Coxiella burnetii in pregnant goats

Hendrik-Jan Roest

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Cover illustration:
The background is a blurred picture of an immunofluorescence staining of the Nine Mile RSA 493 reference strain of *Coxiella burnetii* grown on a Buffalo Green Monkey (BGM) cell culture. The goat is drawn from a picture of one of the Dutch dairy goats from Experiment 1, Chapter 8. The uterus of the goat is reflected by pictures of immunofluorescence staining of different Dutch *C. burnetii* strains on BGM cell cultures. Contributions were made by Marinka Roest, Annemieke Dinkla and Fred van Welie.
The illustration symbolises the strong tropism of *C. burnetii* towards the placenta of pregnant goats when goats are exposed to *C. burnetii*.

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Coxiella burnetii in pregnant goats

Coxiella burnetii bij drachtige geiten
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op
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Hendrik Ido Jan Roest

geboren op 23 januari 1969 te Leiden

Promotor: Prof.dr. J.P.M. van Putten

Co-promotoren: Dr. J.M.J. Rebel
Dr. A. Bossers

Contents

Chapter 1

General introduction

History of the *Q fever agent*

The causative agent of Q fever was discovered simultaneously in Montana, USA and in Queensland, Australia, in the 1930s. A laboratory infection connected the two discoveries. (i) The first isolation of the agent causing Q fever was coincidental to research on Rocky Mountain Spotted Fever at the Rocky Mountain Laboratory in Montana, USA. In an experiment concerning 200 *Dermacentor andersoni* ticks collected at the Nine Mile Creek, Gordon Davis isolated an infectious agent with properties not identified before [1]. The agent could pass filters, was infectious for guinea pigs, Gram-negative, and had an extracellular and intracellular pleomorphic, rickettsia-like appearance [2]. Rolla Dyer, director of the National Institutes of Health in the USA, wanted to convince himself of the new agent described by Herald Cox and worked together with him in the lab. He appeared to contract the first laboratory infection with Q fever [3,4]. Dyer also suggested the link between the new agent and the "Q" virus from Australia [3].

(ii) In Australia, Edward Derrick was assigned to investigate outbreaks of undiagnosed febrile illness among abattoir workers in Brisbane, Queensland, Australia. These outbreaks had been occurring periodically since 1933. He first decided to determine the characteristics of the illness (derrick 1937, reprint 1983). As the illness was not previously described, it was named "Q" fever 'until fuller knowledge should allow a better name' [5]. Referring to the name, first Queensland rickettsial fever was proposed, but was regarded as derogatory to that sovereign state. "X-disease" had already been appropriated in Australia, so Derrick decided that Q (for query)-fever was the appropriate name (according to Macfarlane Burnet [4]). In order to reveal the cause of the disease, several experiments were done, but Derrick failed to detect bacteria. This led him to the (wrong) conclusion that the etiologic agent was a virus. Further studies on the virus of "Q" fever were published by Macfarlane Burnet and Mavis Freeman, also indicating the rickettsia-like properties of the virus [6].

The rickettsia-like properties of the agent resulted in the initial designation of the Q fever agent as *Rickettsia diaporica* (diaporica is derived from the Greek word for having the property or ability to pass through [a filter]) by the American group and as *Rickettsia burneti* (after Burnet) by the Australian group [4]. In 1948, Philip [7] proposed a reclassification into *Coxiella burneti*. In this name both Harold Cox and Frank Burnet are honoured for their contribution to the identification of the Q fever agent. *C. burnetii* has been phylogenetically reclassified from the order of *Rickettsiales* to *Legionellales*, based on the sequence of its 16S rRNA. In the Approved List of Bacterial Names the Q fever agent is now listed as *Coxiella burnetii* (www.ncbi.nlm.nih.gov/books/NBK820/, assess date 24 July 2012).

C. burnetii belongs to the family *Coxiellaceae*, together with the genus *Rickettsiella*. Typical for this family is the intracellular intravacuolar lifestyle in cells of invertebrate or vertebrate hosts. Closest relatives are *Legionella* and *Francisella* species (Figure 1.1, [8]. At this time the phylogeny of the Q fever agent can be summarised as belonging to the kingdom of *Bacteria*, phylum of *Proteobacteria*, class of the *Gammaproteobacteria*, order of the *Legionellales*, family of the *Coxiellaceae*, with the genus *Coxiella* and the only species *burnetii*.

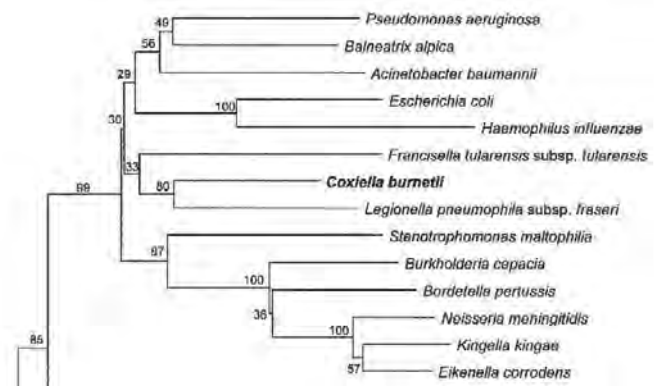

Figure 1.1. Phylogenetic relations within the phylum *proteobacteria*.
Dendrogram based on 16s rRNA sequence comparison of *C. burnetii* with its closest member of the *proteobacteria* phylum (reprint of a part of Figure BXII.y.99 from [8], permission requested).

Bacteriology of *C. burnetii*

Phenotypic properties

C. burnetii is an obligate intracellular Gram-negative bacterium. The pleomorphic rods have a diameter of approximately 0.2-0.4 µm and are 0.4-1.0 µm in length [8]. In the developmental cycle of *C. burnetii* two distinct variants have been identified: a large cell variant (LCV) and a small cell variant (SCV) [9]. The LCV is the metabolically active and replicative entity of the bacterium. During the exponential growth phase generation times were calculated at approximately 11 hours [10]. The LCV transforms into the SCV, which is the spore-like form of *C. burnetii*. In this form the bacterium is highly resistant to environmental stress, such as high temperatures, UV radiation and osmotic pressure. The resistance allows *C. burnetii* to survive in the environment while keeping its infectivity [9]. The SCV can infect host cells, closing the developmental cycle.

Lipopolysaccharide (LPS) is demonstrated in both *C. burnetii* LCV and SCV, although presence of LPS is mainly associated with the SCV [11]. Like several other Gram-negative species, *C. burnetii* can display two different LPS phenotypes. The phase 1 phenotype expresses full length LPS which corresponds to the smooth LPS of other Gram-negative bacteria (e.g. *Brucella* spp. and *Enterobacteriaceae*), while the phase 2 phenotype carries LPS that resembles the rough LPS of other Gram-negative bacteria. Phase 2 LPS lacks the O-antigenic region [12]. Phase 1 bacteria are highly virulent and able to replicate in immunocompetent hosts. This is contrary to phase 2 bacteria which are avirulent and unable to replicate in immunocompetent animals [13,14].

