-Brabant. This proximity to a source excreting high numbers of *C. burnetii* during abortion, with transmission facilitated by dry weather and high numbers of susceptible humans is probably the main cause of the human Q fever outbreak in The Netherlands.
These two factors associated with the new introduction of a more virulent strain or a genetic shift to a more virulent strain could have affected in-herd and between-herd dynamics of Q fever, resulting in the human and animals outbreaks (Roest et al., 2011). The Q fever outbreaks occurred in The Netherlands showed that this infection can easily be missed in the human field as well in the veterinary field and that good monitoring and surveillance systems are necessary to assess the real magnitude of Q fever.
The most important lesson learned from the Dutch Q fever outbreak is that a close cooperation between the human and veterinary fields is essential for responding to outbreaks of zoonotic diseases. Specific disease knowledge and diagnostic tools from both fields are needed to manage outbreaks (Roest et al., 2014).

1.3.3 Q fever in Italy

C. burnetii infection in ruminants has been reported in Italy since 1949 associated to human outbreaks occurred in Marche region in 1949-51 (Moretti 1984), Abruzzo in 1953 (Caporale et al, 1953) and Sicily (Moretti 1984). The infection in ruminants is widespread in all the country as demonstrated by several studies performed in different area of Italy. In the Emilia Romagna region Martini et al. in 1994 found in dairy herds, using CFT, a prevalence rate of 13.1%. In the same region Cabassi et al. (2006) analyzing 650 sera from dairy cattle with abortion and 600 randomly-selected control sera found a seroprevalence equal to 44.9% in the animals which experienced abortion and equal to 22% in the control group. In the Veneto region both a seroprevalence of 18.2% in dairy cows after abortion was reported and the identification of *C. burnetii* in 2.8% of the cattle fetuses tested during the routine diagnostic activity from 2005 to 2008 (Barberio at al., 2009). In the same region a serological study performed on bulk tank milk in the province of Vicenza in 2010 highlighted that among 489 herds tested, 290 (59%) were positives (Barberio et al., 2010). A survey performed in 2007-08 in the Lombardia region using a PCR method for the detection of *C. burnetii* DNA in bulk tank milk of 400 dairy cattle herds, showed that the 40% of the milk samples analyzed were positive (Mannino et al., 2009). In South of Italy *C. burnetii* has been detected in the 11.6% of cattle aborted fetuses , and in the 21,5% of sheep and goats aborted fetuses (Parisi et al., 2006). In water Buffalo in the Campania region a survey performed in 2009 reported a prevalence of 17.5% fetuses positive for *C. burnetii* among 164 examined (Perugini et al., 2009).

In spite of the spread of the infection in ruminants, few Q fever outbreaks in humans have occurred in Italy since the first report of the disease in American soldier in 1945 (Commission on acute respiratory diseases, 1946) at the Grottaglie Air base (near Taranto in the Puglia region).

Others outbreaks were reported between 1946 and 1960 in Abruzzo (Caporale et al., 1953), Campania, Sardinia, Friuli Venezia Giulia (Simeoni, 2009): all these outbreaks were associated with the contact with parturient animals or with the passage of sheep flocks during lambing time. More recently, 4 outbreaks involving several people were reported. The first two occurred in 1987 and 1988 inside an agricultural community for the rehabilitation of drug users in San Patrignano (Rimini, Emilia Romagna). Approximately 40% of the residents were human immunodeficiency virus (HIV) positive and 235 of them presented with clinical evidence of a flulike syndrome that was confirmed to be Q fever. The source of infection was correlated with activities in a sheep farm (Boschini et al., 1999).

The third one occurred in the area of Vicenza in Veneto in 1993 (Manfredi-Selvaggi et al., 1996): a total of 58 human cases were identified in a 5 month period and 48% patients were hospitalized. Three flocks of sheep which passed through the outbreak area between late May and early June were shown to be infected, with prevalence of antibodies ranging between 45 and 53%. The case-control study showed a significant association with exposure to flocks of sheep (Odds ratio = 6.1).

The last one occurred in winter 2003 in a prison population near the city of Como, in the Lombardia region. Overall, 65 of the 600 prison inmates developed the disease. The most probable source of infection has been identified in infected dust diffusion, helped by windy and dry weather, from sheep flock that passed through this area (Starnini et al., 2005).

1.4 PATHOGENESIS

1.4.1 Pathofisiology

In ruminants the most important route of infection from *C. burnetii* is the oral route and the portal of entry is the oropharynx. (McQuiston and Childs, 2002). On the contrary in humans the most important route of infection is inhalation of bacteria-contaminated dust, while the oral route is considered of secondary importance (Porter el al., 2011). Once inhaled or ingested, the extracellular form of *C. burnetii* (SCV or SDC) attaches itself to the cell membrane of monocytes/macrophages, the only known target cells and is internalized into the host cells by phagocytosis. Virulent *C. burnetii* organisms survive inside human monocytes, whereas avirulent bacteria are eliminated (Capo et al., 1999). Phagolysosomes are formed after the fusion of phagosomes with cellular acidic lysosomes. The multiple intracellular phagolysosomes eventually fuse together leading to the formation of a large unique vacuole. *C. burnetii* has adapted to the phagolysosomes of eukaryotic cells and is capable of multiplying in the acidic vacuoles. In fact, acidity is necessary for its metabolism, including nutrients assimilation and synthesis of nucleic acids and amino acids (Rodolakis, 2011). The ability of *C. burnetii* to grow and multiply within phagolysosomes and its propensity to establish persistent infection, are of central importance for the pathogenesis of the disease. The adaptation of *C. burnetii* to intracellular life is linked with acidic pH of its phagosome and both virulent and avirulent bacteria are found in phagosomes. Acidic pH allows the entry of nutrients necessary for *C. burnetii* metabolism and also protects bacteria from antibiotics by altering their activity (Hackstadt and Williams, 1981). Indeed Increasing pH with lysosomotropic agents such as chloroquine restores the bactericidal activity of doxycycline (Raoult et al., 2005).

The mechanisms of *C. burnetii* survival in phagolysosomes are still under study. Mo et al. (1995) and Akporiaye and Baca (1983) identified 3 proteins involved in intracellular survival: a superoxide dismutase, a catalase, and a macrophage infectivity potentiator (Cbmip). Redd and Thompson (1995) found that secretion and export of Cbmip was triggered by an acid pH *in vitro*. Later, studies by Zamboni and Rabonovitch (2003) demonstrated that growth of *C. burnetii* was reduced by reactive oxygen intermediates (ROI) and reactive nitrogen intermediates.

In ruminants after primary multiplication in the regional lymphnodes, an ensuing bacteraemia lasts for at least 7 days at most 21 days and the organism then localizes in the mammary glands and the placenta of pregnant animals (Forland et al., 2010). The natural history of C. burnetii infection in cattle, sheep and goats is that a non-immune (often neo-natal) animal is infected from the environment, often contaminated by parturient or other animals shedding the organism and it undergoes a primary infection with weak clinical signs. However, the organism can persist after initial acute or subclinical disease, being shed in large numbers when a persistently infected female animal becomes pregnant. Experimental studies have shown that, during early pregnancy, the organism may be collected from the liver, spleen, kidney, bone marrow, lymph nodes and the intestine up to 13 weeks of pregnancy. The placenta becomes positive only just before parturition (Harris et al., 2000). At the end of the pregnancy, the placenta allows the organism to multiply to high titles and at parturition it is shed in the placenta through the amniotic and other fluids. Aerosols are liberated and the environment can easily become contaminated with highly resistant bacteria. The level of IgG, climbs at this stage. In subsequent pregnancies, the animal does not excrete again or only at a low title. However, the bacteria may continue to be shed in the milk, particularly of cattle, for long periods of time (Woldehiwet, 2004). Also in animals *C. burnetii* infection may become chronic, like in humans. In goats *C.*

burnetii shedding was found at successive parturitions suggesting that goats could be chronically infected and that the multiplication of the organism may be reactivated during subsequent pregnancies (Berri et al., 2007). In chronically infected ruminants, it is not clearly understood how and where Coxiella persists in the non-pregnant period and which mechanism initiated the bacteria multiplication in the placenta. It is however known that pregnancy results in immunomodulation that may be responsible for the increase multiplication of the organism in the placenta (Polydourou, 1981). A significant correlation was found among *C. burnetii* antibody levels and the concentrations of cortisol, pregnancy-associated glycoproteins (PAG), and plasma progesterone. These findings suggest that the *C. burnetii* infection can modify endocrine patterns throughout gestation and induce placental damage and diminishing PAG levels (Garcia-Ispierto, 2010).

In humans after primary multiplication in the regional lymph nodes, haematogenous spread results in the organism infecting the liver, spleen, bone marrow, the reproductive tract and other organs. Acute infection is usually characterized by atypical pneumonia and hepatitis, and is followed by the formation of granulomatous lesions in the liver and bone marrow, with granulomatous hepatitis being the most frequent indication of infection with *C. burnetii*. The granulomatous lesions have a central open space and a fibrin ring and are referred to as doughnut granulomas (Tissot-Dupont and Raoult, 2007). The formation of granuloma is due to the activation of the cell mediated immunity and tissue granulomas are present only in patients with acute Q fever, where the clinical outcome is usually favorable, suggesting that granulomas play an important role in the resolution of Q fever (Faugaret et al., 2014). Immune control of *C. burnetii* is T-cell dependent but does not lead to *C. burnetii* eradication (Honstettre et al., 2004). *C. burnetii* DNA can also be found in circulating monocytes or bone marrow of people infected months or years earlier (Capo et al., 2003).

C. burnetii infection may become chronic in patients with predisposing conditions, including those with heart valve lesions, vascular abnormalities, and immunosuppression (Fenollar et al., 2001). The latency between acute and chronic infection may last from months to years (Capo and Mege, 2012). It is not clearly understood how and where Coxiella persists during this latency period. In a prolonged Q fever post infection fatigue syndrome (QFS), the bone marrow was identified as potential focus of *C. burnetii* infection from which placenta and other sites such endocardium may be seeded for recrudescent infection (Harris et al., 2000; Marmion et al., 2005). Once established, chronic Q fever is characterized by defective cell-mediated immunity, thus highlighting the major role of cell-mediated immunity in the protection against *C.burnetii* (Angelakis E., Raoult D., 2010). During chronic Q fever the immune response is ineffective (Maurin and Raoult, 1999), and may also be harmful, causing leucocytoclastic vasculitis and glomerulonephritis (Rault, 1990). *C burnetii* continues to multiply despite high concentrations of all 3 classes of antibodies (IgG, M, and A) to phase I and II bacteria. Lymphocyte counts and the CD4-to-CD8 ratio are lowered (Sabatier et al., 1997). Organ biopsies do not show granulomas, but large vacuoles containing *C burnetii* can be detected in infected tissues, such as heart valves and liver and also in aneurysms (Maurin and Raoult, 1999). Monocytes from these patients are not able to kill *C burnetii (*Dellacasagrande et al., 2000), and do not migrate through the endothelium (Raoult et al., 2005). The most prominent lesion of chronic Q fever are endocarditis, aneurisms, osteomyelitis, chronic hepatis, pseudotumors of the lung or of the spleen (Tissot-Dupont and Raoult, 2007).

1.4.2 Immune response

Monocytes and macrophages are the major targets of *C. burnetii* and the intracellular survival of *C. burnetii* organisms requires the subversion of the microbicidal properties of

these cells (Capo and Mege, 2012). *C. burnetii* induces strong remodelling of the actin cytoskeleton when interacting with monocytes and macrophages via avβ3 integrins, with a final effect of reduction of the efficiency of bacteria uptakes (Capo et al., 1999). The internalization pathway is different for phase I (virulent), and phase II (avirulent) forms, because phase II attachment is mediated by both avβ3 integrins and complement receptor CR3. Therefore Phase II internalization is more efficient, resulting in better multiplication, thus explaining why phase II bacteria grow more rapidly than phase I, resulting in a shift from phase I to phase II in the laboratory (Raoult et al., 2005). Virulent *C. burnetii* organisms stimulate transient reorganization of filamentous actin (F-actin) and the formation of pseudopodal extensions that, on one side are associated with phagocytosis impairment and on the other hand are required for virulent *C. burnetii* entry in mononuclear cells (Mege, 2007). The low efficiency of virulent bacteria uptakes is probably critical for the persistence of *C. burnetii* in monocytes and macrophages, but besides low bacterial uptake, also a relative alteration in cytokines production is necessary for the persistence of the bacteria (Capo and Mege, 2012). A key element in promoting the persistence of *C. burnetii* in mononuclear cells is the secretion of IL10. The production of this cytokines has been demonstrated to increase *C. burnetii* replication in monocytes, to down-modulate TNF production and to affect the migration of immune cells to peripheral tissues. Moreover high levels of IL10 secretion has been found in patients with chronic Q fever and murine models have confirmed the key role of this cytokine in bacterial persistence (Maurin and Raoult, 1999).

Also Toll-like receptors (TLRs) play an important role in the immune response: TLR4, which recognizes lipopolysaccharides antigens (LPS), control the immune response against *C. burnetii* through granuloma formation and cytokines production, while TLR2, which recognizes peptidoglycan and lipopeptides, is involved in TNF and IFN-γ production

(Capo and Mege, 2012). The interaction of *C. burnetii* with macrophages, resulting in the reorganization of the cytoskeleton, depends largely from TLR4 and the *C burnetii*-stimulated formation of pseudopodes is prevented by the absence of TLR4 (Mege, 2007). Honestettre et al. (2004) demonstrated, using TLR4-deficient mice, that TLR4 controls the inflammatory response to *C. burnetii* leading to the formation of granuloma, while it has no microbicidal competence. The role of TLR4 in the control of granuloma formation may result from the modulation of the production of cytokines such as IFN-γ and TNF, known to be required for granuloma formation. The engagement of TLR4 leads to the production of type 1 cytokines required for protection against intracellular microorganisms, in contrast to TLR2 engagement that favors the production of type 2 cytokines. Therefore TLR4 is involved in the uptake of virulent *C. burnetii* by macrophages and is necessary for the formation of protective granulomas and the production of IFN-γ and TNF, but do not control the microbicidal activity of macrophages that involves other defense mechanisms (Honestettre et al. 2004).

Also TLR2 are involved in *C. burnetii* infection: Zamboni et al. (2004) showed that TLR2 are involved in TNF and IFN-γ production and that TLR2 activation interferes with *C. burnetii* intracellular replication, as macrophages from TLR2-deficient mice were highly permissive for *C. burnetii* growth compared with macrophages from wild type mice.

The activation of adaptive immune response in human is essential to cure *C. burnetii* infection. In primary infection the uptake of the virulent microrganism by macrophages and dendritic cells leads to the presentation of bacterial antigens to T lymphocytes and to the production by immune cells of IFN-γ and TNF, that induce the apoptosis of infected macrophages (Capo and Mege, 2012). In patients unable to mount a good IFN-γ response, like immunecompromized people or pregnant women, infected macrophages survives and their microbicidal activity is impaired, leading to a high risk to develop chronic Q fever

(Maurin and Raoult, 1999). The combination of IFN-γ production and granuloma formation in patients with primary *C. burnetii* infection suggests a Th1-type polarization of the immune response (Maurin and Raoult, 1999).

The action mechanism of IFN-γ is due to the restoring of phagosome-lysosome fusion in infected macrophages, to the promotion of these cells apoptosis and to the down-modulation of transferrin receptors, leading to a decreased assimilation of iron from the infected cells (Capo and Mege, 2012). Furthermore, during *C. burnetii* infection, monocytes exhibit a M1 type polarization, able to control bacterial replication, while in macrophages *C. burnetii* induces an atypical M2 profile, associated with the release of molecules like IL-10, that is unable to control bacteria replication. The secretion of IFN-γ has been demonstrated to reorients macrophages to a M1 proinflammatory polarization (Capo and Mege, 2012).

Chronic Q fever is characterized by defective cell-mediated immunity, thus emphasizing the major role of cell-mediated immunity in the protection against *C. burnetii*. Lymphocytes from patients with Q fever endocarditis do not proliferate in response to *C. burnetii* antigen, in contrast to lymphocytes from patients with acute Q fever (Koster et al., 1985).

The role of humoral immune response in *C. burnetii* infection has not yet been completely clarified. The current immunological paradigm suggests that humoral response is more effective in extracellular bacterial infection while the major protective immune response against intracellular bacteria occurs in cell mediated immunity. Large amounts of antibodies are produced in humans and animals infected with *C. burnetii*. Antibodies develop within 3-4 weeks from the onset of the disease: in humans the majority of the antibodies after primary infection are directed against phase II antigens and a similar model account for the response to Q fever vaccination, while increased levels of antibodies

directed against phase I antigens are related to chronic infection (Capo and Mege, 2012). Immunoglobulin M antibodies reactive with phase II *C. burnetii* appear rapidly, reach high titers within 14 days and persist for 10–12 weeks (Maurin and Raoult, 1999). Immunoglobulin M antibodies reactive with phase I antigens are usually at a much lower titer during acute infection. Immunoglobulin G antibodies reactive with phase II antigens reach peak titers about 8 weeks after the onset of symptoms, while those reactive with phase I antigens develop only very slowly and remain at lower titers than antibodies to phase II antigens, even after a year. In chronic Q fever, where there is persistence of organisms, the IgG titers to phase I and phase II antigens may both be high, and the presence of IgA antibody to phase I antigen is usually, although not exclusively, associated with chronic infection (Angelakis and Raoult, 2009).

In *C. burnetii* infection, a study from Humpres and Hinrichs (1981) showed that treatment of athymic mice with immune sera against *C. burnetii* had no effect on the bacteria replication within the spleen, suggesting that only cells mediated immunity plays a role in controlling the infection. On the other side two recent studies (Zhang et al, 2007; Shannon et al., 2009) demonstrated that vaccine induced antibodies are able to provide complete protection in immunocompetent mice when infected with *C. burnetii*. Several studies analyzed the ability of anti *C. burnetii* opsonizing antibodies to reduce the survival of the bacteria in mononuclear cells. These studies suggest that, although antibodies are able to increase the ability of phagocytes to uptake Ab-opsonized Coxiella, they did not affect the ability of phagocytes to control the organism replication (Zhang et al., 2012). Moreover Desnues et al. (2009) showed that macrophages incubated with *C. burnetii* cells opsonized with specific IgG antibodies, released higher amounts of IL10 and that *C. burnetii* opsonization increased bacteria replication. Briefly antibodies proved to protect naive mice from *C. burnetii* infection, but their activity is effective only if supported by the cell

mediate response that is essential for the clearance of *C. burnetii* infection (Zhang et al., 2012).

Few studies have been performed in animals concerning immune response and the great majority investigated the humoral immune response only in dairy cows.

Böttcher et al. (2011) observed in an endemically infected dairy herd the presence of 3 different patterns of antibody: phase I negative and phase II positive, phase I positive and phase II negative, phase I and phase II positive. The phase I negative and phase II positive was the predominant pattern found at the first sampling in cows 2-3 years old, but one year later there was an increase in the prevalence of the phase I and phase II positive pattern. This change in serological patterns could demonstrate a transition from acute to chronic infection. Another aspect that was highlighted in this study was that about 60 % of the cows older than 4 years remained seronegative despite the intensive shedding of *C. burnetii* in the herd and the frequent seroconversion in primiparous cows. This phenomenon was explained assuming that at least some of these multiparous cows built up an efficient cellular immunity with low or undetectable levels of antibodies (Böttcher et al., 2011). Another abnormal pattern of humoral immune response in cattle is the presence of seronegative cows, shedding *C. burnetii*, as reported by several authors (Guatteo et al., 2007; Rousset et al. 2009a; Boettcher et al., 2012). Guatteo et al. (2007) found that in this cows the shedding was sporadically or intermittent while cows with a high serological titer were persistent shedders. A possible explanation of this behavior is that also these cows had built up an efficient cellular immunity against *C. burnetii,* so shedding of the bacteria is only sporadic.

Pregnancy has been considered by some authors (Böttcher et al 2011; Nogareda et al., 2012) an important event in determining the outcome of infection by *C. burneti*i in cows, because during pregnancy, a shift of immunity towards TH2-activity with elevated levels

of immunosuppressive IL-10 occurs: TH2-activity drives the immune response towards an unfavorable direction to control an intracellular parasite like *C. burnetii* and IL-10-mediated immune suppression could also activate *C. burnetii* in persistently infected cows during pregnancy. The hypothesis that pregnancy is an important trigger relies on non-immune heifers at the time of first pregnancy (Böttcher et al., 2011).

1.5 CLINICAL SYMPTOMS AND PATHOLOGY

1.5.1 Animals

The natural history of *C. burnetii* infection in cattle, sheep and goats is that a non-immune (often neonatal) animal is infected from the environment, often contaminated by parturient or other animals shedding the organism and undergoes a primary infection with weak clinical symptoms. However, the organism can persist after initial acute or subclinical disease, being shed in large numbers when the persistently infected female animal becomes pregnant (Harris et al., 2000).

There is very little information about the clinical signs of Q fever in domestic animals. Q fever clinical symptoms in animals as in humans are usually non-specific and often relatively mild. Despite the earlier reports of respiratory disease due to *C. burnetii* in sheep, goats and cattle (Babudieri, 1953; Aitken, 1989), the only clinical disease of domestic animals attributable to *C. burnetii* is abortion in naturally and experimentally infected sheep, goats and cattle (Woldehiwet, 2004). Abortion is more frequent in goats and sheep than in cows, while infection in dairy cattle is considered to reduce fertility (Lang et al., 1991). All pregnant ruminants are highly susceptible to infection and the abortions occurs only at the first parturition after infection, while the following gestations terminated normally without any reproductive failures. In goats, *C. burnetii* can induce pneumonia as well as abortion with stillbirth and delivery of weak kids being the most important clinical signs (Berri et al., 2007). Q fever abortions in caprine herd are more important than in sheep flocks, affecting sometime up to 90% of females (Palmer et al., 1983). Furthermore in goats, unlike in sheep, pregnancy subsequent to *C. burnetii* abortion may not be carried to term (Berri et al., 2007).

An investigation by Berri et al. (2007) also showed that *C. burnetii* was excreted in birth products of either affected goats or females that had normal delivery and that they continued to shed the organism long after the outbreak. Although most of the females had a normal delivery, *C. burnetii* was highly excreted at the second kidding season as the bacteria were found in vaginal swabs taken from 94% of the tested goats. In addition, this study showed that goats shed *C. burnetii* at successive parturitions suggesting that these goats could be chronically infected (Berri et al., 2007).

In chronically infected ruminants, it is not clearly understood how and where *C. burnetii* persists in the non-pregnant period and which mechanism initiated the bacteria multiplication in the placenta. Similar latent persistence and recrudescence of Q fever occurs in humans but the organ site for latent infection is still unknown (Berri et al., 2007). However, in a prolonged Q fever post infection fatigue syndrome (QFS), the bone marrow was identified as potential focus of *C. burnetii* infection from which placenta and other sites such endocardium may be seeded for recrudescent infection (Harris et al., 2000; Marmion et al., 2005).

In dairy cattle the others clinical symptoms reported in literature further than abortion are placenta retention, metritis, and mastitis. Positive correlation between seropositivity to *C. burnetii* and placenta retention was found by Lopez-Gatius et al. (2012).

Some authors have reported an increased prevalence of metritis in seropositive animals (To et al., 1998), while others didn't detect such relationship (Muskens et al., 2011). Considering that the detection of *C. burnetii* in the uterus may often happen in healthy animals, also this finding in case of metritis could not be so resolutive to demonstrate a link between metritis and *C. burnetii* infection.

In a study performed on an infected dairy herd in USA, *C. burnetii* shedding in milk was associated with chronic subclinical mastitis in cows demonstrated to be free of infection caused by common aerobic mastitis pathogens (Barlow et al, 2007).

Significant macroscopic pathological lesions reported in animals naturally infected with *C. burnetii* are rare. Usually there are no macroscopic fetal lesions, but the infected placenta can exhibit the following histopathological features: infiltration of the chorionic stroma by mononuclear cells, necrosis of chorionic trophoblasts and focal exudation of fibrin and neutrophils. A significant statistical association was found between these lesions in cases of bovine abortion and the presence of *C. burnetii* demonstrated by immunohistochemical (IHC) test (Bildfell et al., 2000). In another study (Hansen et al., 2011) the microscopic examination of cotyledonary sections from infected placenta showed only minor lesions or absence of lesion at all. The findings suggest that significant *C. burnetii* associated lesions of the bovine placenta at parturition are rare. The absence of severe lesions indicates that placental dysfunction is not a feature of late term placental coxiellosis in cattle and this may explain why increased stillbirth rates have not been reported in *C. burnetii* infected cattle herds (Hansen et al., 2011).

In pregnant goats after experimental infection necrotic and suppurative placentitis were observed from day 130 of gestation. The placenta observed after the abortion were characterized by multifocal necrosis of the chorionic epithelium and severe suppurative inflammation at the base of the villi. The inflammatory exudate was composed mainly of neutrophils, with occasional macrophages (Sanchez et al., 2006). In the same study the only fetal organ in which lesions were observed was the liver, which usually showed mild-to-moderate perivascular hepatitis with neutrophils and lymphocytes surrounding the vessels. Neutrophils sometimes formed foci or appeared as a diffuse infiltrate in the hepatic parenchyma.

Other fetal lesions, described in literature are the presence of peribronchiolar, renal medullary and hepatic portal lymphocytic aggregates (McGavin and Zachary, 2006).

1.5.2 Humans

The main characteristic of Q fever in humans is its clinical polymorphism, therefore the diagnosis is quite difficult and a laboratory confirmation test is needed.

Gender and age also affect the expression of *C. burnetii* infection. Men are symptomatic more often than women despite comparable exposure and seroprevalence (Tissot-Dupont and Raoult, 1992; Maltezou and Raoult, 2002). Moreover, the prevalence of clinical cases in children significantly increases with age and symptomatic Q fever occurs more frequently in people over 15 years old (Maltezou and Raoult, 2002).

Acute Q fever

The incubation period has been estimated to be approximately 20 days (range, 14–39 days). There is no typical form of acute Q fever and the clinical signs vary greatly from patient to patient. The most frequent clinical manifestation of acute Q fever is probably a self-limited febrile illness (91%) which is associated with severe headaches (51%), myalgias (37%), arthralgias (27%) and cough (34%) The main symptoms fever, pulmonary signs, and elevated liver enzyme levels can coexist. Atypical pneumonia is also a major clinical presentation and abnormal chest X rays can be found in 27% of the patients (Tissot-Dupont et al., 2007). Atypical pneumonia is one of the most commonly recognized forms of acute Q fever. Most cases are clinically asymptomatic or mild, characterized by a nonproductive cough, fever and minimal auscultatory abnormalities, but some patients present with acute respiratory distress. Pleural effusion can also appear (Raoult et al., 2005).

Hepatitis is also very frequent in predominately 3 forms: an infectious hepatitis-like form

of hepatitis with hepatomegaly but seldom with jaundice, clinically asymptomatic hepatitis, and prolonged fever of unknown origin with characteristic granulomas on liver biopsy. Q fever hepatitis is usually accompanied clinically by fever and less frequently by abdominal pain, anorexia, nausea, vomiting, and diarrhea (Marrie and Raoult, 1987; Marrie, 1988).

Cardiac involvement is found in 2% of the acute Q fever cases and myocarditis is the leading cause of death (Fournier et al., 2001).

Skin lesions have been found in 5–21% of Q fever patients in different series. The Q fever rash is nonspecific and may correspond to pink macular lesions or purpuric red papules of the trunk (Maurin, 1999).

Among neurological symptoms 3 major entities associated with Q fever have been described: meningoencephalitis or encephalitis; lymphocytic meningitis and peripheral neuropathy (Bernit et al, 2002).

According to the literature, 60% of infected patients are asymptomatic, while 20% develop mild symptoms. The remaining 20% (40% symptomatic) present with a self-limiting flu-like illness with some more severe manifestations including high fever, severe headache, night sweating, nausea, diarrhea, pneumonia,, hepatitis, pericarditis, myocarditis, neurological symptoms and weight loss (Mertens and Samuel, 2007; Angelakis, 2010).

The acute illness spontaneously resolves after 2-6 weeks. However the organism may persist in the bone marrow and the disease can reactivate after appropriate stress. Acute Q fever may develop into chronic illness in 2% of patients (Frode-Forland et al.,, 2010) and can express itself in different forms (Wildman et al., 2002a; Wildman et al., 2002b; Karakousis et al., 2006).

Chronic Q fever

Chronic Q fever may develop many months to years after initial infection. It occurs almost

exclusively in patients with predisposing conditions, including those with heart valve lesions, vascular abnormalities, cancer and immunosuppression. Pregnant women are at high risk of a developing chronic infection (Fenollar et al., 2001). Chronic Q fever manifests mainly as bacterial culture negative endocarditis in up to 48% of cases (Houpikian and Raoult, 2005). Recrudescent granulomatous infection can also occur. Another long term effect of Q fever is the post-Q fever fatigue syndrome (QFS). Up to 60% of patients may experience QFS symptoms which can persist for 6-12 months and then spontaneously resolve (Ayres et al., 1998).

1.6 DIAGNOSIS

1.6.1 Animals

Diagnosis of Q fever based on clinical symptoms or post-mortem examination is almost impossible due to unspecific or missing symptoms or lesions caused by the disease. For this reasons the laboratory diagnosis is the only reliable way to confirm the presence of *C. burnetii* in domestic or wild animals. Several assays have been described for the diagnosis of *C. burnetii* in animals, including both direct identification of the agent and serological testing.

Direct identification of the agent

The most useful samples for the detection of *C. burnetii* in livestock are vaginal mucus, placenta or foetal tissue (Sidi-Boumedine et al., 2010). Samples should be collected from aborted fetuses, placenta and vaginal discharges soon after abortion or parturition. Milk, colostrum and feces samples can also be taken but they are not reliable to detect clinically affected herds or flocks (Sidi-Boumedine et al., 2010). For direct identification of *C. burnetii*, sampling should be targeted at pregnant animals either giving birth normally or aborting. This is because infected female animals, even with normal parturition, are high shedders of *C. burnetii* into birth products (Arricau-Bouvery et al., 2003). The sampling should be carried out as soon as possible after parturition or abortion and more precisely within a week because the shedding level of the bacteria decreases sharply after that time (Sidi-Boumedine et al., 2010), also if *C. burnetii* shedding may persist over several months (Kim et al., 2005; Berri et al., 2007).

Conventional staining techniques (Stamp, Gimenez, Macchiavello, Giemsa and modified Koster) are available within the context of the diagnostic of abortion and are used on tissues from fetus or placenta and on vaginal discharge. These tests have low diagnostic

sensitivities and specificities. They need attention because *C. burnetii* can be confused with *Chlamydophila abortus* or *Brucella* spp (EFSA, 2010).

Detection of *C. burnetii* can also be achieved with IHC. The method uses either indirect immunofluorescence or an immunoperoxidase assay, using polyclonal *C. burnetii* antibodies (either a well characterized antiserum of human origin or a specific antiserum produced in rabbits or guinea pigs). An anti-species (human, rabbit or guinea pig) anti-IgG conjugate labeled with Fluorescein isothiocyanate (FITC) or peroxidase is then used to visualize the bacteria (EFSA, 2010). No specific antibodies for IHC are commercially available (OIE, 2015). IHC in placenta was used by Hansen et al. (2011) with paraffin-embedded section that included a central area of the cotyledon, margin and adjacent chorion laeve. Antibodies against *C. burnetii* were produced by intraperitoneal inoculation of mice with Nine Mile strain and immunoperoxidase used to stain bacteria.

Isolation of *C. burnetii* could be performed from fetuses, placenta, vaginal swabs, milk and feces.

Samples can be refrigerated at +4° C before shipping to the laboratory only if they are delivered at least 24 hours after collection, otherwise they should be frozen at -18° C immediately after sampling and then shipped to the laboratory (Vicenzoni and Barberio, 2013).

Isolation of *C. burnetii* can be done by cell or embryonated chicken egg culture. Such isolation is possible when microscopic examination indicates a large number of *C.burnetii* and a low level of contamination. With heavily contaminated samples, such as placentas, vaginal discharges, feces or milk, the inoculation of laboratory animals may be necessary. Mice and guinea pigs are the most appropriate (EFSA, 2010). *C. burnetii* is classified as a biological agent of level 3: for this reason all methods involving manipulation and replication of live bacteria should be performed in level 3 containment facility (WHO,

2004). However this kind of facility is available only in few laboratories so isolation is usually not adopted in routine diagnostic, but only in specific studies that require the isolation of the microrganism.

Currently the PCR is one of the most sensitive and rapid means for the direct detection of *C. burnetii* and the identification of shedding animals. PCR is adapted to a wide range of samples like vaginal discharge, abortion material, feces and milk (bulk or individual). It is sensitive and rapid and is becoming increasingly common in diagnostic laboratories (Berri et al., 2000; Nicollet and Valognes, 2007).

The level of detection of conventional PCR is related to the sample under investigation (1–500 bacteria/ml of milk; 1 bacteria/mg of feces). Several target genes have been used, such as the multicopy insertion sequence IS1111 or single copy genes encoding various proteins (e.g dismutase [sodB]; com1 encoding a 27 kDa outer membrane protein; heat shock proteins [htpA and htpB]; isocitrate dehydrogenase [icd]; macrophage infectivity potentiator protein [cbmip]) (EFSA, 2010).

The development of real-time PCR technology has recently allowed the quantification of *C. burnetii* in samples using a logarithmic scale (Pfaffl, 2001). Real-time PCR techniques have been described by several authors (Stemmler and Meyer, 2002; Kim et al., 2005; Klee et al., 2006;).

Real-time PCR is considered the most sensitive and rapid mean for the identification of animals shedding *C. burnetii*. The specificity levels of different laboratories for the detection of *C. burnetii* DNA in different spiked matrices (PBS, placenta, milk and aborted fetuses) and of different protocols are comparable (Duquesne et al., 2008; Jones et al., 2011). Regarding sensitivity, PCR tests directed against the multiple-copy target IS1111 (real-time and conventional) were found to be superior to tests detecting single-copy genes. Although a threshold for quantitative real-time PCR is not officially approved at

international level a group of French experts has suggested that abortion in ruminants should be confirmed to be caused by *C. burnetii* when at least 10^4 bacteria per gram of placenta or vaginal swabs are detected (Touratier et al., 2007). In tissues or stomach contents from aborted foetuses, the same group considered that a positive result by quantitative PCR is sufficient to diagnose Q fever as the origin of abortion. For pooled samples, the proposed threshold is 10^3 bacteria per pool. These thresholds are indicative and may be revised especially if new scientific information becomes available (Sidi-Boumedine et al., 2010).

Molecular characterization of strains is crucial to compare genotypes isolated from different animal species, to trace outbreaks and to assess relationships between genotype and virulence of the strains with a special regard to public health (Ceglie et al., 2015).

Several typing methods have been used for the characterization of *C. burnetii* strains, including restriction endonuclease of genomic DNA (Hendrix et al., 1991), pulsed-field gel electrophoresis (Heinzen et al., 1990; Jager et al., 1998).

More recently the availability of complete genome sequences has allowed to apply to this bacterium many highly discriminatory methods, mainly based on molecular techniques like multispacer sequence typing (MST), IS1111-element positioning, infrequent restriction site-PCR (IRS-PCR), multiple loci variable number of tandem repeat analysis (MLVA) and single nucleotide polymorphism (SNP) (Vergnaud and Pourcel, 2006; Massung et al., 2012). Recently, researchers from different countries have applied the MLVA method to *C. burnetii* strains because of its quite high discriminatory power and its applicability either to isolated bacterial strains or directly to DNA extracted from clinical samples (Arricau-Bouvery, 2006; Svraka, 2006). French researchers identified within the *C. burnetii* genome a total of 17 markers different minisatellite and microsatellite to be used

in MLVA genotyping in 2 successive panels. A third panel was implemented during the Dutch *Coxiella* outbreak, involving 6 microsatellites (Tilburg, 2012).

Indirect identification of the agent

Q fever serology is used for different veterinary research objectives throughout the world, but is far from being standardized. There is no officially prescribed test for the serological diagnosis of Q fever. Serological antigens are based on the two major antigenic forms of *C. burnetii*: phase I, obtained from spleens after inoculation of laboratory animals and phase II, obtained by repeated passages in embryonated eggs or in cell cultures. Currently available commercial tests allow the detection of phase II or of both phases II and I anti-*C. burnetii* antibodies (OIE, 2015).

Among the various techniques that can be employed, the 3 most common are CFT, IFA and ELISA.

The CFT was considered the reference test for historical reasons but its diagnostic sensitivity was highly variable (Roest et al., 2011). Several studies showed that the CFT has a low relative sensitivity, but conversely it has a high specificity for the high levels of anti-*C. burnetii* antibodies generated in a Q fever aborted herd or flock (Rousset et al., 2007; Kittelberger et al., 2009; Horigan et al., 2011; Natale et al., 2012; Emery et al., 2014).