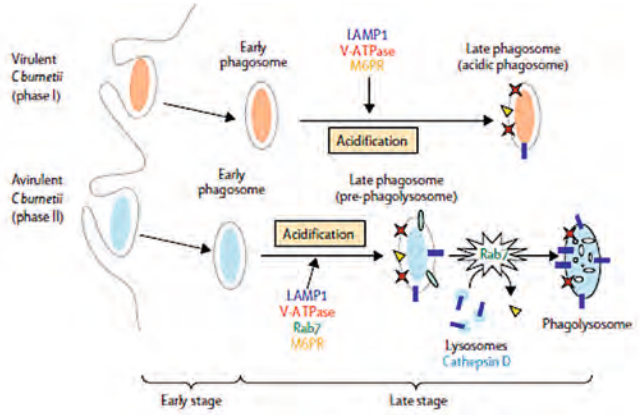

Figure 1.2. *C. burnetii* trafficking in macrophages.
Virulent phase 1 *C burnetii* phagocytosis leads to the formation of early phagosomes that acquire markers of late endosomes and lysosomes (LAMP1, M6PR, and V-ATPase), resulting in the acidification of the phagosome. The final phagosome exhibits the features of late phagosomes, which do not acquire cathepsin D. Avirulent phase 2 *C. burnetii* phagocytosis leads to the formation of early and late phagosomes. Rab7 is involved in the maturation of late phagosomes, which acquire cathepsin D, forming acidic phagolysosomes (Reprinted from The Lancet infectious diseases, [22] Copyright (2005), with permission from Elsevier).

During serial passage in cell culture, phase 1 *C. burnetii* can convert into phase 2 [15]. Both LPS phenotypes can be distinguished via phase specific antibodies. Phase 1 antibodies are directed against the full length LPS of phase 1, whereas phase 2 antibodies are directed against common surface proteins [16]. These surface proteins are also present on the surface of phase 1 *C. burnetii*, but seem to be shielded by the long phase 1 LPS. This may prevent binding of phase 2 antibodies to surface proteins of intact phase 1 bacteria [17]. Unique for *C. burnetii* phase 1 LPS is the presence in the O-chain of galactosaminuronyl-α-glucosamide disaccharide, virenose (vir) and dihydrohydroxystreptose (strep) [12]. Genes have been identified that might be involved in the synthesis of vir and strep. Phase variation seems to be associated with the loss of chromosomal DNA involving a large group of LPS biosynthetic genes, arranged in an apparent O-antigen cluster [18]. This implies a non-reversible process, but it is not known if chromosomal deletions are the only mechanism of phase variation in *C. burnetii*. Although LPS is an important virulence factor, the endotoxic activity of *C. burnetii* phase 1 and phase 2 LPS is 100 to 1000 times less compared to the LPS of *Enterobacteriaceae* [19].

C. burnetii phase 1 LPS plays a role in both host cell internalisation and survival in the phagosome. In phagocytosis of phase 1 bacteria, αvβ3-integrin and integrin associated protein (IAP), as well as Toll-Like receptor 4 (TLR4), are involved. In avirulent phase 2 bacteria, however, attachment is mediated by both αvβ3-integrin and IAP and complement receptor 3, but not

by TLR4 [20-22]. The early phagosomes containing phase 1 bacteria acquire markers of late endosomes and lysosomes, resulting in acidification of the phagosome, but the final maturation step is inhibited by the absence of Rab7. In this acidic vacuole the phase 1 *C. burnetii* escape intracellular killing and are able to replicate. Early phagosomes containing phase 2 bacteria proceed to the formation of early and late phagosomes. In the presence of Rab7, late phagosomes maturate and fuse with lysosomes forming acidic phagolysosomes. This results in effective killing of the phase 2 bacteria [22,23]. Thus, phase 1 *C. burnetii* bacteria are internalised and survive intracellular killing, whereas phase 2 bacteria are efficiently phagocytised and then killed (Figure 1.2, [22]).

The C. burnetii *genome*

The first complete genome sequence of *C. burnetii* was published in 2003. Analysis of the original strain isolated from ticks by Davies and Cox in 1938 (called Nine Mile) revealed a circular genome of 1,995,275 base pairs. Twenty-nine insertion sequence (IS) elements were identified: 21 copies of a unique IS110-related isotype, IS1111, five IS30 and three ISAs1 family elements. These IS elements are dispersed around the chromosome without apparent localized clustering [24]. Analysis of multiple strains showed that the number of IS1111 elements is highly variable between strains. In an analysis of 75 strains the copy number varied between 7 and 110 [25]. Four extra-chromosomal plasmid types have been identified: QpH1 (36 kilo base (kb)), QpRS (39 kb), QpDG (42 kb), and QpDV (33 kb). In one strain, a plasmid seems absent (strain Scurry isolated from a human liver suffering from hepatitis). However, a DNA homologue of a segment of the QpH1 plasmid has been identified on its chromosome [22,26,27]. Initially, *C. burnetii* strains with specific plasmid types were associated with the development of acute or chronic Q fever in humans, but this association was found to be inconstant [28].

The genetic heterogeneity of *C. burnetii* can be assessed with a number of molecular techniques. Different genotyping techniques have been described, mainly based on the identification of differences between selected loci on the genome. An overview of published genotyping techniques for *C. burnetii* is given in Table 1.1. Accurate identification of the agent is important to differentiate between strains and to identify epidemiological markers. These markers are at the basis of the molecular epidemiology that enables the identification of sources of Q fever outbreaks [29]. Eventually, markers may be discovered in the *C. burnetii* genome that identify *C. burnetii* strains posing an increased risk for infection. However, it is not known if virulence-encoding genes are located on or associated with the loci used in the current typing methods. So to date, it is not possible to classify the virulence of strains solely based on the available genotyping methods.

An important characteristic for typing systems is the discriminatory power, i.e. the ability to distinguish between unrelated strains. This is determined by the number of types defined by the test method and the relative frequencies of the types. A single numerical index of discrimination

is suggested by Hunter and Gaston [39]. The Hunter-Gaston Diversity Index (HGDI) is based on the probability that two unrelated strains sampled from a test population will be placed into different typing groups. By comparing the HGDI of a typing system the discriminatory power can be compared. It is important to note that the calculated HGDI depends on the panel of strains (i.e. relatedness of the strains), so for an unbiased comparison of typing methods preferably the same panel should be used. Despite its importance for the quality of typing systems the discriminatory power is not assessed for most of the typing systems available for *C. burnetii*. For RFLP-typing a HGDI of 0.86 is calculated [30]. For the MLVA typing panels 1 and 2 both a HGDI of 0.92 is calculated and for the combined panels and HGDI of 0.99 is calculated [29,40]. It is suggested that a HGDI of >0.90 is desirable to interpret typing results with confidence [39], indicating the MLVA typing system as a useful typing tool for *C. burnetii*. The published MLVA typing method for *C. burnetii* is assumed to be more discriminatory than MST [35,41].

Table 1.1. Overview of published genotyping techniques for *C. burnetii* and year of first publication of the technique for *C. burnetii*.

Abbr.	Stands for	Based on	Publ. year	Ref.
RFLP typing	restriction fragment length polymorphism typing	analysis of the fragments after digestion with specific restriction enzymes	1990	[30,31]
Com1 typing	Com1 encoding genes sequencing	Sequence analysis of the Com1 encoding genes	1997	[32]
Com1/MucZ typing	Com1 and MucZ encoding genes sequencing	sequence analysis of the Com1 and MucZ encoding genes	1999	[33]
MST	multispacer sequence typing	DNA sequence variation in short intergenic regions in the genome	2005	[34]
MLVA	multiple locus variable number tandem repeats analysis	variation in the repeat number in tandemly repeated DNA elements on multiple loci in the genome	2006	[29,35]
IS1111 typing	IS1111 repetitive element PCR-based differentiation typing	identification of different IS1111 insertion elements	2007	[36]
RAPD	randomly amplified polymorphic DNA	analysis of randomly amplified DNA fragments of the genome	2009	[37]
SNP typing	single nucleotide polymorphism typing	Differentiating a single nucleotide difference on a locus in the genome by probes	2011	[38]

Abbr.: abbreviation; Publ. year: year of first publication of the technique for C. burnetii; Ref.: reference

Safety issues

C. burnetii is considered a biothreat agent by its very low infectious dose and high transmissibility. It is listed as a group B bioterrorism agent by the Centres of Disease Control and Prevention (CDC) in the USA ([42], website CDC; http://emergency.cdc.gov/agent/agentlist.asp, assess date 27 July 2012). An infection dose as low as one bacterium is reported for both guinea pigs and humans [43,44]. For this reason, *C. burnetii* is considered a containment level 3 organism by the EU (directive 2000/54/EC of the European parliament and of the council).