In goats a study by Rousset et al. (2007) showed that CFT was less performing than ELISA and IFA, giving a large proportion of dubious results (71%), whereas these same sera gave positive results with ELISA. Moreover, no association was found between positive (or strongly positive) CFT results and Q fever abortion, therefore the authors did not recommend the use of this test for serological screening, because of its low sensitivity. Several reason could explain the poor test performance of CFT. The antigen used for CFT, obtained from Nine Mile strain, utilizes only phase 2 antigens (Porter et al., 2011).

Moreover CFT can fail to detect antibodies when anti-complementary substances are present in the tested sera and can't detect all IgG subclasses. In ruminants for example only IgG1 antibodies are known to fix the complement in CFT (Micusan and Borduas, 1977; Schmeer, 1985) and the presence of IgG2 and IgM antibodies can suppress complement fixation by IgG1 antibodies (Schmeer, 1985).

The IFA adapted as a micro-immunofluorescence technique is the current method for the serodiagnosis of Q fever in humans (Tissot-Dupont et al., 1994). Both phase I and phase II *C. burnetii* antigens are used; phase II antigen is obtained by growing *C. burnetii* Nine Mile reference strain in cell culture, while phase I antigen is obtained from the spleens of laboratory animals. The test can be adapted to the use in veterinary medicine by replacing the human conjugate with a conjugate adapted to the animal species (OIE, 2015). IFA showed in a study on goats (Rousset et al., 2007) a better sensitivity than CFT and a good agreement with ELISA, both qualitatively, in the detection of positive animals and quantitatively, in term of correlation between IFA titers and ELISA optical densitiy . The study also reported that IFA results obtained on sera of aborting goats and of non-aborting goats were significantly different and were associated with occurrence of abortion. Currently, IFA is not commercially available for animals so the test should be prepared in house following the OIE method description. Therefore IFA in respect to ELISA has the disadvantage of being less reproducible between operators and laboratories (OIE, 2015).

ELISA is the most used test in animals for the detection of anti-*C. burnetii* antibodies because is a sensitive technique, easy to perform and standardize.

The ELISA is preferred to IFA and CFT, particularly for veterinary diagnosis, because it is convenient for large-scale screening and the most robust. Ready-to use kits are commercially available and can detect mixtures of anti-phase I and II antibodies (OIE, 2015).

The antigens present in commercial ELISA are of two possible origins: antigens of the American Nine Mile strain of *C. burnetii* isolated from an endogenous tick, or antigens of a strain originating from infected European domestic ruminants. ELISA kits coated by the latter antigens are more sensitive and are advised for serological diagnosis (Rodolakis, 2006). The commercial ELISAs available for veterinary diagnostic purposes detect total antibodies and do not differentiate anti-phase 1 and anti-phase 2 antibodies but a prototype with PhI- and II antigens coated separately is produced on demand by Ideex (Boettcher et al., 2011).

The ELISA commercial kits currently available for the diagnosis of Q fever in ruminants are 3. Comparative analyses of available serological methods have been conducted during a ring trial assessments as part of a EU-funded, Framework 6 project. The IFA and commercially available ELISAs were each reproducible, with comparable diagnostic sensitivity (EFSA, 2010).

The analytical sensitivity of all the different ELISA was found to be 8-16 times higher than that of the best CFT (Roest et al., 2011). Based on recent work, it was found that two commercial ELISAs can display different diagnostic sensitivities (81 and 95%, respectively) using a panel of sera from cattle, goat and sheep (Kittelberger et al., 2009).

In goats a strong association between abortion and the occurrence of strongly positive ELISA results was demonstrated when sera were obtained from goats 15 days after the abortions and not later (Rousset et al., 2007). A study performed in Poland on ruminants (cattle, sheep and goats) demonstrated a moderate relationship between the identification of *C. burnetii* in fetus or placenta by real-time PCR, and ELISA positive results (r = 0.37–0.48) (Niemczuk et al., 2014), while in a similar study performed in Italy a poor relationship was found (r = 0,01-0,16) (Natale et al., 2012).

A study performed on dairy cattle showed that persistent shedders cows had mainly a persistently highly seropositive status and that around 50% of persistently highly seropositive cows were found to be persistent shedders, while non highly seropositive cows were mainly either non- or sporadic shedders (Guatteo et al., 2006).

Diagnostic approach to the disease

The serological methods are useful for carrying out preliminary surveys of infection at herd level but they do not allow for the identification of *C. burnetii* shedding animals. When the positive serological results are found at herd level, the PCR is the method of choice to trace shedders. However, it should not be forgotten that if there is suspicion of infection or shedding of *C. burnetii* despite the absence of serological response, the test for pathogen detection (PCR or culture) should be performed (Niemczuk et al., 2014), because a significant proportion of animals shedding *C. burnetii* bacteria and even some Q fever aborted animals, are found to be seronegative (Guatteo et al., 2007; Rousset et al., 2007; Rousset et al., 2009a; de Cremoux et al., 2012a).

None of the 3 serological available tests can be used to accurately discriminate an abortion case from a normal delivery at individual level. For these reasons Q fever diagnosis in animals should not rely on a unique diagnostic approach. The global clinical and epidemiological context must be taken into account as well as the limitations of diagnostic assays (Porter et al., 2011).

In case of suspect the diagnosis of Q fever should involve the use of multiple techniques and can be interpreted validly only at herd or flock level.

In order to identify *C. burnetii*, sampling should be performed on pregnant animals either giving birth normally or aborting. This is because infected female animals, even with normal parturition, are high shedders of *C. burnetii* into birth products (Arricau-Bouvery et al., 2003). *C. burnetii* shedding may persist over several months (Berri et al., 2005; Kim et

al., 2005). However, the shedding level of the bacteria decreases after parturition or abortion. Thus, sampling should be carried out within the week following abortion or parturition. The identification of the presence of the bacteria in the vaginal mucus of animals having aborted, or in their foetuses, by molecular methods, will then be more reliable. (Sidi-Boumedine et al., 2010).

The serological analyses for the diagnosis of Q fever should be based mainly on the ELISA test. A minimum number of 6 animals having aborted or shown reproductive problems should be taken. These animals should include multiparous and primiparous females which experienced abortions between at least 15 days to a maximum of 3 weeks before. Serology should be used as a complement to the PCR and carried out, preferably, by means of a test using antigens from a ruminant *C. burnetii* isolate (Sidi-Boumedine et al., 2010).

1.6.2 Humans

Q fever clinical symptoms in humans are usually non-specific and often relatively mild; hence, classical differential diagnosis must be supported by laboratory tests for accurate diagnosis of clinical disease. For the direct and indirect diagnosis in humans the methods used are the same described for animals, but the main difference is that in human medicine the diagnosis of Q fever is based on serology, which allows for differentiation between acute and chronic cases (Tissot-Dupont and Raoult, 2007).

Serology in humans can differentiate between acute and chronic infection of *C. burnetii* because phase I and phase II antigens vary depending on the clinical progression of infection. Each phase has a different antigen profile:

- ✓ in acute Q fever, the immune response is primarily driven by IgM and IgG antibodies directed against the avirulent form of *C. burnetii* (phase II).

- ✓ in the chronic form, IgG and IgA antibodies predominate and are directed against both the virulent and avirulent forms of bacteria (phase I).

Acute infection is therefore characterized by elevated phase II antibody levels and it is generally first detectable after the second week of illness.

In chronic Q fever, typically the opposite is true: phase I antigens significantly predominate over phase II.

This happens because antibodies to phase I antigens of *C. burnetii* generally require longer to appear and indicate continued exposure to the bacteria. Thus, high levels of antibody to phase I in later specimens, in combination with constant or falling levels of phase II antibodies and other signs of inflammatory disease, suggest chronic Q fever. Antibodies to phase I and II antigens have been known to persist for months or years after initial infection (Maurin and Raoult, 1999).

The most commonly used serological methods are, like in veterinary medicine:

- ✓ ELISA,
- ✓ CFT
- ✓ IFA.

IFA is commonly considered the reference diagnostic test and is the most frequently used worldwide (Tissot-Dupont and Raoult, 2007). It is accurate, highly sensitive, and specific (Fournier et al., 1998).

Both phase I and phase II *C. burnetii* Nine Mile strain are used as antigens and antibodies of the IgG, IgM, and IgA subclasses can be determined. Sera are screened by microimmunofluorescence at a1:50 dilution with phase II antigens. Positive sera found on screening are serially diluted and then tested on both phase II and phase I antigens for the presence of IgG, IgM, and IgA (Maurin and Raoult, 1999).

A titer > 200 for IgG and > 50 for IgM against phase II antigen indicate a recent Q fever infection, while an IgG titer > 800 against phase I antigen suggests chronic infection

(Fournier et al, 1998). IFA titers usually reach their maximum levels 4 to 8 weeks after the onset of acute disease and then decrease gradually over the following 12 months. The persistence of high levels of anti-phase I antibodies or the reappearance of antibodies after treatment my signal the development of chronic infection (Tissot-Dupont and Raoult, 2007).

1.7. DISEASE CONTROL

There are 3 broad measures to limit transmission and spread of directly transmissible infections in populations: reducing the adequacy of contact between individuals (animal-animal, animal-human, human-human), reducing the proportion of the population susceptible through vaccination and decreasing the infectivity of infected individuals through vaccination or other medical treatment (EFSA, 2010). All 3 measures should be implemented to control the spread of *C. burnetii* infection. The first type of measure is defined as direct prophylaxis, while the second and the third are described as indirect prophylaxis.

It should be highlighted that control measures for *C. burnetii* can only be effectively implemented if cases are detected and confirmed. This requires systematic and reliable classification of units/farms as cases. The basis for such a classification is a commonly agreed case definition. Case detection can be based on suspect case reporting (passive surveillance) or screening (active surveillance) (EFSA, 2010).

Then the choice of a Q fever control strategy will depend on the overall goal of the control effort. This could, for example, be limited to avoiding severe cases or focus on problem farms, or at the other end of the spectrum, attempt complete eradication of *C. burnetii* infection in the entire population (EFSA, 2010).

1.7.1 Direct prophylaxis measures

The main concept behind direct prophylaxis is that the optimal control strategy may require a combination of several control interventions. This control strategy should be applied through measures to:

- ✓ Reduce the number of contacts per unit time. A broad range of measures are commonly applied to limit contact during animal disease control, including quarantine, test and slaughter, livestock movement control, changes in farm management etc.
- ✓ Reduce the transmission potential per contact. Due to the feature of the infectious agent, it is generally difficult to influence the transmission potential within populations. However, these measures can be very important to limit the transmission potential per indirect contact between different flocks (hygienic measures).
- ✓ Reduce the number of different farms in contact through trade restrictions (EFSA, 2010).

All these measures are not specific to Q fever only and the efficacy of each to reduce the infectious pressure or the *C. burnetii* transmission between animals and herds remains unknown. Case control studies allowing the identification of risk factors for Q fever outcome may not be reliable enough, due to the uncertainty about the true status of the control. A survey conducted in almost 100 infected dairy herds reported 2 main factors associated with an increase of seroprevalence: the introduction of more than 10 animals in the herd per year and the absence of disinfection of the calving pen after each calving (Taurel et al., 2009).

Control measures should pay attention to the bedding material as a source of *C. burnetii* transmission among animals and from animals to humans, as described in several studies (Manfredi_Selvaggi, 1986; Arricau-Bouvery et al., 2001; Berri et al., 2004; Starnini et al., 2005). In The Netherlands, spread of manure from infected herds was forbidden for at least 90 days after suspicion of infection (Schimmer et al., 2008). The effectiveness of this measure must be evaluated and modified if necessary. Arricau-Bouvery et al. (2001)

performed decontamination of feces of experimentally infected goats with calcium cyanamid. However there are considerable differences in methods of manure management, consequently a specific standardized protocol has not yet been established for bedding manure treatment and decontamination.

The following systems were assessed for effectiveness:

- ✓ Deep litter systems, where goats are kept indoors on straw litters, straw is added regularly and removed only 3-4 times a year. The manure is usually moved to another location in or out of the farm.
- ✓ Slurry treated with cyanamide calcium,
- ✓ Manure composting for a period of time with or without covering (EFSA, 2010).

Composting is a manure fermentation process that kills bacteria as the temperature rises. Traditional composting consists in piling manure within concrete walls and fermentation for 3 months. The inside temp is estimated to be a minimum of 50° C and this process will lead to a minimum of $4.3x10^{-7}$ reduction of C. burnetii.

Manure must be covered and composted or treated with lime or calcium cyanamide 0.4% before being spread on the field; spreading of manure should never be performed when the wind blows (Arricau-Bouvery and Rodolakis, 2004).

Abortions have been identified as an important risk factor for herd or flock status. Consequently, husbandry practices that control the exposure of animals to infectious doses of *C. burnetii*, such as segregation of areas for calving, lambing and kidding as well as removal of placenta and abortion materials, are useful to reduce bacterial exposure (EFSA, 2010).

The removal of these materials that have the potential to contain very high numbers of *C. burnetii* to specific rendering plants could reduce the environment contamination. The parturition pens must be disinfected as well as every utensil used for delivery (Arricau-

Bouvery and Rodolakis, 2005).

In the context of Q fever, the introduction of infected animals into naïve herds could be avoided if there were control of movement of animals of defined status between herds of defined status. However, this strategy is reliant on the reliable classification of animals and herds (for example, a farm-level certification system including testing scheme, cut offs, status definition etc). Given the diagnostic quality of currently available diagnostic tests however, considerable uncertainty is likely to remain as to the true status of an animal or herd (EFSA, 2010).

A number of Q fever outbreaks in humans have been associated with the shearing of sheep (Hellenbrand et al., 2001; Hellenbrand et al., 2005). Sheep wool can be heavily contaminated with infected birth products. Dust containing *C. burnetii* is produced during shearing and bacterial DNA can be found in the air of barns where sheep have been shorn (Schulz et al., 2005). In such situations, shearing personnel should wear protective filter masks and the fleece should be kept wet or even disinfected. (EFSA, 2010).

Drinking milk containing *C. burnetii* can result in sero-conversion although it remains unclear as to whether, and if so, to what extent, clinical disease can result from the consumption of milk or dairy products or of other foods containing *C. burnetii* (EFSA, 2010). Therefore pasteurization at 72 °C during 15" or sterilization of milk from infected flocks is regularly recommended even if the oral route is not the main one (Arricau-Bouvery and Rodolakis, 2005).

1.7.2 Indirect prophylaxis measures

Vaccination

Current vaccines used in humans and animals include formalin-killed, whole-cell vaccine preparations (WCV) (Marmion et al., 1990) and chloroform methanol-extracted bacterial residue (CMR) (William et al, 1986; Waag et al., 1997). A WCV from the Henzerling

strain (Q vax, CSL Limited, Parkville, Victoria, Australia) has been commercially available and used for human vaccination in Australia since 1989 (Arricau-Bouvery and Rodolakis, 2005). The vaccine has been licensed in Australia to protect at-risk slaughterhouse employees and veterinary professionals. The vaccine can only be given to people not previously in contact with *C. burnetii*, as vaccinating subject that have already mounted an immunological response may lead to serious adverse reaction like systemic symptoms of inflammation. In the Netherland, during the epidemic of 2007-2011, 1354 people were vaccinated, all from the defined high risk patient group, people with heart or severe vascular disease (Van der Hoek et al., 2012).

In animals the most effective vaccines are those composed of inactivated whole phase I bacteria (Arricau-Bouvery and Rodolakis, 2005).

An inactivated non-adjuvanted phase I *C. burnetii* antigen Nine Mile strain vaccine (Coxevac, CEVA Santé Animal, France) is available for goats and cattle in Europe.

In goats the efficacy of vaccination with phase I and phase II vaccines was compared, using this phase I (COXEVAC™) vaccine and a phase II vaccine (CHLAMYVAX FQ™), in pregnant animals, experimentally infected with a dose of *C. burnetii* sufficient to cause abortion or premature birth in 85% of the goats in the control group (Arricau-Bouvery et al., 2005). the phase I vaccine significantly protected goats against the development of *C. burnetii* infection causing abortions, as it reduced placental colonization, eliminated milk shedding and strongly reduced vaginal and fecal shedding of *C. burnetii* particles. In contrast, the phase II vaccine did not showed any difference compared to the control group (Arricau-Bouvery et al., 2005).

Vaccination with Coxevac is targeted in all goats >3 months of age and should be performed at least 3 weeks prior to breeding (EMA, 2010). Booster vaccines are recommended every 280 days thereafter, but the exact duration of immunity has yet to be

determined (de Cremoux et al., 2012b). The immunogenicity of commercially available vaccines in open does and ewes may be more effective than in those pregnant for long-term control of within-herd *C. burnetii* spread (Porter et al., 2011). However, the efficacy to prevent shedding from infectious animals needs to be assessed if vaccination is to be a method of public health intervention.

In a systematic review and metanalysis about phase I vaccine (O'Neill et al., 2013) all publications reviewed but one (Hogerwerf et al., 2011) reported preventive vaccination exclusively to reduce the risk of shedding or clinical effects of *C. burnetii* infection following vaccination. During the Dutch Q fever outbreak, Hogerwerf et al. (2011) reported farms vaccinated reactively to reduce shedding and clinical effects or to prevent infection or subsequent clinical effects.

Previous studies have shown that shedding is greatest at the first and second parturitions after infection in both goats and sheep, with most animals becoming infected in the first year of life (Hogerwerf et al., 2011; Porter et al., 2011).

Vaccination with the phase I vaccine was also associated with prevention and/or reduction of shedding in ruminants (Hogerwerf et al., 2011), especially when applied on primiparous and/or susceptible goats (De Cremoux et al., 2012b) or susceptible non-pregnant dairy cows (Guatteo et al., 2008). Non-pregnant and uninfected dairy cows when vaccinated had a 5 times lower probability of becoming a shedder than an animal receiving a placebo. On the contrary cows vaccinated when pregnant had a similar likelihood of becoming shedder as an animal receiving the placebo. The authors explained this outcome of their study assuming that pregnancy had an adverse effect on the immune response of the cow (Guatteo et al., 2008). However, several studies reported that phase I vaccines failed to prevent shedding of *C. burnetii* in naturally infected prior to vaccination in cows, goats and sheep (Guatteo et al., 2008; Rousset et al., 2009b; Astobiza et al., 2010a), highlighting the

role of the vaccine in protecting uninfected but not in treating infected animals.