Working with containment level 3 organisms requires dedicated biosafety level 3 laboratories and animal facilities, as well as experienced and trained personnel. Emergency procedures and medical monitoring of personnel should be implemented to guarantee the safety of the people working with agents such as *C. burnetii*. Although a human Q fever vaccine is available in Australia (not registered in the Netherlands), its use to prevent Q fever in laboratory employees is still under debate.

Isolation and cultivation of *C. burnetii*

Techniques

Successful isolation and cultivation of *C. burnetii* in laboratory animals, embryonated eggs and cell culture were already achieved in the first studies on the Q fever agent [1-3,6,45]. Guinea pigs were the first laboratory animals to be used in Q fever research. The animals develop hyperthermia of over 40°C at five to twelve days post inoculation and *C. burnetii* can be isolated from the spleen, indicating systemic infection [46,47]. In mice, the susceptibility to infection with phase I *C. burnetii* varies between mouse strains. A/J mice are highly sensitive to the agent, resulting in a 100% morbidity and a 70% mortality, while resistant mice strains show neither morbidity nor mortality. Yet, both sensitive and resistant mice strains show a generalized *C. burnetii* infection, and antibody production is similar [48]. We successfully used the *C. burnetii* resistant Swiss Random OF1 mice strain for the isolation of *C. burnetii* from tissues with low *Coxiella* load and/or when tissues were probably contaminated with other bacteria. Tissue suspensions were intraperitoneally inoculated and after 7 to 21 days the mice were euthanized to isolate *C. burnetii* from the spleen. To minimise the use of mice, suspected samples were first screened by a *C. burnetii* specific PCR [Chapter 3] to assess the *C. burnetii* load prior to inoculation of the mice. Thus, mice were used in specific cases both for propagation of *C. burnetii* and as a filter for contaminants.

In embryonated eggs *C. burnetii* grows almost exclusively in the yolk sac endoderm cells. This propagation technique involves the inoculation of a tissue suspension containing *C. burnetii* into 6 to 7 day-old embryonated chicken eggs via the yolk sac. The yolk sac is harvested after 10 to 15 days ([46], OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2012, http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.01.12_Q-FEVER.pdf. Access date 26 July 2012). However, this culture system is time-consuming and extensive experience is needed to harvest the yolk sac. In addition, culture results are hard to monitor and the results are comparable to cell culture isolation techniques. Therefore, we did not implement this technique in our lab for routine culturing of *C. burnetii.*

The cell culture system is currently the most widely used *in vitro* system to isolate and cultivate *C. burnetii*. A number of cell lines can be used for *in vitro* cultures [49]. In our experiments we used Buffalo Green Monkey (BGM) cells, a Vero cell line. In the isolation of *C. burnetii* from infected tissues, filters with pore sizes of 1.2 µm and 0.45 µm were used. Although this final filter step decreases the number of *Coxiella* bacteria by a factor 10 [50], it is necessary for some tissue material to avoid contamination of the monolayer with other bacteria. A culture medium without antibiotics is used, to avoid negative influence on the growth of the *Coxiella* bacteria. This method has been successfully applied to isolate Dutch outbreak strains of *C. burnetii* (Figure 1.1, [51, Chapter 8]).

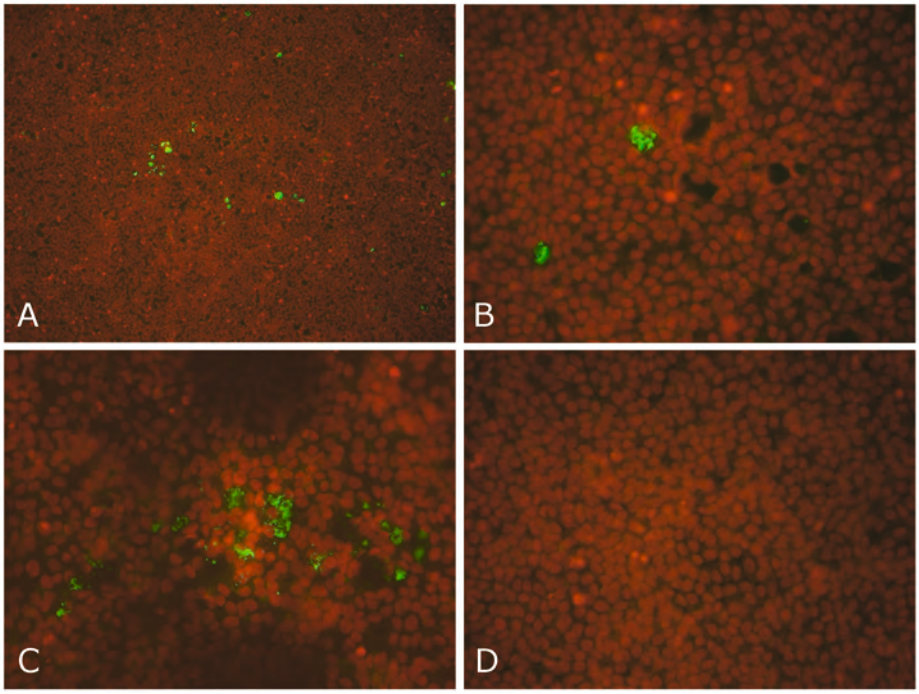

Figure 1.3. Dutch *C. burnetii* strains on Buffalo Green Monkey cell culture system.
Results of the immunofluorescence staining of a Buffalo Green Monkey (BGM) cell culture using a *C. burnetii* specific monoclonal antibody (MAB313-oregon green, Squarix). Presence of *C. burnetii* is indicated by the green dots. The nuclei of the BGM cells are stained brownish with propidium iodide [51].

A: The first Dutch *C. burnetii* strain isolated from the placenta of a goat (strain X09003262-001, 100x magnification)

B: Close-up of Figure 1.3 A (400x magnification)

C: *C. burnetii* strain isolated from the placenta of a sheep (400x magnification)

D: Non-infected BGM cells (negative control) (400x magnification)

A recent major improvement in the methods of isolation and cultivation of *C. burnetii* is the introduction of a host cell free medium based on the composition of the acidic phagosome environment in which *C. burnetii* replicates [52,53]. The major advantage of the use of a chemically defined growth environment is the absence of host cell genetic material, which is particularly helpful in the genetic analysis of *C. burnetii*. In our laboratory we were able to isolate and grow different strains in the modified acidified citrate cysteine medium (ACCM, [52], both in broth and plate format.

Quality control in C. burnetii *cultivation*

A visual check on isolate purity and contamination is hampered by the intracellular nature of *C. burnetii*. Bacterial contamination of cell cultures is readily visible by the change in clarity of the culture medium, especially when culture medium without antibiotics is used. However, several intracellular bacteria, such as *Chlamydia* spp., *Simkania negevensis* and *Mycoplasma* spp., are also able to contaminate cell cultures. To control these kind of contaminations, it is advisable to implement regular checks for these contaminants in quality control (QC) protocols. To detect possible cross-contamination between cultures with different strains, regular checks on the identity of the strains by genotyping techniques should also be part of the QC.