Coxevac is registered for use in non pregnant cows to reduce excretion of *C. burnetii*. Under field conditions however it is frequently used out of label in pregnant cows to prevent shedding in a contaminated environment. In these terms the vaccine safety on pregnant animals it's fundamental. Guatteo et al. (2008) observed in their study that the use of Coxevac in pregnant animals had no impact on pregnancy in cattle.

Therefore vaccination cannot be considered as a classical treatment and a significant reduction of shedding in infected animals was not demonstrated. Also based on current diagnostic tests, it is not possible to serologically distinguish vaccinated and naturally-infected animals. Nevertheless, vaccination on infected herds allowed to prevent the appearance of clinical signs and to significantly decrease the zootechnical losses to Q fever (Guatteo et al., 2008; EFSA, 2010; Guatteo et al., 2012).

Antibiotic treatment

Antibiotic treatment is used effectively in humans to reduce clinical symptoms associated with Q fever. Doxycycline, 100 mg twice daily for 14 days (Dumler et al., 2002) is recommended for acute illness. Antibiotic treatment lessens the time in which the patient has fever, (Gikas et al., 2001) and hastens recovery from pneumonia (Marrie, 2003). For endocarditis has been recommended a 18 months treatment of doxycycline (100 mg, twice daily) and hydroxychloroquine (200 mg, three times daily) (Maurin and Raoult, 1999). Chloroquine raises the pH in the phagolysosome increasing the efficacy of doxycycline ((Raoult et al., 2005).

In animals, antibiotic treatment did not show to be effective to prevent shedding of bacteria or limiting the duration of bacterial excretion in sheep (Astobiza et al., 2010b) and goats (Blain, 2006).

In cattle antibiotic therapy is also frequently implemented in routine practice either at

drying off to prevent late abortion or at calving to prevent the shedding peak. Like in humans, because of its activity and its intracellular diffusion, tetracycline are the most used antibiotic (Taurel et al., 2014). The use of tetracyclines was associated with a prevention of vaginal shedding at calving in dairy cows, when injected once at drying off (Taurel et al., 2012).

The most used protocol consists of two injections of Oxytetracycline (20 mg/kg) during the last month of gestation (Arricau-Bovery and Rodolakis, 2005).

In a study comparing the effectiveness of vaccination and/or antibiotics at different regimens to prevent and limit *C. burnetii* shedding at calving in dairy cows, the use of tetracycline was associated with a lower risk of being detected shedder at calving, but had no significant effect on the bacterial load shed (Taurel et al., 2012). On the basis of the update knowledge, the antibiotic treatment in domestic ruminants was considered not effective to substantially reduce either the level or the duration of bacterial shedding (EFSA, 2010).

1.8 TABLES AND FIGURES OF CHAPTER 1

Table 1: Prevalence of *C. burnetii* (animal and herd level) in cattle in Europe and Turkey (EFSA, 2010).

Country	Year of stud	No. tested		% positive		Test[a]	Reference
		Cattle	Herds	Cattle	Herds		
Albania	1999	552		8.5		ELISA	Cekani et al., 2008
	1995-1997	311		10.9		ELISA	Cekani et al., 2008
Bulgaria	2002	3,006		8.2		CFT	Martinov 2007a
	2003	3,714		6.5		CFT	Martinov 2007a
	2004	120		20.8*		IFA	Panaiotov et al.,
	2004	3,188		9.7		CFT	Martinov 2007a
	2005	3,026		8.1		CFT	Martinov 2007a
	2006	2,932		10.6		CFT	Martinov 2007a
	1989-2006	95,737		5.4		CFT	Martinov 2007a
	1977-1988	20,086		11.8		CFT	Martinov 2007a
	1950-1976	4,749		19.8		CFT	Martinov 2007a
Cyprus	NA	75		24.0		IFA	Psaroulaki et al.,
Denmark	2008		100		59.0	ELISA-milk	Agger et al., 2010
	2007		742		57	ELISA-milk	Bodker and Christoffersen
Italy	1998	544***	21	13	nd	IFA	In Arricau-Bouvery and
		155***	6	2	nd	IFA	In Arricau-Bouvery and
		486	26	20	nd	IFA	In Arricau-Bouvery and
	NA	650		44.9**		ELISA	Cabassi et al., 2006
	NA	600		22.0		ELISA	Cabassi et al., 2006
Germany	1998-2000	1,167	*105*	1.4 to	nd	ELISA	Sting et al., 2002
	1998	21,191	544	8	nd	ELISA	In Arricau-Bouvery
	1996-1997	826	*38*	14.3** 0.6	nd	ELISA	Sting et al., 2000
	1992-1993	500	*NA*	7.6	nd	CFT	Wittenbrink et al., 1994
		665	*39*	9.6	76.9		
		383**	*33*	19.3	78.8		
		612 Bulls	*1*	5.6	100		
	1991	1,095	*21*	11.8	81	ELISA	Rehacek et al., 1993
	1989-1990	3,500	*155*	13.3	57.4	ELISA	Klemt and Krauss,
Netherlan	1987	1,160**	*234*	21	37	ELISA	Muskens et al., 2007
Spain	2006-2007	79		35.4		IFA	Ruiz-Fons et al.,
	2008-	626		6.7		ELISA	Ruiz-Fons et al.,
	2008-		42		42.9	ELISA	Ruiz-Fons et al.,
Turkey	2006-2008	92		16.3		ELISA	Ceylan et al., 2009
	2005	230		9.6		ELISA	Seyitoglu et al.,
	1998	416	48	6	nd		In Arricau-Bouvery and
UK (NI)	2009	5,182		6.2		ELISA	McCaughey et al.,
	2009		273		48.4	ELISA	McCaughey et al.,

(a) Indirect Immunofluorescence assay (IFA), Enzyme Linked Immunosorbent assay (ELISA), Complement fixation test (CFT), Microagglutination test (MAT)
* Investigation in relation to a human outbreak
** Investigation in relation to clinical symptoms in the population (animals)
*** The study was conducted to compare animals kept indoors (544) and outdoors (155) NA - not available; ML- most likely

Table 2: Prevalence of *C. burnetii* (animal and herd level) in sheep in Europe and Turkey (EFSA, 2010).

SHEEP							
Country	**Year**	**No. tested**		**%positive**		**Test**	**Reference**
		Sheep	Flocks	**Sheep**	Floc		
Albania	1999	292		12.3		ELISA	Cekani et al., 2008
	1995-1997	350		8.9		ELISA	Cekani et al., 2008
Bulgaria	2002	1,819		12.7		CFT	Martinov 2007a
	2003	1,811		8.3		CFT	Martinov 2007a
	2004	100		21.0*		IFA	Panaiotov et al.,
	2004	1,258		14.1		CFT	Martinov 2007a
	2005	1,911		15.2		CFT	Martinov 2007a
	2006	1,925		8.4		CFT	Martinov 2007a
	1950-1976	17,088		16.7		CFT	Martinov 2007a
	1977-1988	16,593		18.8		CFT	Martinov 2007a
	1989-2006	99,189		4.8		CFT	Martinov 2007a
	NA	153		56.9**		CFT	Martinov. 2007b
Croatia	2004	182		11.0*		CFT	Medic et al., 2005
Cyprus	NA	481		18.9		IFA	Psaroulaki et al.,
Germany	NA		*95*		2.7		Runge and Ganter,
	1998	1,346		1.3		ELISA	In Arricau- Bouvery and
	1999	100	1	57		ELISA	In Arricau- Bouvery and
		3,460		8.7		ELISA	Sting et al., 2002
Greece	NA	554		10.5		IFA	Pape et al., 2009a
Italy	1999-2002	7,194	675	9/38		ELISA	In Arricau- Bouvery and
Netherlan	1987	3,603		3.5		ELISA	In Muskens et al.,
	2008	12,363		2.4		ELISA	Van den Brom and P.
Spain	1999-2003		148	8.8**		PCR	Oporto et al., 2006
	1999-2003		148	2.7**		CFT	Oporto et al., 2006
	1999-2003	38		42.1**		CFT	Oporto et al., 2006
	2005	34		67.6**		ELISA	Garcia-Perez et al.,
	2005	1,011		8.9		ELISA	Garcia-Perez et al.,
	2005		154	22.1		ELISA	Garcia-Perez et al.,
	2007-2008	1,379		11.7		ELISA	Ruiz-Fons et al.,
			46	34		ELISA	Ruiz-Fons et al.,
Turkey	NA	465		21.1**		ELISA	Karaca et al., 2009
	2001-2004	743	42	20	83	ELISA	Kennerman et al.,
	1998	411		10.5		IFA	In Arricau- Bouvery and
UK (NI)	NA 2009?	1,022		12.3		ELISA	McCaughey et al.,
	NA 2009?		58	62.1		ELISA	McCaughey et al.,

(a) Indirect Immunofluorescence assay (IFA), Enzyme Linked Immunosorbent assay (ELISA), Complement fixation test (CFT), Microagglutination test (MAT)
* Investigation in relation to a human outbreak
** Investigation in relation to clinical symptoms in the population (animals)
*** The study was conducted to compare animals kept indoors (544) and outdoors (155) NA - not available; ML- most likely

Table 3: Prevalence of *C. burnetii* (animal and herd level) in goats in Europe and Turkey (EFSA, 2010).

GOATS							
Country	Year	**No. tested**		**% positive**		**Test** [(a)]	**Reference**
		Goats	**Herds**	**Goats**	**Her ds**		
Albania	1999	260		4.2		ELISA	Cekani et al., 2008
	1995-	443		8.8		ELISA	Cekani et al., 2008
Bulgaria	2002	677		11.8		CFT	Martinov, 2007a
	2003	1,044		7.4		CFT	Martinov, 2007a
	2004	50		40.0*		IFA	Panaiotov et al.,
	2004	1,016		21.7		CFT	Martinov, 2007a
	2005	832		11.1		CFT	Martinov, 2007a
	2006	359		19.2		CFT	Martinov, 2007a
	1950-	1,417		20.5		CFT	Martinov, 2007a
	1977-	1,791		10.8		CFT	Martinov, 2007a
	1989-	54,175		7.6		CFT	Martinov, 2007a
Cyprus	NA	417		48.2		IFA	Psaroulaki et
France	2006	359		36.0		ELISA	Dubuc-Forfait et
	2006		42	88.1		ELISA	Dubuc-Forfait et
	2006	75		65.3**		ELISA	Chaillon et al.,
	2008	1,057		32.0		ELISA	Dubuc-Forfait et
	2008	42		88.1		ELISA	Dubuc-Forfait et
Germany	1998	278		2.5		ELISA	In Arricau-Bouvery and
Greece	NA	61		6.6		IFA	Pape et al., 2009a
Italy	1999-2002	2,155	104	13	47	ELISA	In Arricau-Bouvery and Rodolakis 2005
Netherlands	1987	498		1 goat		ELISA	Muskens et al., 2007
	2008	3,409		7.8		ELISA	Van den Brom and P.
Poland	NA, ML	98		79.6**		MAT	Platt-Samoraj et al., 2005
Spain	2007-	115		8.7		ELISA	Ruiz-Fons et al.,
	2007-		11		45.5	ELISA	Ruiz-Fons et al.,
Turkey	2006-	92		5.4		ELISA	Ceylan et al., 2009
UK (NI)	NA, ML	54		9.3		ELISA	McCaughey et al., 2010
	NA, ML		7		42.9	ELISA	McCaughey et al., 2010

(a) Indirect Immunofluorescence assay (IFA), Enzyme Linked Immunosorbent assay (ELISA), Complement fixation test (CFT), Microagglutinating test (MAT)
* Investigation in relation to a human outbreak
** Investigation in relation to clinical symptoms in the population (animals)
*** The study was conducted to compare animals kept indoors (544) and outdoors (155) NA - not available; ML- most likely

Table 4: Estimated prevalence of Coxiella burnetii infection in people, based on studies conducted in Bulgaria, France, Germany and the Netherlands, 1982–2010 (Georgiev, 2013).

Country	Year of	Number	Sample	%	Test
BG	1993–2000	14,353	RG	15.0	CFT, MIFT
BG	1995–1997	224	BD	38.0	MAT, MIFT
BG	2001–2004	5,207	RG	18.0	CFT, MIFT
BG	2004	104	HO (PW)	7.7	IFA
DE	2002	255	HO	22.0	NA
FR	1982–1990	22,496	RG	23.0	NA
FR	1988	924	BD	4.0	IFA
FR	1995	790	BD	1.0	IFA
FR	1995–1996	785	NA	5.0	IFA
FR	1996	620	BD	3.0	IFA
FR	1996	12,716	NA	0.2	IFA
FR	1996	208	RG	71.0	IFA
FR	2002–2003	376	RG (PW)	2.6	IFA
FR	2002–2003	91	RG (CA)	5.5	IFA
FR	2002–2003	578	HO	14.7	IFA
NL	1982	222	RG	83.8	NA
NL	1983	359	BD	24.0	NA
NL	2006–2007	5,654	GP	2.4	ELISA, IFA
NL	2007–2009	2,004	HO (PW)	9.1	IFA
NL	2009	543	BD	12.2	ELISA, IFA

BD: blood donors; BG: Bulgaria; CA: cardiac abnormalities; CFT: complement fixation test; DE: Germany; ELISA: enzyme-linked immunosorbent assay; FR: France; GP: general population; HO: humans in outbreak areas; IFA: indirect immunofluorescence assay; MAT: microagglutination test; MIFT: microimmunofluorescence test; NA: information not available or not specified; NL: Netherlands; PW: pregnant women; RG: risk group.

Figure 1: Number of notified human Q fever cases with a known first day of illness according to the week of onset of symptoms, from 1 January 2007 to 11 May 2010 (Roest et al., 2013).

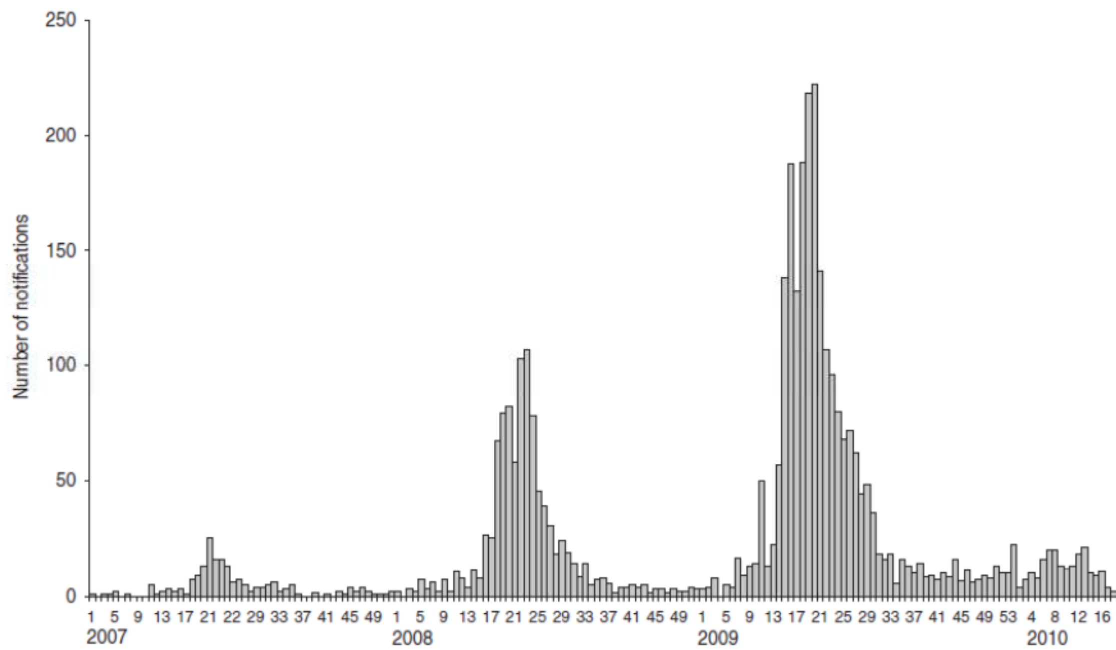
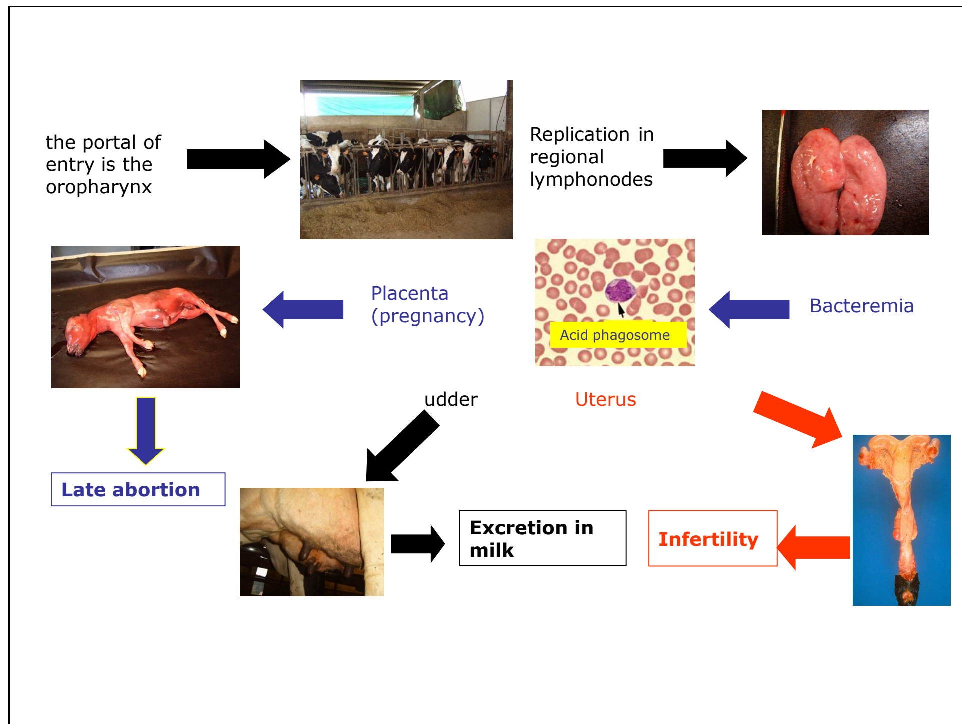


Figure 2: pathogenesis of *C. burnetii* infection in ruminants: entry, dissemination and different outcome of the infection (modified from Barberio and Vicenzoni, 2013).



CHAPTER 2: *Coxiella burnetii* infection in dairy cows and goats: assessment of diagnostic methods, and evaluation of immune response in shedders

2.1 MATERIALS AND METHODS

2.1.1 Analytical methods

Complement fixation test

The CFT was performed in agreement with the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE, 2015). A commercial antigen was used, (Siemens Healthcare Diagnostic Products, Erlangen, Germany) that was a mixture of Nine Mile and Henzerling in phase II strains. Hamster complement was used (BioMérieux, Lyon, France) at a dilution corresponding at 2 unit (U). briefly the analytical procedure was the following:

After predilution to 1:10 with Veronal buffer, cattle sera were inactivated for 30' at 58° C and goats sera at 60° C. The antigen and the complement were then diluted in Veronal buffer. Then 25 μL of each sera were dispensed in microtitre plates for CFT test and anticomplementarity test (ACP). In CFT test plate sera were mixed with 25 μL of antigens and 25 μL of complement in each well; in ACP plate sera were mixed with 25 μL of Veronal buffer and 25 μL of complement. After agitation the plates were incubated at +5°C for 18 hours. Then 25 μL of hemolytic system, previously diluted and sensitized in waterbath at 37°C for 15', were added to each well. After agitation plates were incubated at 37°C for 30' centrifugated for 5' at 2.000 r.p.m., and then reading was performed.

The cut-off title was fixed at the titer of 1:10.

ELISA

Sera and milk samples were analyzed with a commercial ELISA test, (LSIVET Ruminants milk/serum Q fever, Lissieu, France), according to manufacture instructions. The kit was an indirect ELISA with antigens in phase I and phase II isolated from domestic ruminants. Serum samples were prediluted 1:400, while milk samples were prediluted 1:20. The test results were calculated using the s/p ratio (ODsample-ODneg)/(ODpos-ODneg). Sera samples were classified as negatives if s/p ≤ 0.4, weak positives (+) if > 0.4 ≤1, medium positives (++) if > 1 ≤2, high positives (+++) if > 2 ≤3, very high positives (++++) if > 3. Milk samples (BTM) were classified as negatives if S/p ≤ 0.3, weak positives (+) if > 0.3 ≤1, medium positives (++) if > 1 ≤2, high positives (+++) if > 2.

Molecular assays

Molecular assays were performed using a real-time PCR (r-t PCR) commercial kit (ADIAVET® COX REALTIME, Adiagène Saint Brieuc, France). Pre-treatment and sample processing were performed according to the manufacturer's instructions. The extraction of Coxiella DNA from different matrices was performed according to the manufacturer's instructions (QIAamp DNA mini kit, Qiagen), as follows:

- From milk: 400 µL of individual milk or BTM was transferred to a 1.5 mL microtube after vortexing.
- From vaginal swabs: 1 ml of sterile water was added to the vaginal swab, the sample was vortexed for 30 s, and 200 µL of the supernatant was transferred to a 1.5 mL microtube.

The instrument employed for r-t PCR was a Roche LightCycler 2.0 or an Applied Biosystems 7900 HT Fast Real- Time System. The diagnostic sensitivity of the r-t PCR analysis of the BTM samples was determined to be sufficient to detect 1 shedder cow in a group of 250 milking cows.

The amplification program is clarified in table 1 and was the same if Life Technologies or Roche instruments were used.

Control samples with defined cycle threshold (ct) cycle values were used to verify the quality of the analysis performed. An internal positive control sample was coamplified for each sample tested to detect the presence of PCR reaction inhibitors in the sample.

The individual milk samples that tested positive in the r-t PCR assay were divided into two classes depending on the ct cut-off value of 31. For this method, ct = 31 represents a concentration of 10^3 bacteria/ml (ADIAVET® COX REALTIME, validation data sheet, November 2010). Animals whose milk samples had ct values ≤ 31 were classified as high shedders, whereas animals with ct values > 31 were classified as low shedders.

2.1.2 Study design

2.1.2.1 Assessment of the efficacy of r-t PCR assays on BTM for the detection of infected dairy herds and flocks

The sensitivity and specificity of a single r-t PCR test on BTM were evaluated using a control-case study in dairy cattle and goat farms. The first step was the identification of infected and negative farms, in which a sample of BTM was taken for a r-t PCR test to detect *C. burnetii* DNA.

Dairy cattle

In dairy cattle the infected herds, according to the literature (Sidi-Boumedine et al, 2010; Guatteo et. al., 2011), were defined as farms with:

- clinical symptoms of Q fever like abortions or infertility,
- at least one positive r-t PCR result confirming the presence of the agent of Q fever on specimens from affected animals,
- a prevalence ≥ 20%.

Negatives farms were defined as farms with:

- a regular surveillance and diagnosis of abortions,
- all the r-t PCR test on abortions or vaginal swabs negative for *C. burnetii*,

a prevalence < 20%.

The herds enrolled in the study were farms that undergo to a regular surveillance and diagnosis of abortions in the monitoring plan of Regione Veneto for bovine abortions. All Aborted foetuses accompanied by the dam's blood sample were delivered to the diagnostic laboratory of Istituto Zooprofilattico delle Venezie (IZSVe) and submitted to a panel of laboratory tests. The sera were tested for antibodies against Brucella, Neospora, IBR, Leptospira, BVD, Q fever, Chlamydophila, and the foetuses were tested for the detection of the following agents: bacteria, *Neospora caninum*, BVD virus, Chlamydophila, and *C. burnetii.*

All the farms with a *C. burnetii* r-t PCR positive abortion, were reached by means of their veterinary practitioners, and sampled. Among the farms with negative r-t PCR and serology were selected 73 herds having the following features:

- ✓ perform a regular surveillance and diagnosis of abortions including laboratory diagnosis performed at the IZSVe,
- ✓ be included in the dairy herd improvement (DHI) control system performed by the Italian Breeders Association (AIA).

These negative herds were then tested with a prescreening test to detect the presence of antibodies against *C. burnetii* in a BTM sample collected from the employs of breeders association during the DHI controls. The samples were frozen at -20° C after the collection, shipped to the IZSVe laboratory and tested with ELISA for the detection of anti-*C. burnetii* antibodies. Only farms with ELISA negative result were included in the study.

In all the herds, positive and negative, included in the trial, to assess the prevalence of *C. burnetii* infection, 13 cows were randomly selected and a blood sample (nearly 10 ml) was taken from the tail vein of each cow using a vacuum tube (Vacutainer, Becton Dickinson, Franklin Lakes, NJ, USA). The number of cows to be tested were 13 because it allows to detect with a confidence level of 95% at least 1 positive cow if the prevalence is ≥ 20%.

The samples were stored in a bag refrigerated with eutectic plates, transported to the laboratory, centrifugated at 3.000 r.p.m. for 10', and the sera were stored at -20° C. All the samples were tested with ELISA and CFT.

At the same time a 60 ml of milk sample were taken from the bulk tank, that was previously mixed for 5' using the automatic tank stirrer. The milk was stored in a refrigerated bag with eutectic plates, transported to the laboratory, split in 2 tubes and stored at -20° C. One tube was used for r-t PCR test to detect *C. burnetii* DNA and the other kept frozen in case the analysis had to be repeated.

Dairy goats

In dairy goats the infected flocks, according to the literature (Sidi-Boumedine et al, 2010; Guatteo et. al., 2011), were defined as farms with:

- clinical symptoms of Q fever like abortions or infertility,
- at least one positive r-t PCR result confirming the presence of the agent of Q fever on specimens from affected animals,
- a prevalence ≥ 15%.

Negatives flocks were defined as farms with:

- a regular surveillance and diagnosis of abortions;
- all the r-t PCR test on abortions or vaginal swabs negative for C. burnetii,
- a prevalence < 15%.

The Flocks enrolled in the study (29) were selected in the province of Trento, Vicenza and

Belluno, among dairy flocks with at least 20 goats. All the flocks enrolled in the study were regularly visited every 15 days from November to April: all the abortions were registered, vaginal swabs were taken from goats that aborted and also from animals with regular kidding, to perform *C. burnetii* r-t PCR. All the flocks enrolled were sampled to evaluate the seroprevalence, and BTM for r-t PCR was taken.

Vaginal swabs (Becton Dickinson, Franklin Lakes, NJ, USA) were taken from the vagina after cleaning and disinfection of the vulva with a disinfecting solution (5% Virkon S™, Dupont USA). After sampling the swabs were placed in sterile tube, stored in a refrigerated bag, transported to the laboratory, and stored at -20° C. Afterward they were tested with r-t PCR test to detect *C. burnetii* DNA.

In all the flocks included in the trial, to assess the prevalence of *C. burnetii* infection, 18 goats were randomly selected and a blood sample (nearly 10 ml) was taken from the jugular vein of each goat using a vacuum tube (Vacutainer, Becton Dickinson, Franklin Lakes, NJ, USA). The number of goats to be tested were 18 because it allows to detect with a confidence level of 95% at least 1 positive cow if the prevalence is ≥ 15%.

The samples were stored in a bag refrigerated with eutectic plates, transported to the laboratory, centrifugated at 3.000 r.p.m. for 10' and the sera were stored at -20° C. All the samples were tested with ELISA and CFT.

At the same time a 60 ml of milk sample were taken from the bulk tank, that was previously mixed for 5' using the automatic tank stirrer. The milk was stored in a refrigerated bag with eutectic plates, transported to the laboratory, split in 2 tubes and stored at -20° C. One tube was used for r-t PCR test to detect *C. burnetii* DNA and the other kept frozen in case the analysis had to be repeated.

2.1.2.2 Evaluation of the dynamics of the antibodies response, and the *C. burnetii* excretion in infected animals

Dairy cattle

Among the infected dairy farms involved in the study were selected 4 herds with:

- ✓ at least 1 abortion and the BTM positive to r-t PCR for *C. burnetii* DNA,
- ✓ an average number of lactating cows > 100 and < 400,
- ✓ the approval of the farmer to participate at the study and his commitment to don't interrupt the trial before the end of the study.

In each of the enrolled herds was performed a first screening collecting from each of the lactating cows a sample of blood from the tail vein using a vacuum tube (Vacutainer, Becton Dickinson, Franklin Lakes, NJ, USA), and a sample of milk taken from the foremilk of each quarter, prior of the attachment of the milking group. Milk samples were collected according to the National Mastitis Council guideline for milk sample and handling (National Mastitis Council, 1999).

The samples were stored in a bag refrigerated with eutectic plates and transported to the laboratory. At the laboratory blood samples were centrifugated at 3.000 r.p.m. for 10',the sera were stored at -20° C, and afterward were tested with ELISA and CFT for the detection of antibodies against *C. burnetii*. Milk samples were split in 2 tubes, stored at -20° C, and afterward 1 tube was used for r-t PCR test to detect *C. burnetii* DNA, and the other kept frozen in case the analysis had to be repeated.

According to the test results, were established 4 groups of nearly 10 cows to be sampled 3 times every 2 months. The cows were sorted among the groups as follow:

- ✓ group 1: cattle ELISA negative and PCR negative (ELISA-PCR-);
- ✓ group 2: cattle ELISA negative and PCR positive (ELISA-PCR+);
- ✓ group 3: cattle ELISA positive and PCR negative (ELISA+PCR-);

- ✓ group 4: cattle ELISA positive and PCR positive (ELISA+PCR+).

From these cows at each control was taken a sample of blood, milk and feces and tested, serum with ELISA and CFT for antibodies detection, milk and feces with r-t PCR for *C. burnetii* DNA detection.

the 4 groups of cows were created considering also these elements:

- ✓ days in milk: when possible cows at the beginning of lactation were chosen,
- ✓ CFT results: when possible in the group ELISA+ PCR+ and ELISA+ PCR- where introduced cows that were also CFT positive.

Dairy goats

Among the infected flocks enrolled in the r-t PCR assessment only 2 farmers approved the participation to the study, so a 3rd infected flock was included in the study, despite the number of lactating goats was only 12.

In each of the enrolled flocks was performed a first screening collecting from each of the lactating goats a sample of blood from the jugular vein using a vacuum tube (Vacutainer, Becton Dickinson, Franklin Lakes, NJ, USA), and a sample of milk taken from the foremilk of each halves, prior of the attachment of the milking group. Milk samples were collected according to the National Mastitis Council guideline for milk sample and handling (National Mastitis Council, 1999).

The samples were stored in a bag refrigerated with eutectic plates and transported to the laboratory. At the laboratory blood samples were centrifugated at 3.000 r.p.m. for 10’,the sera were stored at -20° C and afterward were tested with ELISA and CFT for the detection of antibodies against *C. burnetii*. Milk samples were split in 2 tubes, stored at -20° C, and afterward 1 tube was used for r-t PCR test to detect *C. burnetii* DNA and the other kept frozen in case the analysis had to be repeated.

According to the test results, were established 3 groups to be sampled 3 times every 2

months. The goats were sorted among the groups as follow:

- ✓ group 1: goats ELISA negative and PCR negative (ELISA-PCR-);
- ✓ group 2: goats ELISA positive and PCR negative (ELISA+PCR-);
- ✓ group 3: goats ELISA positive and PCR positive (ELISA+PCR+).

From these goats at each control was taken a sample of blood, milk and feces and tested, serum with ELISA and CFT for antibodies detection, milk and feces with r-t PCR for *C. burnetii* DNA detection.

2.1.3 Herds and flocks features

Dairy cattle

- ✓ Herd 1: the total number of cattle was 214, the breed Italian Holstein, on average the number of milked cow in the year was 107. The housing system was freestall barns and the cows were milked in a herringbone parlour. Abortion and metritis cases were reported < 5%, but poor fertility rate was one of the main problem of the farm.
- ✓ Herd 2: the total number of cattle was 215, the breed Brown, on average the number of milked cow in the year was 107. The housing system was freestall barns and the cows were milked in a tandem parlour. Several late abortions with detection of *C. burnetii* occurred in the farm in the last 16 months, and poor fertility rate was one of the main problem of the farm.
- ✓ Herd 3: the total number of cattle was 239, the breed Italian Holstein, on average the number of milked cow in the year was 100. The housing system was freestall barns and the cows were milked in a herringbone parlour. Abortion and metritis cases were reported < 5%, but poor fertility rate was one of the main problem of the farm.
- ✓ Herd 4: the total number of cattle was 606, the breed Italian Holstein, on average the number of milked cow in the year was 294. The housing system was freestall barns and

the cows were milked in a herringbone parlour. Several late abortions with detection of *C. burnetii* occurred in the farm in the last 6 months, and poor fertility rate was one of the main problem of the farm.

Dairy goats

- ✓ Flock 1: the average number of milked goats was 80, the breed were Alpine and Saanen. The goats were housed in a concrete building with pens separated according to the functional state of the animals: lactating, dryed-off, young goats. Bedding material used was straw and each pen was in communication with an external paddock. Goats were milked in a parallel milking parlour. Milk was in part sold to a dairy company and in part used in the farm for cheese production, that was sold directly to the consumers. Abortion cases were reported < 5%.
- ✓ Flock 2: the number of milked goats was 12, the breed were Alpine and Saanen. The goats were housed in a concrete building separated in 2 pens, 1 for lactation goats, and the other for the young and dryed-off goats. Bedding material used was straw and each pen was in communication with an external paddock. Goats were milked in a small parallel milking parlour (3 places). In the summer the goats were sent to a mountain farm where they were kept grazing with other goats from other farms. Also in the summer milking was performed in a parallel milking parlour. During the winter milk was sold to a dairy company, in the summer was used in the mountain farm for cheese production that was sold directly to the consumers. Several abortion cases were reported in the last year (> 15%).
- ✓ Flock 3: the average number of milked goats was 180, the breed were Alpine and Saanen. The goats were housed in a concrete building with pens separated according to the functional state of the animals: lactating, dryed-off, young goats. Bedding material used was straw and each pen was in communication with an external paddock. Goats

were milked in a parallel milking parlour. All the milk was used to produce cheese in a authorized dairy plant annexed to the farm, and the product was sold to shops or directly to the consumers. Abortion cases were reported < 5%.

2.1.4 Statistical analysis

The evaluation of the performance of r-t PCR in BTM was done by estimation of sensitivity and specificity according to the definition of Martin et al., (1987).

The statistical association between the serological status of the cows in infected herds and the occurrence of *C. burnetii* shedding in milk was evaluated using the logistic regression and the statistic of Wald assuming alfa = 0.05: only p value < 0.05 were considered statistically significant. The level of association was estimated by mean of the Odds ratio (OR). The OR estimates the different probability of a serological positive cow to be shedders of *C. burnetii* in milk compared to a serological negative cow. The OR was estimated comparing the results of r-t PCR test in milk of each cow with the result of CFT test and ELISA test. Furthermore was estimated the OR of the comparison between results of r-t PCR test in milk and the serological status only of the cow with a high positive response to the ELISA test. For this purpose a different ELISA cut-off was used (s/p >2). each OR was estimated with the confidence level (CI) at 95%. The estimation of the agreement between ELISA and CFT was performed using the Cohen's Kappa coefficient. All the statistics was performed by mean of SPSS Statistics vers. 21 (IBM).

2.2 RESULTS

2.2.1 Assessment of the efficacy of a r-t PCR assay on BTM for the detection of infected dairy herds

Dairy cattle

C burnetii was detected in fetuses or placenta of 17 dairy cattle farms that submitted their samples for the diagnosis of abortion to the IZSVe laboratories,. The great majority of these herds (16) had a within-herd seroprevalence for *C. burnetii* antibodies ≥ 20% (table 2). The r-t PCR on BTM gave a positive result in 9 herds among 17. Of these BTM positive herds, 8 had a within-herd seroprevalence ≥ 20%, and only 1 herd had a seroprevalence < 20%. To check if this peculiar result was due to a recent infection, all the cows of the herd were tested for *C. burnetii* serology using ELISA after 2 months and also the BTM sample was repeated. BTM was confirmed as positive to r-t PCR also in the second sample and out of 52 sampled cows only 5 (9,6%) had a positive result to ELISA test.

The herds classified as infected, according to the definition stated in the study design, were 16: only in 8 (50%) of these herds the BTM was found positive to r-t PCR.

If the within-herd prevalence was assessed using the CFT instead of ELISA, the herds classified as infected turned out to be 12 instead of 16. In 8 (67%) of these herds the BTM was found positive to r-t PCR, while in other 4 herds the BTM was negative to r-t PCR (table 3).

To find out farms uninfected from *C. burnetii,* 73 herds were selected among these with a surveillance control plan on abortions, based on routine laboratory diagnosis of abortions. All the samples submitted by these herds were negative both at r-t PCR and serological test of the cows that aborted. Nevertheless in 51 (70%) of these herds, BTM was found positive

for antibodies against *C. burnetii* and they were removed from the study.