Q fever in animals

Host range

C. burnetii can infect a wide range of animal species. As mentioned above, the bacterium was initially isolated from the tick *Dermacentor andersoni* [1]. Since these first reports, *C. burnetii* has been detected in over 40 tick species. In the Netherlands prevalence of *C. burnetii* in ticks is low (0.2%, [54]. Several bird species can also become infected with *C. burnetii*, as experimentally shown [55-58]. Natural infections have been reported in domestic birds and in wild birds [59,60]. In terrestrial as well as in marine wildlife the presence of *C. burnetii* has been confirmed in roe deer, wild boars, rodents, European hare, pacific harbour seal, a Steller sea lion, Northern fur seals and harbour porpoises [60-65]. In Dutch wildlife *C. burnetii* has been detected in roe deer and rats [Chapter 5, 66]. *C. burnetii* can also be present in cats and dogs. Seroprevalences vary between 19% and 42% for cats and up to 22% for dogs [67-70]. In the Netherlands, seroprevalences in cats and dogs of 10% and 13%, respectively, have been measured [Chapter 2].

In domestic ruminants *C. burnetii* infections are widespread. Seroprevalence levels are estimated up to 82% in cattle. In sheep and goats average seroprevalences are slightly lower compared to cattle, with values of up to 73% [71]. Prevalence of *C. burnetii* on cattle herd level as measured from bulk tank milk samples ranges between 32 and 94% [72-74]. The seroprevalence and agent prevalence in Dutch cattle is comparable to what is reported in the literature. Seroprevalences in sheep and goats in the Netherlands are estimated at 2.4% and

7.8%, respectively [75]. This is in the lower range of what is internationally known. In 19% of the Dutch bulk tank milk samples from dairy sheep and in 33% of the bulk tank milk samples from dairy goats, *C. burnetii* DNA was detected [76].

Pathogenesis of Q fever and immune response in animals

Little is known about the pathogenesis of Q fever in domestic animals. Under laboratory conditions, *C. burnetii* inoculation of both guinea pigs and mice results in a systemic infection, including pneumonia, hepatitis and splenomegaly [48,77,78]. The severity of pathological changes depends on the strain. Splenomegaly is thought to be an indicator for the virulence of *C. burnetii* strains in guinea pigs and mice [77]. Also the inoculation route seems to influence pathogenesis. In mice, intranasal inoculation is mainly associated with pneumonia, whereas intraperitoneal inoculation is mainly associated with hepato-splenomegaly [79]. In inoculated pregnant mice, *C. burnetii* is abundantly present in both the foetal and maternal parts of the placenta [80]. In goats this is different [81, Chapter 8].

In pregnant goats, the trophoblasts of the allantochorion are target cells of the *C. burnetii* in which multiplication occurs. *C. burnetii* antigen is barely detected in adjacent maternal parts of the placenta. In other maternal organs *C. burnetii* DNA, but no viable bacteria, can be found at some time points during pregnancy [81]. Pathogenesis is not yet investigated in relation to the excretion routes of *C. burnetii* in domestic ruminants. This knowledge can help in understanding the excretion to improve diagnosis and intervention.

In mice, both humoral and cellular immune responses are important in limiting the infection. Macrophages and other mononuclear cells are believed to be the major target cells during *C. burnetii* infection [82]. T-cells are suggested to be critical for clearance of *C. burnetii* after infection. B-cells are important for the prevention of tissue damage [14]. Antibodies can be detected as early as 14 days post inoculation for anti-*C. burnetii* phase 2 antibodies and 21 days in the case of anti-*C. burnetii* phase 1 antibodies [83]. This is comparable to what was found in goats. Antibody levels start to increase around 21 days post inoculation, although in published work no differentiation has been made between antibodies directed against phase 1 and directed against phase 2 *C. burnetii* [84]. However, knowledge on the phase specific immune response might be helpful in the early diagnosis of *Coxiella* infections, such as in the laboratory diagnosis of Q fever in humans. In human Q fever diagnosis, tests are used that differentiate between phase 1 and phase 2 specific antibodies of the IgM and IgG subclasses. With combinations of phase specific and immunoglobulin subclass specific titres, acute and chronic Q fever can be diagnosed [85,86]. In addition, tests that measure cellular immunity can be used in the diagnosis of chronic Q fever [87]. In veterinary research, attempts are made to uses phase specific ELISAs to investigate herd dynamics of Q fever [88]. However, titre dynamics of phase specific subclasses of immunoglobulins, as well as cell-mediated immunity in domestic ruminants, are still largely unknown. More detailed information on this can potentially help in the diagnosis of Q fever and will be helpful in improving vaccines.

Clinical presentation

The most important clinical presentations of Q fever in animals that are relevant for its zoonotic properties are abortion and stillbirth. Field observations clearly demonstrate *C. burnetii* as a cause of abortion and stillbirth in goats, sheep, cattle and cats [46,71,89-94]. Abortion occurs most frequently at the end of gestation, without preceding clinical symptoms [95]. However, it is not clear if infection always leads to abortion. Experimental infections in pregnant goats resulted in abortion in all animals [81,84,96], but experimental infections in pregnant sheep did not result in any abortions [97,98]. Experimental infection of a pregnant cow resulted in abortion [46]. The cause of these differences in pregnancy outcome after infection is unknown.

In non-pregnant animals, *C. burnetii* infection is virtually asymptomatic. In cattle, *C. burnetii* infection is associated with metritis and reproduction problems [46,59]. In dairy goat herds where Q fever abortions occurred, metritis in does can be present. Weak kids were reported with low body weight and high mortality. Rearing of apparently healthy kids can be complicated by respiratory and digestive tract disorders [99].

Excretion routes

Knowledge of the excretion of *C. burnetii* from infected animals is crucial in understanding the transmission routes and risks of human infection. Abortions in *C. burnetii* infected domestic ruminants are accompanied by massive excretion of the bacteria and spread into the environment. This is the most important excretion route of *C. burnetii*, as up to 109 organisms per gram placenta tissue are excreted [84]. It is suggested that with the birth of healthy calves, kids or lambs from infected animals fewer bacteria are excreted [100]. *C. burnetii* has also been detected in faeces, vaginal mucus and milk of infected domestic ruminants [84,90,93,101]. In goat herds, both in aborting and non-aborting goats, *C. burnetii* DNA has been detected in faeces, vaginal mucus and/or milk [93]. In cattle, also variable excretion via faeces, vaginal mucus and milk has been reported, sometimes independent of an abortion history. Sixty-five per cent of cows seem to shed *C. burnetii* by only one route, evenly distributed over the three routes. Cows that excrete *C. burnetii* by all three routes seem scarce [101]. Comparison of the three excretion routes in cattle, goats and sheep show that milk shedding is more frequent in cattle and goats. Ewes shed more and longer in vaginal mucus than goats [95]. Both sheep and goats can shed *C. burnetii* in subsequent pregnancies [91,102]. Based on these results, it is suggested that testing of animals based on only one biological sample may lead to false negative results. Thus, while excretion of high numbers of *C. burnetii* with birth products during abortion is evident, the importance of and the correlation between the excretion routes of *C. burnetii* via faeces, vaginal mucus and milk is much less well established and warrants further investigation.