Therefore only 22 herds were sampled: 12 (55%) showed a within herd prevalence ≥ 20% and 10 (45%) < 20%. The r-t PCR detected *C. burnetii* DNA only in the BTM of 6 herds (table 2): all these herds had a within-herd prevalence ≥ 20%. In these 22 herds the ELISA test for antibodies detection on BTM was repeated on the new samples taken for r-t PCR. Out of the 22 BTM samples tested, 5 turned positive to the ELISA test and 4 of them belonged to farms with a within-herd prevalence ≥ 20%. Out of the 6 samples positive to the r-t PCR, 3 were also positive for antibodies against *C. burnetii* and 2 remained negative.

The herds classified as uninfected, according to the definition stated in the study design, were 10: of these herds none had a positive r-t PCR result in the BTM sample.

If the within-herd prevalence was assessed using the CFT instead of ELISA, the herds classified as uninfected were 17 instead of 10. Of these herds in 2 (12%) the BTM was found positive to r-t PCR, while in other 15 herds (88%) the BTM was negative to r-t PCR (table 3).

The number of infected herds was 16 and the number of uninfected herds was 10, while 13 herds could not be classified as infected or uninfected due to the lack of one or more features (within-herd prevalence or presence/absence of *C. burnetii* abortions). The sensitivity and specificity of BTM r-t PCR, calculated using these data, were respectively equal to 0.5 and 1 (table 4).

If the within-herd prevalence was assessed using the CFT instead of ELISA, the herds classified as infected were 12 and those classified as uninfected 17: the sensitivity and specificity of BTM r-t PCR, were respectively equal to 0.7 and 0.9 (table 5).

The ELISA test used for the detection of seropositive cows in the herds has different levels of positive s/p value cut-off, that allows to distinguish among positive animals the

following groups: weak positives (+) if > 0,4 ≤1, medium positives (++) if > 1 ≤2, high positives (+++) if > 2 ≤3, very high positives (++++) if > 3.

Taking into account the possibility that the majority of false positive results in the ELISA test are included in the group of weak positives (Guatteo, 2007), we estimated the within-herd seroprevalence, assuming as positive only the samples with a s/p value > 1, excluding all the weak positive samples. Using this cut-off to assess of the within-herd prevalence, no difference was found in the classification of the infected herds: all the 16 farms previously classified as infected, had at least 1 cow with a s/p value > 1 (table 6).

In the classification of uninfected herds, the use of this cut-off allowed to increase the number of herds included in the definition of uninfected herds from 10 to 14. Indeed in 4 of the herds with negative abortions for *C. burnetii*, the within-herd seroprevalence change from ≥ 20% to < 20%, as only 1 weak positive sera was found in these herds. All these 4 farms were negative to r-t PCR in the BTM sample, therefore the sensitivity and specificity of a single test on BTM by mean of the r-t PCR, were exactly the same as those obtained with the previous ELISA cut-off, respectively 0,5 and 1 (table 7).

Dairy goats

The dairy goat flocks followed during the study were located mainly in the province of Trento (18), but there were also 8 flocks in the province of Vicenza and 3 in the province of Belluno. In the farms kidding were scattered throughout the observation time, (November – April), but the great majority occurred between December and February.

Out of the 29 flocks sampled in the study, 15 (51%) had goats shedding *C. burnetii* from vagina mucus after abortions and also after normal parturition (table 2). In all flocks *C. burnetii* DNA was detected in at least 2 or more goats after kidding. No abortion storms occurred during the trial, but only single cases of abortions, which occurs more often in the enzootic form of the disease. Most flocks with vaginal swabs positive for *C. burnetii*

were located in the province of Trento (13), and only 2 in the province of Vicenza.

Among these flocks only 9 (60%) had a within-flock seroprevalence for *C. burnetii* antibodies ≥ 15% (table 8). The r-t PCR on BTM gave a positive result in 8 flocks out of 15. Of these BTM positive flocks, 7 had a within-flock seroprevalence ≥ 15%, and only 1 flock had a seroprevalence < 15%.

The flocks classified as infected, according to the definition stated in the study design, were 9: only in 8 (89%) of these flocks the BTM was found positive to r-t PCR.

If the within-flock prevalence was assessed using the CFT instead of ELISA, the flocks classified as infected were only 2 instead of 9 and only in 1 flock the BTM was found positive to r-t PCR (table 9).

The flocks in which all the vaginal swabs collected tested negative for *C. burnetii* DNA, detection were 14 (49%). All these flocks had a within-flock seroprevalence < 15% and the BTM negative *C. burnetii* (table 8), thus all the 14 flocks were classified as uninfected. The same result was obtained if the within-flock seroprevalence was assessed using the CFT instead of ELISA. Therefore, the number of infected flocks was 9 and the number of uninfected flocks was 14, while 6 flocks could not be classified as infected due to the low within-herd seroprevalence. The sensitivity and specificity of the BTM r-t PCR, calculated using these data, were respectively equal to 0.8 and 1 (table 10).

If the within-flock prevalence was assessed using the CFT instead of ELISA, the flocks classified as uninfected were 13 instead of 14, because in 1 flock a high number of CFT positives and ELISA negatives sera (14 among 18) was detected.

As in dairy cattle, the within-flock seroprevalence was then estimated, assuming as positive only the samples with a s/p value > 1 and excluding all the weak positive samples. Using this cut-off for the assessment of the within-flock seroprevalence, the flock classified as infected were 7 instead of 9. The BTM r-t PCR was positive in all these

flocks. No difference of course was found in the classification of the uninfected herds, because all the sera tested were negative to the ELISA (table 11). Differently from what happened in dairy cows, the use of this cut-off reduced the number of flocks included in the definition of infected from 9 to 7. The sensitivity of BTM r-t PCR moved from 0.8 to 1, while the sensitivity remained unchanged equal to 1 (table 12).

2.2.2 Evaluation of the dynamics of the antibodies response and the *C. burnetii* excretion in infected animals

Dairy cattle

In the first sampling performed on the 4 infected dairy herds, 608 lactating cows were sampled. Table 13 and 14 show the results of the analysis performed on milk and blood samples, collected from each cow. The number of cows shedding *C. burnetii* in milk was 48 (7.9%), the number of seropositive cows to ELISA test was 181 (29.8%) and the number of seropositive cows to CFT test was 65 (10.7%).

The ELISA test allows to distinguish among positive animals the high positive ones: this was the reason to calculate also the number and percentage of cows with an s/p cut-off > 2, that include only high and very high positive animals. The number of high positive cows was 85 (14%).

The percentage of positive cows to r-t PCR among herds ranged from 4.4% to 13.1%: if we consider the number of positive cows of each herd, this value was very similar and ranged from 10 to 14. The percentage of seropositive cows to the ELISA test was more variable among herds, ranging from 21.5% to 48%, while the percentage of CFT seropositive cows ranged from 8% to 15.9%.

Animals with r-t PCT positive milk samples were sorted in 2 groups according to their ct: those with ct values ≤ 31 were classified as high shedders of *C. burnetii*, the others as low

shedders. The number of high shedders was 12 (table 14), equal to 2% of the total number of animals and 25% of the PCR positive cows. It should be highlighted that all 12 high shedder cows were classified as high positive to ELISA test, while only 5 of these cows were positive to CFT test.

After the first sampling, according to the results of ELISA and r-t PCR, the cows were grouped as follows and sampled 3 other times every 2 months:

- ✓ group 1: 49 cows ELISA negative and PCR negative (ELISA-PCR-);
- ✓ group 2: 14 cows ELISA negative and PCR positive (ELISA-PCR+);
- ✓ group 3: 43 cows ELISA positive and PCR negative (ELISA+PCR-);
- ✓ group 4: 34 cows ELISA positive and PCR positive (ELISA+PCR+).

Table 15 shows the size of each group at each time of sampling: it is interesting to note that among the r-t PCR positive cows, 14 were seronegative to ELISA test. Furthermore all these cows were also negative to CFT. During the study, 31 cows were culled before the last sampling: these cows have been culled because of health problems, mainly infertility, metabolic disease and lameness. It is very difficult to evaluate whether the persistence of the *C. burnetii* infection could be related to the culling of these animal, due to the high replacement rate of intensive dairy farms in the Veneto area, but the group from which the majority of animals were culled, was the ELISA+PCR+, as showed in table 16.

In group 1, ELISA-PCR-, 3 cows belonging to different herds, at 2nd, 3rd and 4thsampling, changed their status and became shedders of *C. burnetii* in milk only in 1 single sampling, but did not develop an antibodies response. In the same group, 7 cows became slightly seropositive but at the following sampling, they turned back to seronegative status, thus it is very likely that this variation was due to an aspecific response to the ELISA test rather than to a seroconversion.

The evolution of group 2, the cows ELISA-PCR+, was quite interesting. The majority of

these cows, 12 out of 14, stopped the shedding of *C. burnetii* in milk after the first sampling and from the 3rd one, all cows tested negative at the r-t PCR on milk. Despite this, it was expected that over the time these cows would produce antibodies against *C. burnetii* due to the presence of the infection, whereas they remained seronegative for all the study time except for 1 cow, which showed a transitory seroconversion at the 3rd sampling.

In group 3, the cows ELISA+PCR-, 14 cows became shedders in milk at the 2nd sampling and 4 of these cows remained positive to r-t PCR in milk for all the time of the study. Other 2 cows became seronegative at the 3rd sampling, but turned again seropositive at the 4th sampling, and other 2 cows became seronegative at the last sampling.

In group 4, cows ELISA+PCR+, all cows remained seropositive throughout the study time, even if it should be considered that 50% of the cows of this group were culled before the end of the trial. Regarding the shedding of *C. burnetii* by these cows, 24 of them were found positive at least at 2 consecutive samplings and 9 cows were positive at 3 consecutive samplings.

The DNA of *C. burnetii* was detected in the feces of 9 cows only . All 9 cows had only 1 single positive sample without persistence of shedding during the time of the study (table 17). Among these 9 positive samples, 8 had a high ct, ranging from 37.12 and 38.86 with cows belonging all to the same herd, while only 1 sample from a different herd, had a lower ct equal to 29.30 Of these 9 cows, 5 were ELISA-PCR -, 1 was ELISA-PCR+, 1 was ELISA+PCR-, and 2 ELISA + PCR +. The cow with the lower ct was ELISA–PCR-. Of these cows, 3 tested positive during dry-off and the remaining 6 had a negative PCR test in milk.

The results of the serological and molecular test performed at the first sampling were compared to evaluate the relation between the serological status of the cows in infected herds and the occurrence of *C. burnetii* shedding in milk. Tables 18 and 19 show the output

data of this comparison: the percentage of agreement between r-t PCR in milk and CFT was only 35.4% for positive samples, while for negative samples it was 91.4%. The percentage of seronegative cows (at the CFT test) shedding *C. burnetii* in milk, was 64.6%. The OR of the comparison was 5.8 (table 20), in other words the probability that a CFT seropositive cow would be a *C. burnetii* shedder in milk is 5.8 time greater than for a CFT seronegative cow. The percentage of agreement between r-t PCR in milk and ELISA was 70.8% for positive samples, while for negative samples it was 73.8%. The percentage of seronegative cows (at the ELISA test) shedding *C. burnetii* in milk, was 29.2%. The OR of the comparison was 6.8 (table 20).

The agreement between FDC and ELISA, measured with Cohen's K was 0.42, a level of agreement defined as fair.

In a study by Guatteo et al. (2007) it was described that cows with a high serological titer (S/p>2), had a greater probability of being persistent shedders and the authors proposed this ELISA cut-off, to screen cows in order to detect persistent shedders. For this reason we checked whether using this cut-off value it was possible to increase the probability to detect cows shedding *C. burnetii* in milk.

The output data of this comparison are displayed in table 21: the percentage of agreement between r-t PCR in milk and ELISA decreased from to 70.8% to 54.2% for positive samples, while for negative samples it increased from 73.8% to 89.5%. The number of ELISA seropositive cows with PCR negative results decreased from 26.3% to 10.5%, but the percentage of seronegative cows shedding *C. burnetii* in milk, rose from 14 % to 22%. The OR increased from 6.8 to 10.03 (table 20).

A better evaluation of the use of serology for herds screening to identify suspect persistent shedders of *C. burnetii* is possible considering all 4 samples taken during the study, from cows with PCR positive milk samples. Therefore the cows with at least 2 positive milk r-t

PCR tests were identified as persistent shedders and a new comparison was performed to evaluate the association between the serological status of the cows and the persistent shedding of *C. burnetii* in milk.

Table 22 shows the output data of this comparison: the percentage of ELISA seropositive cows with PCR negative was 27.2% and only 2 (8.3%) out of 24 persistent shedder cows were seronegative at the ELISA test. The OR was extremely high, 29.4 (table 20). If the cut-off of ELISA test was raised up to > 2, including only the high seropositive cows, the percentage of ELISA seropositive cows with PCR negative was 11.6% and the number of persistent seronegative shedder cows was 7 (29.2%). The OR was 18.4 (table 20).

Dairy goats

The results of serological and molecular tests performed are displayed in table 22. At first sampling in the 3 infected flocks 257 lactating goats were sampled. Graph 1 shows the results of the analysis performed on milk and blood samples collected from each goat. The number of goats sampled from each flock was very different, ranging from 12 to 171, due to the different size of the flocks. The goats shedding *C. burnetii* in milk were 59, the 23% of the sampled animals, the ELISA seropositive goats were 177 (69%) and the CFT seropositive ones were 11 (4%). The percentage of *C. burnetii* shedders in milk was very different among the 3 flocks, ranging from 100% of goats in flock 2 to 1% in flock 3, while the percentage of seropositive goats was more uniform and above 60% in all 3 flocks, ranging from 65% to 83%. In flocks 1 and 2 none of the sampled goats was positive to CFT test, in flock 3 only 6% of animals exhibited the presence of complement fixing antibodies.

After the first sampling, according to the results of ELISA and r-t PCR, the goats were grouped as follows and sampled 3 other times every 2 months:

- ✓ group 1: 15 goats ELISA negative and PCR negative (ELISA-PCR-),

- ✓ group 2: 17 goats ELISA positive and PCR negative (ELISA+PCR-),
- ✓ group 3: 22 goats ELISA positive and PCR positive (ELISA+PCR+).

Differently from cows, only 1 ELISA-PCR+ goat was found (flock 2), so it was impossible to create a group with a single animal, but even this goat was sampled 3 times during the trial. Moreover while in flock 1 and 2 animals originated from all groups, in flock 2, where all goats were PCR positive, the only present group was ELISA+PCR+.

At 2nd sampling in flock 1 and 2 the number of goats shedding *C. burnetii* in milk had sharply decreased and only 1 positive goat was found in either flocks, while in flock 3 the 2 milk shedders were still found positive at r-t PCR. At this sampling in flock 1 the feces of 15 goats were found positive at r-t PCR. Of these goats 7 belonged to the ELISA+ PCR+ group, 5 to the ELISA+ PCR- group and 3 to the ELISA-PCR- group. The only goat that was still shedding *C. burnetii* in milk was also shedding in feces. All the ct of the r-t PCR positive feces were > 35, therefore the bacterial load of *C. burnetii* excreted with feces was very low. Further 1 goat with positive r-t PCR in feces was found in flock 2: this goat was in the group ELISA+PCR+ but at this sampling her milk was negative at the r-t PCR.

At 3rd sampling all milk samples in flocks 1 and 2 were negative, while in flock 3 the same 2 goats were still shedding *C. burnetii* in milk. In flock 1, out of the 15 goats shedding *C. burnetii* in feces at 2nd sampling, 9 were still shedding it in feces and 4 new goats were positive in feces. In flock 2 a new goat was found positive at r-t PCR in feces.

At 4th sampling in flock 1, 2 goats belonging to the ELISA+PCR- group were found positive at r-t PCR on milk, in flock 2 no milk positive goats were found and in flock 3 only 1 goat was still positive. In flock 1 5 goats were still shedding *C. burnetii* in feces, 2 belonging to the ELISA+PCR group + and 3 to the ELISA+PCR- group.

The pattern of *C. burnetii* excretion in feces was very different between flock 1 and the

other 2 flocks. In flocks 2 and 3 this way of shedding was only sporadic and limited to respectively 2 and 1 goat, while in flock 1 several goats started to shed *C. burnetii* in feces. Out of 15 fecal shedders found at 2nd sampling, 3 remained positive for 3 consecutive samplings, 6 for 2 and 10 at only 1 sampling. Among the goats shedding *C. burnetii* in feces for 2 or 3 samplings, some belonged to the ELISA+ PCR+ and other to the ELISA+ PCR- groups: no seronegative goats were found shedding in feces, as seen in flock 3.

The great majority of seropositive goats remained in this status until the end of the study, only 3 goats out of 39 (7%) were negative at ELISA test at last sampling, while 3 goats out of 15 (20%) became positive at ELISA test. The evaluation of the serological status of the goats with CFT was very difficult: in flocks 1 and 2 at 1st sampling no positive animal were found, at the 2nd one a high proportion of goats, respectively 35% and 25%, were found positive, but at last sampling all goats where negative again. In flock 3 at 1st sampling 6% of goats were positive, at the 2nd all the goats were negative, at the 3rd 90% of goats were positive, at 4th sampling 25% of goats were positive. These remarkable variations in a relative short time were unpredictable and not associated with *C. burnetii* shedding.

2.3 DISCUSSION

2.3.1 Assessment of the efficacy of r-t PCR assays on BTM for the detection of infected dairy herds and flocks

The criteria for the definition of *C. burnetii* infection are much easier to apply to a single animal than to a herd or flock. For this reason the definition of infected or uninfected herd or flock used in this study was is very precise and has considered all elements that demonstrated the active circulation of the etiological agent in herds or flocks, taking in account other studies published on this subject (Sidi-Boumedine et al, 2010; Guatteo et. al., 2011).

The results of the test performed in dairy cows highlighted that when *C. burnetii* abortions occurred in a herd *the within-herd* seroprevalence is greater than > 20%, even if a less sensitive test, as CFT, is used. When ELISA test was used, it was possible to evaluate the results with a cut-off that did not include the weak positive samples, without any change in the number of infected detected herds . These findings confirmed that in infected herds the circulation of *C. burnetii* usually triggered a strong antibodies response in cows. The situation found in 1 single herd remained unexplained, where, despite the presence of abortions and PCR positive BTM, most of cows tested seronegative even several months later: the only possible explanation is the presence of few seronegative cows shedding *C. burnetii* in milk, phenomenon already described in cows infected by *C. burnetii* (Rodolakis, 2006).