Diagnostics

The occurrence of abortion is obviously not specific for Q fever. Evidence of a *C. burnetii* infection as a cause of the abortion requires laboratory confirmation. *C. burnetii* can be detected in

infected placenta material via immunohistochemistry (IHC), using *C. burnetii* specific antibodies. When *C. burnetii* is the cause of abortion, high numbers of bacteria are usually observed in the trophoblasts of the allantochorion [81,99, Chapter 8]. *C. burnetii* can also be detected with specific polymerase chain reaction (PCR) tests. Specific and sensitive detection of *C. burnetii* can be achieved with primers and probes targeting the IS1111 element. This element has multiple copies in the genome of *C. burnetii*, resulting in an increased sensitivity compared to primers and probes that target single copy genes [25]. PCR can be used to detect *C. burnetii* DNA in a wide range of samples, including placenta tissues, faeces, vaginal mucus and milk (OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2012, http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.01.12_Q-FEVER.pdf, access date 30 July 2012). Apart from histopathology and PCR, several indirect diagnostic methods of Q fever detection are available. *C. burnetii* specific antibodies in serum can be measured using the complement fixation test (CFT), enzyme-linked immunosorbent assay (ELISA) and immunofluorescence assay (IFA) test. The CFT is most widely used in veterinary laboratories, although commercially available ELISAs to detect *C. burnetii* antibodies in ruminants are increasingly used. Compared to ELISA, the CFT lacks sensitivity to detect *C. burnetii* specific antibodies in ruminant serum [103-105]. Several reports indicate that *C. burnetii* can be shed by cattle, sheep and goats without a detectable serological response. Although this could be due to the delay in the serological response after a primary infection, it may indicate that serology is not a useful tool to diagnose abortions at the individual level [90,93,106]. This lack of antibody response is not well explained yet, but can be caused by a lack of sensitivity of the used test or can be caused by a lack of understanding of the mechanism of excretion of *C. burnetii*.

Current protocols to diagnose Q fever on herd level in cattle and small ruminant herds recommend a combination of both direct and indirect techniques. It is recommended that samples from abortion material and/or from vaginal mucus of two to six animals are investigated by PCR within eight days after abortion. In addition, serum samples from at least six to ten animals should be investigated, especially when PCR results are negative. These animals should preferably have delivered at least 15 days prior to sampling [107]. PCR targeting the IS1111 genetic element is the most sensitive technique to detect *C. burnetii*, whereas ELISA is the most sensitive technique to detect *C. burnetii* specific antibodies.

Disease control

To control Q fever in domestic ruminants two methods are available: antibiotic treatment and vaccination. In case of acute Q fever in humans doxycycline is the antibiotic of choice. For chronic Q fever long-term treatment with doxycycline and hydrochloroquine is recommended. Although thorough evaluations of these therapies are lacking, they are considered to be effective [108]. The effect of antibiotics to treat Q fever in animals is poorly studied. A few studies suggest a reduction in abortion rate and in the number of bacteria excreted at parturition [109,110]. However, this effect could not be observed in small ruminants in the Netherlands treated with

oxytetracycline [99]. In dairy cattle, a beneficial effect of antibiotic treatment complementary to vaccination with a phase 1 *C. burnetii* inactivated vaccine was observed [111]. Because the effect of antibiotic treatment is not clearly evidence-based and prudent use of antibiotics is needed to avoid microbial resistance, it is advised that antibiotics should not be used for treatment of Q fever in animals.

Vaccination with a phase 1 *C. burnetii* inactivated vaccine is reported to be effective in preventing abortion, as well as in reducing bacterial shedding in goats and cattle [96,112,113]. Vaccination in goats and cattle is most effective in primiparus animals and reduces both the prevalence of shedders, as well as the bacterial load [112-114]. However, vaccination might not be effective in goats or cattle that are already infected, nor in pregnant cattle [113,114]. Other drawbacks of the vaccine are the complicated production process, the need for dedicated BSL3 facilities for production and the impossibility to differentiate between infected and vaccinated animals (DIVA). Contrary to vaccination with a phase 1 *C. burnetii* vaccine, the current phase 2 *C. burnetii* vaccine is not effective [96]. This result indicates that currently Q fever in domestic ruminants can best be prevented by vaccination with a phase 1 *C. burnetii* vaccine. A new generation of vaccines that are easier to produce (e.g. recombinant vaccines) may overcome the main drawbacks of the current vaccine, but these are not available yet.

Q fever in humans

Pathogenesis of Q fever and immune response

Humans usually acquire Q fever by inhalation of environmental *C. burnetii*. Alveolar macrophages and other mononuclear phagocytes are thought to be the primary target cells of the pathogen [82]. In these cells *C. burnetii* survives intracellular killing and is able to replicate. A bacteraemia will lead to systemic infection with involvement of the liver, spleen, lungs and bone marrow [47]. *C. burnetii* infection often leads to protective granulomatous lesions in these organs. The TLR 4 innate immune receptor also plays a role in the formation of these granulomas, as well as in the production of interferon (IFN)-γ and tumor necrosis factor (TNF) by T-cells [20,82]. It is suggested that IFN-γ mediates *C. burnetii* killing via phagosome maturation and phagosome alkalinisation [23]. Both anti-*C. burnetii* phase 2 IgM and IgG antibodies develop within 3 weeks post infection, accompanied by low levels of anti-phase 1 IgM [115]. Antibody-mediated immunity seems to be important, as infection of dendritic cells with antibody-opsonized bacteria resulted in the increased expression of maturation markers and inflammatory cytokines in mice [82]. Thus, in humans both humoral and cellular immunity play a role in *C. burnetii* infection. In pregnant women, adverse pregnancy outcome has been associated with Q fever [47], however, no evidence for this has been found during the recent Dutch Q fever outbreak [116].

Clinical presentation

Infection can manifest as three main clinical presentations: acute Q fever, chronic Q fever and the post-Q fever fatigue syndrome (QFS). Following exposure to *C. burnetii* almost 60% of the Q fever cases are asymptomatic. Among the 40% symptomatic acute Q fever patients, the majority will present a non-specific, self-limiting illness. More severe clinical symptoms include fever, headache, chills, atypical pneumonia and hepatitis [5,22,47]. In the Netherlands, the recent acute Q fever outbreak showed a mortality rate of 1.2% within approximately one month after hospitalization of patients. All lethal cases suffered severe underlying medical conditions [117]. Acute Q fever is diagnosed in the laboratory following (i) a positive *C. burnetii* specific PCR, (ii) the presence of IgM phase 2 antibodies in serum accompanied by clinical symptoms, or (iii) a fourfold increase of the IgG phase 2 antibody titre. These laboratory findings are also the notification criteria in the Netherlands [118].

Chronic Q fever can develop from a primary infection in about 1 to 5% of the patients. Chronic Q fever can become manifest years after initial infection. Clinical symptoms include non-specific fatigue, fever, weight loss, night sweats and hepato-splenomegaly, as well as endocarditis [22,86]. After the recent outbreak in the Netherlands, vascular complications appeared to be more common than endocarditis, but endocarditis may not have become apparent yet [108]. Laboratory diagnosis of chronic Q fever includes the detection of the Q fever agent by PCR or a high titre of *C. burnetii* phase 1 IgG [86].

QFS is another long-term presentation of Q fever. Contrary to chronic Q fever, *C. burnetii* cannot be detected in QSF patients. Furthermore, antibody levels against the bacteria are low or negligible. Symptoms of QFS include prolonged fatigue, arthralgia, myalgia, blurred vision and enlarged painful lymph nodes [47]. Acute Q fever with hospitalisation was found to be a risk factor for QFS [119]. The cause of the development of chronic Q fever or QFS in certain individuals is still unknown.

Epidemiology of *C. burnetii*

In humans, Q fever is essentially an airborne infection resulting from the inhalation of contaminated aerosols [43,120]. Aerosols can become contaminated with *C. burnetii* during abortion and parturition of infected pregnant ruminants [98,121]. Environmental contamination resulting in contaminated aerosols may also follow excretion of *C. burnetii* via faeces, vaginal mucus or possibly milk of animals. This contributes to the occupational hazard of Q fever. However, contaminated aerosols are able to travel up to 5 km and infect humans. Windy, warm and dry weather can facilitate this transport [122-124]. Also other factors, such as vegetation and soil moisture, seem to be relevant in the dispersion of the bacteria [125]. So, direct contact with animals is not a prerequisite for acquiring Q fever, as several outbreaks demonstrate [123,126-

128, Chapter 2]. It is assumed that most animals also become infected via inhalation [129], although oral uptake in an heavily infected environment cannot be excluded.

Domestic ruminants appear to be the main source of infection for human Q fever. As indicated earlier, abortion in infected pregnant goats, sheep and cattle, but also normal parturition in infected sheep, result in the massive excretion of *C. burnetii* into the environment. Q fever in cattle seems to result in fewer abortions, probably resulting in a lower risk. Companion animals should also be considered as a source for human Q fever, since several human outbreaks were related to parturient cats and dogs [94,130-132]. The role of horses and wildlife as a source of Q fever for humans is not clear. In overview, the epidemiology of Q fever can be summarized in a transmission model as presented in Figure 1.4 (adapted from [133]).

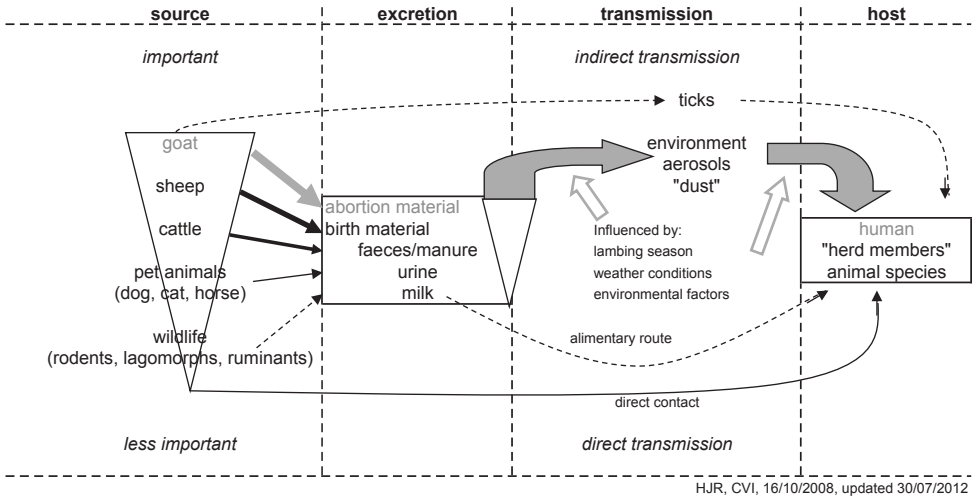

Figure 1.4. Transmission model for Q fever.
Overview of the possible transmission routes of *C. burnetii* from the animal reservoir to the human (and animal) hosts. The boldness of the arrows indicate the importance of the route, dotted lines indicate possible contributions. The most probable transmission route of *C. burnetii* in the Dutch Q fever outbreak is indicated in grey (adapted from [133]).

Q fever outbreaks

Q fever has a major public health impact when outbreaks occur. Outbreaks are reported frequently and worldwide, involving up to 415 laboratory-confirmed human cases per outbreak. Even higher numbers of human cases are reported, but the attribution to Q fever is unclear as cases are not always laboratory-confirmed [95,103,108]. Outbreaks are usually geographically

localised and restricted to one episode. Sheep are identified as the source in the majority of the outbreaks, with goats as 'second best'. Remarkably, outbreaks linked to sheep have also been reported after normal lambing [134,135]. Only a few outbreaks of Q fever have been related to infected cattle and cats [95,103,108]. Source identification, however, is mainly based on epidemiological examinations. In most outbreaks, confirmation of the identity of the *Coxiella* strain involved in both host and source, for example by genotyping, is lacking. This is a major drawback in the identification of sources, as sources of Q fever are multiple and *C. burnetii* can be transmitted over larger distances. Thus, identification of the source and confirmation of the relation with human disease is preferably done by genotyping of the involved *C. burnetii* strains.

Aim and outline

The aim of this thesis is to investigate the epidemiology and pathogenesis of *C. burnetii* infection in goats in the recent large outbreak of Q fever in the Netherlands. Main objectives are to characterize *C. burnetii* outbreak isolates from infected goats and other animals using genotyping and to understand the pathogenesis, excretion patterns, and the immune responses during experimental infection of pregnant goats.

Chapter 2 presents the background for the studies presented in this thesis. The history of Q fever in the Netherlands is described, as well as the factors that may have contributed to the recent unprecedented outbreak of Q fever. An overview is given of the onset of the outbreak and of the actions that were taken to manage the outbreak. Possible causes and the magnitude of the Dutch Q fever outbreak are discussed. **Chapter 3** describes the genetic background of the *C. burnetii* present in the goats that presumably caused the outbreak. This information was necessary not only to link the source to the host, but also to aid the outbreak management team in getting control over the outbreak. In **Chapters 4**, **5** and **6**, the different potential reservoirs of *C. burnetii* are investigated. Isolates from sheep, goats and cattle were compared to human isolates via genotyping (**Chapter 4**). In addition, we investigated roe deer as representative for wildlife (**Chapter 5**), as well as cats, dogs, pigs and horses (**Chapter 6**), for the presence of *C. burnetii*. In **Chapter 7** the lack of knowledge about the genetic background of *C. burnetii* in dairy cattle is addressed by the genetic analysis of *C. burnetii* DNA present in consumer milk products from 18 European and 10 non-European countries.

To study the pathogenesis, excretion and immune response against *C. burnetii* in goats, experimental infections in pregnant goats were performed. **Chapter 8** describes the pathogenesis and excretion of *C. burnetii* in pregnant goats. To closely mimic the field situation, a natural infection route, as well as a Dutch outbreak isolate, were used for inoculation. The humoral and cellular immune response after *C. burnetii* inoculation, which may give clues to improve the diagnosis of Q fever in goats, is described in **Chapter 9**.

In the general discussion (**Chapter 10**), the major findings presented in this thesis are discussed in relation to the Dutch Q fever outbreak and in the perspective of the international literature. Implications of the findings for the field and the control of Q fever, as well as topics for future investigation, are discussed.