The results obtained with r-t PCR on BTM showed that a high number of infected herds (50%) is misclassified as uninfected with only a single test performed. These results, taking in account that r-t PCR is a very sensitive method (EFSA 2010), demonstrated that *C. burnetii* shedding in milk is one of the possible outcomes of this infection in dairy herds

(Guatteo, 2006), but not always and during the whole period, there were cows shedding *C. burnetii* in milk, therefore a single BTM sample could not be able to detect all infected herds. Conversely the specificity of the BTM r-t PCR was excellent and all the uninfected herds have been correctly classified. Hence the combination of r-t PCR and seroprevalence assessment could be a useful tool to detect uninfected herds.

Another aspect that should be considered in dairy herds is the use of BTM to detect antibodies against *C. burnetii*. In this study all herds with negative abortions were tested with ELISA using BTM, before the assessment of the seroprevalence using a randomized sample of cows. We observed that among the 22 herds with negative abortions, 12 (55%) had a seroprevalence ≥ 20%, thus the ELISA test on BTM was not able to detect many herds with a seroprevalence that is consistent with the presence of *C. burnetii* infection in the herd (Sidi-Boumedine et al, 2010; Guatteo et. al., 2011).

The prevalence of *C. burnetii* infection in dairy goat flocks in North-eastern Italy was unknown at the beginning of this study. The results of tests performed on sampled flocks highlighted that the 30% of flocks were infected, according to the definition of infected flock used in this study. Of course this data are only an indication of the possible existing prevalence, because the object of the study was not to perform an assessment of the infection prevalence and the flocks sample was not designed for this purpose.

The absence of specific clinical symptoms in infected ruminants have been described in literature (Arricau-Bouvery and Rodolakis, 2005), but especially in caprine flocks Q fever abortions can affect sometime up to 90% of females (Palmer et al., 1983). In this study we observed only the occurrence of sporadic abortions in infected flocks and in many cases the detection of *C. burnetii* was performed from vaginal swabs of goats that had delivered normal and vital kids.

Among the flocks with a *C. burnetii* positive vaginal swab, only 60% showed also a seroprevalence ≥ 15%, a very different outcome from the one observed in dairy cattle. It is quite difficult to explain this result, because in flocks affected by circulation of *C. burnetii* at the time of kidding, we would expected a high seroprevalence in goats and the absence of severe clinical symptoms like "storming abortions" usually means that the disease was enzootic in the flock and not a new infection (Berri et al, 2001; Berri et al, 2007).

We hypothesized that the *C. burnetii* strains circulating in the flocks were low pathogenic so they infected only few goats after shedding at parturition, but further studies are needed to confirm this hypothesis.

All flocks with negative BTM at r-t PCR had also a seroprevalence < 15%. The CFT test showed a lower sensitivity in goats compared to cattle. Out of 9 infected flocks with a seroprevalence ≥ 15 using ELISA test, only in 2 of them CFT positive goats were found. The use of ELISA test with a cut-off that did not include the weak positive samples, affected remarkably the classification of infected flocks. The number of infected flocks decreased from 9 to 7, thus the use of this cut-off in not advisable in goats.

The sensitivity of r-t PCR on BTM was equal to 0.8, much better than in cattle and the specificity was excellent. The different sensitivity of BTM PCR between cattle and goats is probably due to the different pattern of the reproduction cycle in the two species. In goats kidding are seasonal, concentrated in few months, the shedding of *C. burnetii* in milk occurred mainly after parturition and milk samples have been collected during this time. In dairy cattle calving are widespread throughout the year therefore, in absence of persistent shedders, until the calving of a new infected cow, a single BTM sample can produce a negative result even in an infected herd.

2.3.2 Evaluation of the dynamics of the antibodies response and the *C. burnetii* excretion in infected animals

The results of the tests performed in dairy herds highlighted a remarkable difference between the amount of cows shedding *C. burnetii* and the seropositive ones.

As showed in table 13 and 14 the seroprevalence evaluated by means of ELISA testing was very high, while the shedding of *C. burnetii* occurred in up to a maximum of 13% of cows. This result, in agreement with the outcome of other studies (Guatteo et al., 2007; Sidi-Boumedine et al., 2010), highlighted that cows remained seropositives for long time after the infection, when the shedding of *C. burnetii* had stopped several months before.

The number of cows shedding *C. burnetii* in milk was lower than the one reported in other studies (Guatteo et al., 2007; Guatteo et al., 2011), where the percentage of shedding cows was close to 40%. Among these shedding cows it is interesting to observe that the percentage of high shedders was very low (0-4%). One possible explanation could be the time elapsed between the abortion of positive cows and the first sample of milk or that the disease was enzootic in all herds sampled during the study, so that a balance between cows and etiological agent might have established. As reported in another study (Guatteo et al., 2007), we observed that all these high shedding cows excreted the bacterium in milk throughout the time they remained in the herd, thus they seemed to have an important role in maintaining the infection in the herd (Guatteo et al., 2007). Therefore detection and culling of these cows would be advisable, but of course to perform repeated r-t PCR tests on individual milk samples of all cows is a very expensive strategy. Hence we observed that all the high shedding cows were also high positive ELISA reactors, therefore a strategy based on a two step screening, first ELISA and then r-t PCR on the milk of high ELISA positive cows, could be useful to detect the high persistent shedders of the herd.

The occurrence of seronegative cows shedding *C. burnetii* in milk has been reported in some study (Rodolakis, 2006; Guatteo et al., 2007; Guatteo et al., 2012) and this event has been explained as a transitory phase that occurs at the beginning of the infection, or as a lack of sensitivity to the serological test (Rodolakis 2006). In this study 14 seronegative cows shedding *C. burnetii* in milk were found. Out of these cows, 13 were present till the end of the study and only 1 cow showed a transitory seroconversion at 3rd sampling. In this case both hypothesis used to explain this outcome of *C. burnetii* infection are unsuitable because we are not at the beginning of the infection and the ELISA test detected correctly the antibodies in all the other cows shedding *C. burnetii* in milk. A possible explanation of this event could be the presence in these cows of a strong cell-mediated immune response with low or absent production of antibodies, due to the efficacy of the cell-mediated response.

The excretion of *C. burnetii* in these cows lasted for a very short time: at 2nd sampling 12 cows were negative at r-t PCR test on milk and from the 3rd sampling all cows in the group were negative. For this reason these cows might not seem very relevant for the epidemiology of the infection.

The group of ELISA- PCR- cows provided a figure of the widespread patterns of the infection among lactating cows. In this group 2 cows tested positive at the r-t PCR in milk respectively at 3rd and 4th sampling but they didn't seroconvert; at the same time 7 cows had a transitory seroconversion only at 2nd sampling. The hypothesis of a strong cell-mediated immune response with low or absent production of antibodies could also be used to explain this event. This hypothesis is supported by the findings of a study performed in Germany (Schumacher et al., 2011) using the γ Interferon production test, that showed the presence of seronegative cows with strong cell-mediated immune response against *C. burnetii*. Thus we can hypothesized that some cows after infection with *C. burnetii* develop mainly a

strong cell-mediated response that avoid the persistence of the bacterium in the cow and therefore the production of antibodies in these animals is very low and transient.

In the group of ELISA+PCR- cows we observed that almost 40% of them had at least 1 positive milk sample with r-t PCR. This result matches the findings reported in other studies (Guatteo et. al 2007; Rodolakis, 2006) on the occurrence of discontinuous excretion in milk, where a single PCR test was not enough to detect all shedding cows. The opposite happened in the ELISA+PCR+ group : these cows became negative at the PCR in milk over the time and at the end of the study, only 6 cows were still positive. In all cows of this group serology was positive throughout the study.

The excretion of *C. burnetii* in feces was merely occasional: only 9 cows were found positive and many of them were ELISA-PCR-. This result matched the findings of Guatteo et al. (2007) and highlighted the poor relevance of cows in the widespread of the bacterium in the environment, compared to other ruminants, especially sheep, that can shed large amounts of *C. burnetii* in the feces (Rodolakis et al., 2006).

The detection of cows excreting *C. burnetii* is an important but difficult task in the diagnosis of the infection in dairy herds, due to the different ways of shedding and to discontinuous excretion of the bacterium. The use of repeated molecular analysis on different biological substrate could achieve this result, but this approach is economically unsustainable. The use of serological test only is, on the other hand, unreliable because the presence of antibodies lasts for a long time after the infection (Sidi-Boumedine et. al., 2010).

In this study we evaluated the association between the serological response of the cow to ELISA or CFT tests and the occurrence of *C. burnetii* shedding in milk. We select the milk as substrate to evaluate the shedding, because a previous study demonstrated that it is the only biological excretion with a fair level of agreement between PCR and serology (Natale

et al., 2012). This better level of agreement is probably due to the long lasting shedding of *C. burnetii* in milk and not in other biological excretions (EFSA, 2010; Guatteo et al., 2007). The level of statistical association was evaluated by estimation of the OR, a parameter that measures the different probability of a serological positive cow to be shedders of *C. burnetii* in milk compared to a serological negative cow. The results obtained highlighted a high statistically significant association, especially for the detection of persistent shedders of both ELISA and CFT, with values of OR equal to 6.8 for CFT and 29.4 for ELISA. The great majority of persistent shedding cows were positive at the ELISA test, 22 out of 24 and this explains the very high value of the OR, but the number of cows that tested positive to ELISA was too high (181) to adopt this test as a prescreening to select cows for r-t PCR on milk. On the other side the OR of the CFT was too low to use this test for prescreening, due to the high number of persistent shedder (14) that were negative to CFT.

Cows with a high serological titer (s/p>2) to ELISA have a greater probability of being persistent shedders (Guatteo et al. (2007), for this reason we checked whether using this cut-off value would reduce the number of positive cows, while keeping a good chance to detect the ones shedding *C. burnetii* in milk. Using this criteria to detect seropositive cows, a high statistical association (OR 8.4) was found between serological results and r-t PCR in milk and out of 24 persistent shedding cows only 7 had an ELISA s/p value ≤ 2. Moreover the number of seropositive cows using this cut-off were 85, a number that might enable the use of the test as a prescreening to select the cows to test with r-t PCR in milk.

The evaluation of the *C. burnetii* excretion patterns in 3 goat flocks has provided several interesting data that can be compared with those observed in the dairy herds.

The results of the study on flocks highlighted the great variability in the features of *C. burnetii* excretion in goats, also if we must take into account the difference of the size

among the flocks. The shedding of *C. burnetii* in milk at 1stfirst sampling involved all the goats in flock 2, 60% of the goats in flock 1 and only 1% in flock 3. The percentage of seropositive goats with ELISA test was high and quite similar among flocks, therefore the disease had probably already been present in the farm since at least the previous reproductive season. The great variability in the number of goats shedding in milk could be due to different management procedures and this could explain the low percentage of shedders in flock 3, which had better quality housing, facilities and health management. These management features can contribute to reduce the widespread of the infection within the herd and thus reduce also the number of infected goats.

After 2 months from the first sample, the excretion of *C. burnetii* in milk decreased dramatically also in flock 1 and 2, highlighting a big difference with the dairy herds. In cows the number of shedders in milk took longer to decline and at the end of the study there were some cows still positive to r-t PCR in milk.

Also in fecal excretion of *C. burnetii* there were some differences among the 3 flocks: in flock 2 and 3 the detection of positive goats was sporadic while in flock 1 the number of fecal shedder was greater and relatively long-lasting. This difference among flocks seems to be more connected with individual factors, like the type of strain and the response of the animal, than to management and housing systems.

Among the goats shedding *C. burnetii* in feces, there were some seropositive animals but showing PCR-negative milk and also some seronegative ones, thus this excretion manner might be in goats the only way of *C. burnetii* shedding, as it happens in sheep (EFSA, 2010).

It should be emphasized that all the ct were > 31, therefore the bacterial load per gram of feces was low, aspect of great importance because small ruminant feces for their physical features could be very dangerous for the spill-over of the infection from animals to

humans, as demonstrated in several of the outbreaks occurred in the last decades (Arricau-Bouvery et al., 2001; Berri et al., 2004; EFSA, 2010; Manfredi_Selvaggi, 1986; Starnini et al., 2005).

The serological response to the ELISA test highlighted a high percentage of positive animals, enforcing the evidence already obtained in the study on BTM r-t PCR performance, that infected flocks have a seroprevalence as high as the one of dairy herds. Therefore for diagnostic purpose is always useful to evaluate the seroprevalence of goats flocks when Q fever infection is suspected.

The serological response to CFT was very peculiar: at 1st sampling the percentage of positive goats was low or even 0, in the following samplings there were many positive results, but only for one single sampling and then the goats returned negatives.

These results confirmed in part the lack of sensitivity of CFT compared to ELISA (OIE, 2015), but moreover they highlighted the low reliability of CFT in goats, due to this great variability in the serological response. Further studies are needed to clarify whether this test response is associated with specific immunological features of humoral response or it depends on the kind of *C. burnetii* strains involved in the herd infection.

2.4 CONCLUSIONS

This study focused on the evaluation of diagnostic methods used for the detection of the *C. burnetii* infection in cattle and goats. The diagnosis of the infection should involve the use of multiple techniques and can be validly interpreted only at herd or flock level. For this purpose both serological and molecular assays were evaluated, the dynamics of the antibodies response were studied and the ways of *C. burnetii* excretion in cows and goats.

The assessment of diagnostic methods lead to the conclusion that detection of infected herds or flocks needs the simultaneous use of assays able to detect the bacterium and the presence of antibodies, since the assessment of the seroprevalence has proved very helpful to recognize the presence of active infections in farms, especially in goats flocks.

The use of r-t PCR on BTM alone was assessed in the study because it could be a useful and cheap tool for detection of infected herds or flocks. However the research outcome suggested that the results of this assay should be carefully evaluated to avoid misinterpretations. In dairy herds a single r-t PCR test on BTM is reliable only in case of positive result, while in case of negative result, the test has a 50% failure probability. In this situation it is advisable to check the within herd seroprevalence and to repeat the test after 1-2 months. In goats flocks, if the BTM is collected just at the end of the kidding season, when the probability of having *C. burnetii* shedding in milk is highest, the test's sensitivity reaches the value of 80%, therefore negative results are more reliable. In this case, to increase sensitivity, it is enough to check the seroprevalence of the flock without repeating the BTM sample that, after 2 months, could be negative because the excretion of *C. burnetii* in milk has stopped.

The detection of persistent shedders in dairy herds could be an important tool to reduce the risks of transmission among animals. Serological tests could provide useful information on

the cow's status and thus they can be used as a screening tool of the herd before testing with r-t PCR the milk, with the aim to reduce the cost of analysis. The results obtained in this study highlighted the possibility to use the ELISA test to screen the herd and then analyze with r-t PCR only the milk of high positive cows. This allowed to detect the great majority of the persistent shedders present in the herds.

The excretion of *C. burnetii* in dairy cows after the time of calving occurred mainly in milk and the bacterium was found in feces only sporadically. During the study some cows showed persistent shedding in milk throughout the 6 months of observation and others shed *C. burnetii* discontinuously or only occasionally. In this study we also found some seronegative cows shedding *C. burnetii* in milk that did not show any seroconversion until the end of the trial. The occurrence of seronegative cows shedding *C. burnetii* in milk has been reported in some studies (Rodolakis, 2006; Guatteo et al., 2007; Guatteo et al., 2012) and this event has been explained as a transitory phase that occurs at the beginning of the infection or as a lack of sensitivity of the serological test (Rodolakis 2006). The results of this study suggest a different explanation for this event because we followed the cows for 6 months and the test was performing correctly with all the other cows shedding *C. burnetii* in milk. We hypothesized that some cows after infection with *C. burnetii* develop mainly a strong cell-mediated response that avoid the persistence of the bacterium in the cow and therefore the production of antibodies in these animal is very low and transient. Further investigation are needed to increase the knowledge of the immune response in ruminants, because they could provide important explanations about the behavior of the bacterium in these animals.

C. burnetii excretion in caprine flocks was more diversified than in cattle, especially the shedding of the bacterium in feces occurred with a higher probability, while the excretion in milk lasted on average only 1 month, even if few long-lasting shedders were found in 1

goat flock as well.

The serological response to the ELISA assay was high in all flocks tested, enforcing the evidence that infected flocks have a seroprevalence high as the one of the dairy herds. Oppositely CFT test showed a poor sensitivity compared to ELISA and we observed great a variability in the serological response of goats to CFT, therefore the test results had poor reliability.

2.5 TABLES, FIGURES AND GRAPHS

Table 1: amplification program used for carry out the r-t PCR execution

R-T PCR PROGRAM FOR COXIELLA BURNETII			
	Slope		Acquisition
Step 1: HOT START	20	50°C / 2 min. and after 95°C / 10 min.	None
Step 2: DENATURATION	1,5	95°C / 15 sec	None
Step 3: ANNEALING/	1,5	60°C / 1 min	Single
CYCLES N°from Step 2 to Step 3		45	//
COOLING	20	40°C / infinite	None

Table 2: Results of ELISA serological test on blood samples for the within-herd seroprevalence assessment and BTM r-t PCR test, performed in dairy cattle herds, sorted by presence (herds with *C. burnetii* abortion) or absence (herds without *C. burnetii* abortion) of abortions with a positive r-t PCR for *C. burnetii*. Sera were defined positive when ELISA s/p value was > 0.4.

	herds with C. burnetii abortions	%	herds without C. burnetii abortions	%
Herds number	17	-	22	-
Herds with seroprevalence < 20%	1	6%	10	45%
Herds with seroprevalence ≥ 20%	16	94%	12	55%
Herds with BTM PCR +	9	53%	6	27%
Herds with seroprevalence ≥ 20% BTM PCR +	8	47%	6	27%
Herds with seroprevalence < 20% BTM PCR -	8	47%	6	27%
Herds with seroprevalence < 20% BTM PCR +	1	6%	0	0%
Herds with seroprevalence < 20% BTM PCR -	0	0%	10	45%

Table 3: Results of CFT serological test on blood samples for the within-herd seroprevalence assessment and BTM r-t PCR test, performed in dairy cattle herds, sorted by presence (herds with *C. burnetii* abortion) or absence (herds without *C. burnetii* abortion) of abortions with a positive r-t PCR for *C. burnetii*.

	herds with C. burnetii abortions	%	herds without C. burnetii abortions	%
Herds number	17	-	22	-
Herds with seroprevalence < 20%	4	24%	17	77%
Herds with seroprevalence ≥ 20%	12	71%	5	23%
Herds with BTM PCR +	9	53%	6	27%
Herds with seroprevalence ≥ 20% BTM PCR +	8	50%	4	18%
Herds with seroprevalence < 20% BTM PCR -	4	25%	1	5%
Herds with seroprevalence < 20% BTM PCR +	1	6%	2	9%
Herds with seroprevalence < 20% BTM PCR -	3	19%	15	68%

Table 4: Contingency table showing the ability of a single r-t PCR test performed on BTM to detect *C. burnetii* infection in a herd. The herd status, infected or uninfected has been defined according to within-herd seroprevalence and presence/absence of *C. burnetii* abortion. Within-herd seroprevalence was estimated using ELISA test with a s/p value cut-off > 0.4.