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Chapter 2

The Q fever epidemic in the Netherlands: history, onset, response and reflection

H.I.J. Roest[1]
J.J.H.C. Tilburg[2]
W. van der Hoek[3]
P. Vellema[4]
F.G. van Zijderveld[1]
C.H.W. Klaassen[2]
D. Raoult[5]

1 Department of Bacteriology and TSEs, Central Veterinary Institute of Wageningen UR, Lelystad, The Netherlands.
2 Department of Medical Microbiology and Infectious Diseases, Canisius Wilhelmina Hospital, Nijmegen, The Netherlands.
3 Centre for Infectious Disease Control, National Institute for Public Health and the Environment, Bilthoven, The Netherlands
4 Department of Small Ruminant Health, Animal Health Service, Deventer, The Netherlands
5 URM, Université de la Mediterranée, CNRS-IRD UMR 6236, Faculté de Medecine, Marseille, France

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Abstract

The 2007–2009 human Q fever epidemic in The Netherlands attracted attention due to its magnitude and duration. The current epidemic and the historical background of Q fever in The Netherlands are reviewed according to national and international publications. Seroprevalence studies suggest that Q fever was endemic in The Netherlands several decades before the disease was diagnosed in dairy goats and dairy sheep. This was in 2005 and the increase in humans started in 2007. Q fever abortions were registered on 30 dairy goat and dairy sheep farms between 2005 and 2009. A total of 3523 human cases were notified between 2007 and 2009. Proximity to aborting small ruminants and high numbers of susceptible humans are probably the main causes of the human Q fever outbreak in The Netherlands. In general good monitoring and surveillance systems are necessary to assess the real magnitude of Q fever.

Introduction

Q fever is a zoonosis caused by *Coxiella burnetii*, an intracellular Gram-negative bacterium that is prevalent throughout the world [1]. Domestic ruminants are considered to be the main reservoir for Q fever in humans [2], although other animal species, including pet animals, birds and reptiles, may also be responsible for human cases. Transmission to humans is mainly accomplished through inhalation of contaminated aerosols. The main clinical symptom of Q fever in goats and sheep is abortion and in cattle reduced fertility. With abortion, 1 000 000 000 *C. burnetii*/g placenta can be excreted [3]. Duration of shedding of *C. burnetii* by infected livestock varies depending on the excretion route and species. In milk, *C. burnetii* can be excreted for 8 days in ewes and up to 13 months in cattle. In faeces, *C. burnetii* can be excreted up to 8 days after lambing in ewes and up to 20 days in goats [4]. Goats may shed *C. burnetii* in two successive kidding periods [5]. Most animal species carrying *C. burnetii* show no symptoms at all [4]. In humans infection with *C. burnetii* remains asymptomatic in ~60% of infected persons. In symptomatic patients, acute Q fever usually presents as a flu-like, self-limiting disease, atypical pneumonia or hepatitis. Infection in pregnancy may lead to adverse pregnancy outcomes, such as spontaneous abortion or premature delivery. About 1–5% of all Q fever cases may progress into a chronic infection, often leading to life-threatening endocarditis [4,6,7,8,9].

Since 2007, a Q fever outbreak has been ongoing in The Netherlands and this is referred to as the largest outbreak of Q fever ever reported in the literature [10]. However, Q fever is not an entirely new disease in The Netherlands. In this review we give an overview of the history of Q fever in The Netherlands, both in animals and in humans, the emergence of the disease and the control measures that have recently been taken. In conclusion, we put the outbreak in an international perspective.

Q fever becomes endemic, 1956–2005

Situation of Q fever in humans between 1956 and 2005

In the 1950s a comprehensive survey of the global distribution of Q fever was commissioned by the World Health Organization. In The Netherlands, between 1951 and 1954, almost 10 000 human sera were tested for Q fever with a complement-fixation test and all were negative [11,12]. In 1956, the first three human cases were reported in The Netherlands [13]. Two patients might have been linked to imported cattle or a visit abroad, while in the third patient there was no obvious cause. In 1958 and 1967 two human Q fever cases were described associated with the handling of imported wool [14,15].

The notification of infectious diseases started in The Netherlands in 1865. The law changed several times and the list of notifiable diseases became longer with every change. Q fever was made notifiable in 1975, although it was a very rare disease at that time [16]. Between 1975 and 2006 the annual number of cases increased from 0 to 32 per year (Figure 2.1). Thirty-three of the

cases notified between 1979 and 1983 were investigated more thoroughly. Twenty-two (67%) of these patients were probably infected in The Netherlands through contact with animals or animal products [17]. A survey in 1982/1983 among 432 persons, considered to be at high risk because of close contact with animals and animal products, showed high percentages (58% in taxidermists to 84% in veterinarians) of seropositive responders with mainly IgG antibodies against *C. burnetii* phase-II antigen, indicating that Q fever had become endemic in The Netherlands [18].

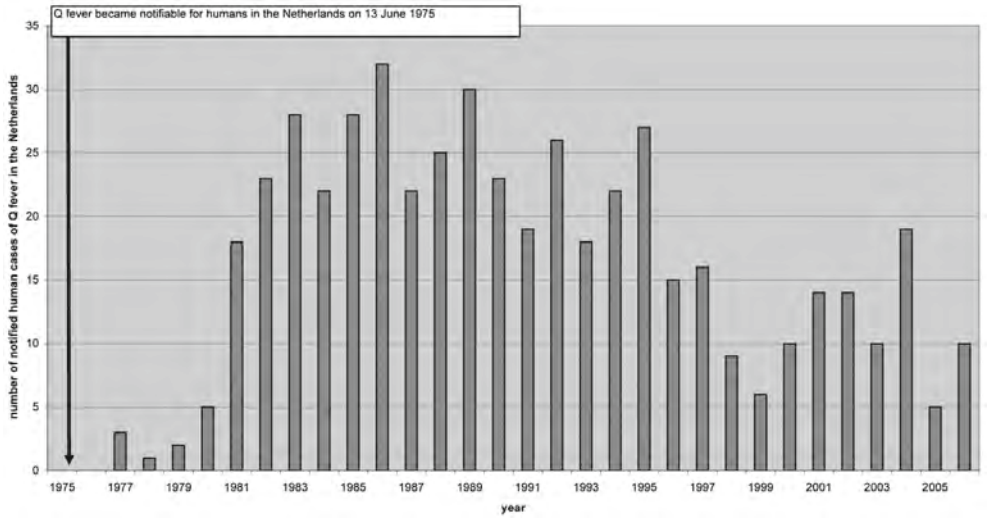

Figure 2.1. Number of notified human cases in The Netherlands between 1975 and 2006.

The increase in human cases between 1977 and 1983 (Figure 2.1) was assessed by Richardus *et al*. [18] by testing serum samples from persons, not considered to be at high risk, taken in 1968, 1975, 1979, and 1983 with an indirect immunofluorescence test (IFT) for specific IgG antibodies against *C. burnetii* phase-II antigen. In adults an average seroprevalence of 46% in 1968 and of 48% in 1983 was found. In children seroprevalences varied from on average 54% in 1975 to 28% in 1979 and 1983. These results showed no significant increase in the percentage of infected persons over the years 1968–1983. The increased number of notified cases since 1980 was explained by the introduction of a sensitive indirect immunofluorescence test for IgM antibodies against *C. burnetii*. In the same study [18] occupational groups with a high risk of infection showed a significantly higher proportion of seropositives compared to the low-risk control group, in 1983 84%of 221 veterinarians and 68% of 94 residents of dairy farms were positive, whereas in the control group on average 29% tested positive for IgG antibodies against phase II of *C. burnetii*. The age distribution suggested an early onset of infection as antibody percentages over all age groups between 1 and 64 years were comparable [18]. It remains unclear why seroprevalence in the non-high-risk groups, varying between 30% and 50% in the 1980s, was high while on average 23 clinical Q fever cases were detected. Protective immunity from childhood [19] and under-

diagnosis [20] could have played a role, but the serological test methods that were used in the 1980s were developed in-house and are no longer available, so the sensitivity and specificity of these tests can no longer be verified.