			Herds status		Total
			Uninfected	Infected	
PCR BTM	Negative	Number	10	8	18
		% (refered to herds status)	100%	50%	69%
	Positive	Number	0	8	8
		% (refered to herds status)	0%	50%	31%
Total		Number	10	16	26
		% (refered to herds status)	100%	100%	100%

Table 5: Contingency table showing the ability of a single r-t PCR test performed on BTM to detect *C. burnetii* infection in a herd. The herd status, infected or uninfected has been defined according to within-herd seroprevalence and presence/absence of *C. burnetii* abortion. Within-herd seroprevalence was estimated using CFT test.

			Herds status		Total
			Uninfected	Infected	
PCR BTM	Negative	Number	15	4	19
		% (refered to herds status)	88%	33%	66%
	Positive	Number	2	8	10
		% (refered to herds status)	12%	67%	34%
Total		Number	17	12	29
		% (refered to herds status)	100%	100%	100%

Table 6: Results of ELISA serological test on blood samples for the within-herd seroprevalence assessment and BTM r-t PCR test, performed in dairy cattle herds, sorted by presence (herds with *C. burnetii* abortion) or absence (herds without *C. burnetii* abortion) of abortions with a positive r-t PCR for *C. burnetii*. Sera were defined positive when ELISA s/p value was > 1.

	herds with C. burnetii abortions	%	herds without C. burnetii abortions	%
Herds number	17	-	22	-
Herds with seroprevalence < 20%	1	6%	14	64%
Herds with seroprevalence ≥ 20%	16	94%	8	36%
Herds with BTM PCR +	9	53%	6	27%
Herds with seroprevalence ≥ 20% BTM PCR +	8	47%	6	27%
Herds with seroprevalence < 20% BTM PCR -	8	47%	2	9%
Herds with seroprevalence < 20% BTM PCR +	1	6%	0	0%
Herds with seroprevalence < 20% BTM PCR -	0	0%	14	64%

Table 7: Contingency table showing the ability of a single r-t PCR test performed on BTM to detect *C. burnetii* infection in a herd. The herd status, infected or uninfected has been defined according to within-herd seroprevalence and presence/absence of *C. burnetii* abortion. Within-herd seroprevalence was estimated using ELISA test with a s/p value cut-off > 1.

			Herds status		
			Uninfected	Infected	Total
PCR BTM	Negative	Number	14	8	22
		% (refered to herds status)	100%	50%	73%
	Positive	Number	0	8	8
		% (refered to herds status)	0%	50%	27%
Total		Number	14	16	30
		% (refered to herds status)	100%	100%	100%

Table 8: Results of ELISA serological test on blood samples for the within-flock seroprevalence assessment, and BTM r-t PCR test, performed in dairy goat flocks, sorted by presence (flocks with *C. burnetii* + vaginal swabs) or absence (flocks with *C. burnetii* - vaginal swabs) of *C. burnetii* in vaginal swabs. Sera were defined positive when ELISA s/p value was > 0.4.

	Flocks with C. burnetii+ vaginal swabs	%	Flocks with C. burnetii- vaginal swabs	%
Flocks number	15		14	
Flocks with seroprevalence ≥ 15%	9	60%	0	7%
Flocks with BTM PCR +	8	47%	0	0%
Flocks with seroprevalence ≥ 20% BTM PCR +	7	47%	0	0%
Flocks with seroprevalence < 20% BTM PCR -	2	13%	0	0%
Flocks with seroprevalence < 20% BTM PCR +	1	7%	0	0%
Flocks with seroprevalence < 20% BTM PCR -	5	33%	14	100%

Table 9: Results of CFT serological test on blood samples for the within-flock seroprevalence assessment, and BTM r-t PCR test, performed in dairy goat flocks, sorted by presence (flocks with *C. burnetii* + vaginal swabs) or absence (flocks with *C. burnetii* - vaginal swabs) of *C. burnetii* in vaginal swabs.

	Flocks with C. burnetii+ vaginal swabs	**%**	**Flocks with C. burnetii- vaginal swabs**	**%**
Flocks number	15		14	
Flocks with seroprevalence ≥ 15%	9	60%	1	7%
Flocks with BTM PCR +	8	47%	0	0%
Flocks with seroprevalence ≥ 20% BTM PCR +	7	47%	0	0%
Flocks with seroprevalence < 20% BTM PCR -	2	13%	1	7%
Flocks with seroprevalence < 20% BTM PCR +	1	7%	0	0%
Flocks with seroprevalence < 20% BTM PCR -	5	33%	13	93%

Table 10: Contingency table showing the ability of a single r-t PCR test performed on BTM to detect *C. burnetii* infection in a flock. The flock status, infected or uninfected has been defined according to within-flock seroprevalence and presence/absence of *C. burnetii* in vaginal swabs. Within-flock seroprevalence was estimated using ELISA test with a s/p value cut-off > 0.4.

			Flocks status		Total
			Uninfected	Infected	
PCR BTM	Negative	Number	14	2	16
		% (refered to flocks status)	100%	22%	70%
	Positive	Number	0	7	7
		% (refered to flocks status)	0%	78%	30%
Total		Number	14	9	23
		% (refered to flocks status)	100%	100%	100%

Table 11: Results of ELISA serological test on blood samples for the within-flock seroprevalence assessment and BTM r-t PCR test, performed in dairy goat flocks, sorted by presence (herds with *C. burnetii* + vaginal swabs) or absence (herds with *C. burnetii* - vaginal swabs) of *C. burnetii* in vaginal swabs. Sera were defined positive when ELISA s/p value was > 1.

	Flocks with C. burnetii+ vaginal swabs	**%**	**Flocks with C. burnetii- vaginal swabs**	**%**
Flocks number	15		14	
Flocks with seroprevalence ≥ 15%	7	47%	0	0%
Flocks with BTM PCR +	8	53%	0	0%
Flocks with seroprevalence ≥ 20% BTM PCR +	7	47%	0	0%
Flocks with seroprevalence < 20% BTM PCR -	0	0%	0	0%
Flocks with seroprevalence < 20% BTM PCR +	1	7%	0	0%
Flocks with seroprevalence < 20% BTM PCR -	7	47%	14	100%

Table 12: Contingency table showing the ability of a single r-t PCR test performed on BTM to detect *C. burnetii* infection in a flock. The flock status, infected or uninfected has been defined according to within-flock seroprevalence and presence/absence of *C. burnetii* in vaginal swabs. Within-flock seroprevalence was estimated using ELISA test with a s/p value cut-off > 1.

			Flocks status		Total
			Uninfected	Infected	
PCR BTM	Negative	Number	14	0	14
		% (refered to flocks status)	100%	0%	67%
	Positive	Number	0	7	7
		% (refered to flocks status)	0%	100%	33%
Total		Number	14	7	21
		% (refered to flocks status)	100%	100%	100%

Table 13: Results of serological test (ELISA and CFT) for *C. burnetii* antibodies detection, performed on the blood sample of each lactating cow at first sampling, in the 4 dairy herds enrolled in the trial for the evaluation of the dynamics of the antibodies response and the *C. burnetii* excretion in infected animals. For ELISA test a double cut-off has been used: s/p value > 0.4, to include all the positive samples and s/p value > 2 to include only the high and very high positive samples.

Herd	N° cows tested	CFT	CFT (%)	ELISA s/p >0.4	ELISA s/p >0.4 (%)	ELISA s/p >2	ELISA s/p >2 (%)
Herd 1	107	17	15.9%	42	39.3%	17	15.9%
Herd 2	107	12	11.2%	23	21.5%	4	3.7%
Herd 3	100	8	8.0%	48	48.0%	34	34.0%
Herd 4	294	28	9.5%	68	23.1%	30	10.2%
Total	**608**	**65**	**10.7%**	**181**	**29.8%**	**85**	**14.0%**

Table 14: Results of the r-t PCR test for *C. burnetii* DNA detection performed on the milk of each lactating cow at first sampling in the 4 dairy herds enrolled in the trial for the evaluation of the dynamics of the antibodies response, and the *C. burnetii* excretion in infected animals. The last 2 columns highlight the number and percentage of high shedders of C. burnetii in milk (ct value ≤ 31).

Herd	N° cows tested	PCR +	PCR + (%)	PCR + Ct≤31	PCR + Ct≤31 (%)
Herd 1	107	11	10.3%	4	3.7%
Herd 2	107	14	13.1%	0	0.0%
Herd 3	100	10	10.0%	4	4.0%
Herd 4	294	13	4.4%	4	1.4%
Total	**608**	**48**	**7.9%**	**12**	**2.0%**

Table 15: Size of the 4 groups of cows established according to the results of ELISA and r-t PCR in milk, at each time of sampling.

Group	T1	T2	T3	T4	Totale
ELISA -_PCR-	49	48	41	39	177
ELISA -_PCR+	14	14	13	13	54
ELISA +_PCR-	43	43	40	38	164
ELISA +_PCR+	34	29	21	19	103
Totale complessivo	140	134	115	109	498

Table 16: Variation along the time of the serological and shedding status of the cows during the study at each sampling: changes of status at each sampling (T2-T3-T4) are highlighted by the gray fill of the cells and the character in bold. The last 2 columns list the cows dried-off or culled at each sampling.

GROUP 1	SAMPLING TIME	ELISA -_PCR-	ELISA -_PCR+	ELISA +_PCR-	ELISA +_PCR+	DRY-OFF	CULLED
ELISA -_PCR-	T1	49	0	0	0	0	-
	T2	40	***1***	***7***	0	0	1
	T3	34	***1***	0	0	6	7
	T4	37	***1***	0	0	1	2
GROUP 2	SAMPLING TIME	ELISA -_PCR+	ELISA -_PCR-	ELISA +_PCR-	ELISA +_PCR+	DRY-OFF	CULLED
ELISA -_PCR+	T1	14	0	0	0	0	-
	T2	2	***8***	0	0	4	0
	T3	0	***12***	1	0	0	1
	T4	0	***11***	0	0	2	0
GROUP 3	SAMPLING TIME	ELISA +_PCR-	ELISA -_PCR-	ELISA -_PCR+	ELISA +_PCR+	DRY-OFF	CULLED
ELISA +_PCR-	T1	43	0	0	0	0	-
	T2	26	0	0	***14***	3	0
	T3	24	***2***	0	***7***	7	3
	T4	26	***2***	0	***5***	5	2
GROUP 4	SAMPLING TIME	ELISA +_PCR+	ELISA -_PCR-	ELISA -_PCR+	ELISA +_PCR-	DRY-OFF	CULLED
ELISA +_PCR+	T1	34	0	0	0	0	-
	T2	17	0	0	***9***	3	5
	T3	8	0	0	***4***	9	8
	T4	6	0	0	***13***		2

Table 17: List of the cows with positive PCR test for the detection of *C. burnetii* DNA in feces. The table shows for each cow: the herd, the number of samplings that tested positive, the results of ELISA test (expressed as s/p ratio) and PCR in milk at the same sampling and the cow group with reference to the test results of the first sampling.

Herd	Sampling Number	Animal ID	*ELISA test result	**Milk PCR result	Feces PCR result (Ct)	Group
2	2	490	0.05	0	29.3	ELISA -_PCR-
1	4	467	0.00	0	37.15	ELISA -_PCR-
1	2	603	0	0	37.2	ELISA -_PCR-
1	4	660	0.04	d	38.23	ELISA -_PCR-
1	3	672	0	0	38.27	ELISA -_PCR-
1	2	570	0	0	37.12	ELISA -_PCR+
1	4	666	2.62	d	38.86	ELISA +_PCR-
1	2	638	1.94	0	38.05	ELISA +_PCR+
1	3	650	4.41	d	37.14	ELISA +_PCR+

*The results are reported as s/p value ratio, positive cows have a s/p > 0.4
**d= dried-off cows, 0 = negative result to the PCR

Table 18: Contingency table showing the comparison between the shedding of *C. burnetii* in milk measured by r-t PCR and the serological status of the cows, measured with ELISA and CFT test. Data are expressed in absolute value and in percentage.

	CFT				ELISA s/p > 0,4		
PCR	-	+	Total	**PCR**	-	+	Total
-	512	48	560	-	413	147	560
+	31	17	48	+	14	34	48
Total	543	65	608	**Total**	427	181	608
%	-	+		**%**	-	+	
-	91.4%	8.6%	-----	-	73.8%	26.3%	-----
+	64.6%	35.4%	-----	+	29.2%	70.8%	-----

Table 19: Contingency table showing the comparison between the shedding of *C. burnetii* in milk measured by r-t PCR and the serological status of the cows, measured with ELISA with 2 different cut-off: s/p > 0.4 and s/p > 2 (only high positive cows). Data are expressed in absolute value and in percentage.

	ELISA s/p > 2				ELISA s/p > 0,4		
PCR	-	+	Total	**PCR**	-	+	Total
-	501	59	560	-	413	147	560
+	22	26	48	+	14	34	48
Total	523	85	608	**Total**	427	181	608
%	-	+		**%**	-	+	
-	89.5%	10.5%	-----	-	73.8%	26.3%	-----
+	45.8%	54.2%	-----	+	29.2%	70.8%	-----

Table 20: assessment of the statistical association between the serological status of the cows in infected herds and the occurrence of *C. burnetii* shedding in milk, comparing the results of ELISA with 2 different cut-off (s/p > 0.4 and s/p > 2) and of CFT. The association is evaluated including all the shedders cows and considering only the persistent shedder cows. The level of association was evaluated by mean of the Odd ratio (95% Confidence interval CI is reported). All the association tested had a p value = 0.00.

	All cows shedding *C. burnetii* in milk		Persistent shedder cows	
Test	**OR**	**CI 95%**	**OR**	**CI 95%**
CFT	5.8	3.01 - 11.33	6.8	2.91 - 16.20
ELISA S/p >0,4	6.8	3.56 – 13.07	29.4	6.83 - 126.47
ELISA S/p >2	10.03	5.35 - 18.81	18.4	7.37 - 46.04

Table 21: Contingency table showing the comparison between the persistent shedding of *C. burnetii* in milk (cows with at least 2 positive milk PCR test) and the serological status of the cows at first sampling, measured with ELISA with 2 different cut-off: s/p > 0.4 and s/p > 2 (only high positive cows). Data are expressed in absolute value and in percentage.

	ELISA s/p > 2				ELISA s/p > 0,4		
PCR	**-**	**+**	**Total**	**PCR**	**-**	**+**	**Total**
-	516	68	584	**-**	425	159	584
+	7	17	24	**+**	2	22	24
Total	523	85	608	**Total**	427	181	608
%	-	+		**%**	-	+	
-	88.4%	11.6%	-----	**-**	72.8%	27.2%	-----
+	29.2%	70.8%	-----	**+**	8.3%	91.7%	-----

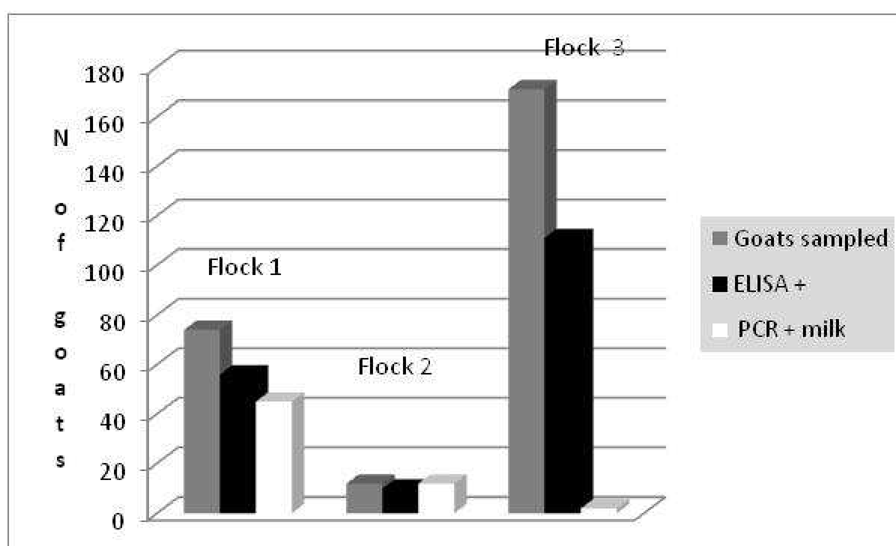
Tabella 22: results of serological (ELISA and CFT) and molecular (r-t PCR) tests performed on blood, milk and feces of each lactating goat at 1st sampling in the 3 dairy flocks enrolled in the trial for the evaluation of the dynamics of the antibodies response and the *C. burnetii* excretion in infected animals.

	Flock 1				Flock 2				Flock 3			
Time of sampling	**T1**	**T2**	**T3**	**T4**	**T1**	**T2**	**T3**	**T4**	**T1**	**T2**	**T3**	**T4**
Goats sampled	74	20	19	19	12	12	12	11	171	23	21	21
ELISA +	56	17	16	15	10	9	10	9	111	12	13	13
CFT +	0	7	2	0	0	3	0	0	11	0	19	5
PCR + milk	45	1	0	2	12	1	0	0	2	2	2	1
PCR + feces	-	15	13	5	0	1	1	0	0	1	0	0

Table 23: results of serological (ELISA and CFT) and molecular (r-t PCR) tests performed on blood, milk and feces of each lactating goat at each of the 4 sampling in the 3 dairy flocks. Data are expressed as percentage of goats positive to each test with reference to the total number of goat sampled at each sampling.

	Flock 1				Flock 2				Flock 3			
Time of sampling	**T1**	**T2**	**T3**	**T4**	**T1**	**T2**	**T3**	**T4**	**T1**	**T2**	**T3**	**T4**
ELISA +	76%	85%	84%	79%	83%	75%	83%	82%	65%	52%	62%	62%
CFT +	0%	35%	11%	0%	0%	25%	0%	0%	6%	0%	90%	24%
PCR + milk	61%	5%	0%	11%	100%	8%	0%	0%	1%	9%	10%	5%
PCR + feces	-	75%	68%	26%	0%	8%	8%	0%	0%	4%	0%	0%

Graph 1: Summary of the results of *ELISA* and r-t PCR test performed at 1st sampling sorted by flock: the bars highlight the total number of goats sampled (gray bars), the number of ELISA test positive goats(black bars) and the number of r-t PCR goats positive on milk (white bars).



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