Situation of Q fever in animals between 1956 and 2005

Surveys in the early 1950s indicated that the bovine population was free of Q fever at that time [11,12]. Between 1981 and 1987 the endemic state of Q fever in farm animals was confirmed by seroprevalence studies in cattle, sheep and goats [21,22]. In 1981, 55% of 20 sampled cattle on one farm were tested positive. In 1987, 10% of 1320 non-dairy cattle, 21% of 1160 dairy cattle and 3% of a total of 494 dairy heifers were seropositive. At the herd level, about 36% were found positive with an average of 35% seropositive animals per herd. The occurrence of *C. burnetii* in cattle herds seemed to be associated with abortions, as 77% of the herds with abortions had a mean seroprevalence for Q fever of 39%. For the 31% of the herds without abortions, the mean seroprevalence was only 17%. In sheep 3.5% (127/3603 sheep sera from 191 flocks) of the animals were found positive with a herd prevalence of 27%. In goats 2/594 sera from individual goats from 54 flocks were positive. In 1992 prevalence in 220 representative cat sera was 10% and in 400 representative dog sera 13% (D. J. Houwers *et al*. unpublished data).

Table 2.1. Number of dairy goat and dairy sheep farms with confirmed Q fever abortions.

	Year					
	2005	**2006**	**2007**	**2008**	**2009**	**total**
Dairy sheep farms		1		1		2
Dairy goat farms	2	6	7	7*	6	28

* farms with animals at two lactations

Transition period, 2005–2007

Changing situation of Q fever in animals since 2005

Clinical Q fever in animals was diagnosed in The Netherlands for the first time in 2005 [23]. In two dairy goat herds with abortion problems *C. burnetii* was detected by immunohistochemistry (IHC) on placentas [24]. From 2005 to 2007 Q fever abortions were diagnosed on 15 dairy goat farms and one dairy sheep farm (Table 2.1). Abortions, with herd rates up to 60%, were seen mainly in the final month of pregnancy without signs of general illness, although some goats were temporarily a little sluggish with reduced appetite. After abortion some goats showed symptoms of endometritis. Full-term kids were weak, with low body weight and high mortality. In several apparently healthy kids the rearing period was complicated by respiratory and digestive tract disorders. Treatment of pregnant goats with oxytetracyclines did not reduce the abortion rate [24]. Retrospectively, *C. burnetii* could be detected by IHC in one preserved dairy goat's placenta from a farm with abortion problems in 2001. In dairy cattle herds *C. burnetii* antibodies were

detected on 57% of 344 farms using ELISA on bulk tank milk (BTM) samples during 2005–2006. It was calculated that on 35% of the farms at least 30% of the cattle could be positive [25].

Changing situation in humans since 2007

In 2007, individual human Q fever cases were reported, occurring after visits to dairy goat farms with abortion problems [24,26]. The first documented outbreak of Q fever in The Netherlands was described by Karagiannis *et al*. [27] and Van Steenbergen *et al*. [28]. A total of 168 human cases were notified in 2007 (Figure 2.2). The epidemic became apparent by a number of indicators: first, a medical microbiologist reported two patients admitted to hospital with severe pneumonia, who did not respond to standard antibiotic therapy. Four days later, a general practitioner informed the Municipal Health Service (MHS) of an unusual number of ten patients with atypical pneumonia in his practice. A similar report by another general practitioner from the same area was received 2 weeks later. Finally, an increase in the number of notifications for Q fever was noticed in the national registration system of the National Institute for Public Health and the Environment (RIVM). No specific source could be identified, but it was postulated that the high number of abortions on the surrounding dairy goat farms might be the source of the human Q fever cases in the province of Noord-Brabant. Airborne transmission of contaminated dust particles could have been facilitated by the unusually hot and dry weather in the spring of 2007.

In 2007, the outbreak was concentrated around a single village and in this village a case-control study was performed [29]. Contact with manure, hay, and straw proved to be a risk factor. Moreover, people living in the eastern part of the village close to ruminant farms, of which one dairy goat farm had a recent history of abortion problems, were at higher risk than people living in other parts of the village. Contact with animals and consumption of raw milk products were not significant risk factors in the multivariable analysis.

Notification criteria for a confirmed human Q fever case were a clinical presentation with fever or pneumonia or hepatitis and confirmation of the diagnosis in the laboratory by at least a fourfold rise in IgG antibody titre against *C. burnetii* in paired sera or the presence of IgM antibodies against phase II or antibodies against *C. burnetii* phase I [30]. A probable case was defined as clinical signs with a single high antibody titre [31].

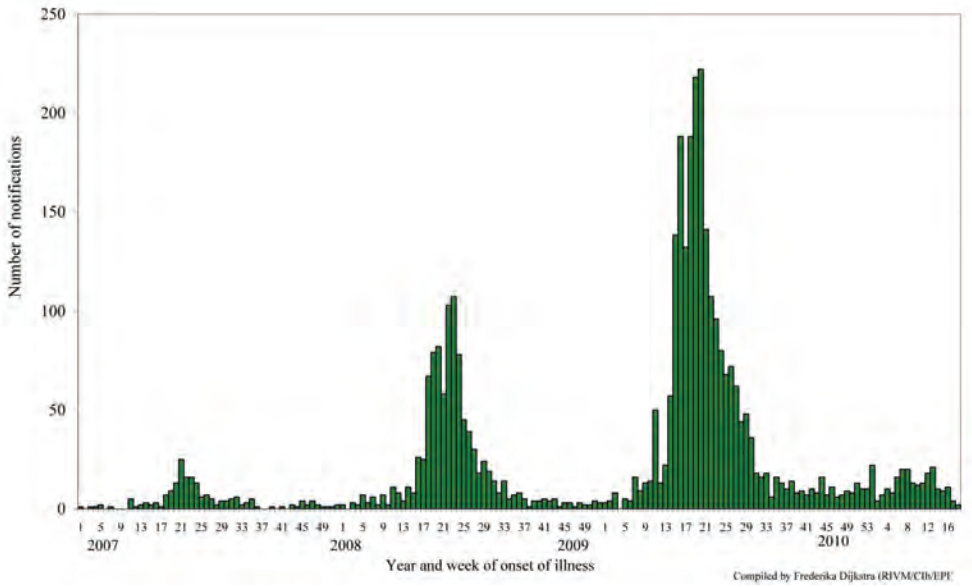

Figure 2.2. Number of notified human Q fever cases with a known first day of illness according to the week of onset of symptoms, from 1 January 2007 to 11 May 2010. 2007 (n=168), 2008 (n=1000), 2009 (n=2355), 2010 (n=208). Total number of human cases in 2007–2009 (n=3523) (compiled by F. Dijkstra).

Response phase, 2008–2010

Continuation of the human outbreak

In 2008, it soon became clear that the 2007 outbreak was not an isolated incident. In May 2008 an outbreak of Q fever occurred in a psychiatric care institution in Nijmegen, province of Gelderland, ~15 km from the 2007 outbreak area [32]. At least 28 in-patients, employees, and visitors had laboratory-confirmed Q fever illness and several patients of the institution developed atypical pneumonia. A small flock of sheep without clinical symptoms of Q fever was present at the location. Patients had close contact with lambs, including cuddling as part of the patients' therapy sessions. Furthermore, on a dairy goat farm close to the city of Nijmegen, a large number of goats unexpectedly aborted their offspring. Q fever was confirmed by PCR on vaginal swabs. The farmer showed no clinical symptoms indicative for Q fever. The farmer's wife suffered from only moderate flu-like symptoms, including fever and coughing, and for both persons Q fever was confirmed by PCR on serum, throat swabs, urine and faecal samples. To determine the genetic relatedness between the human and animal clinical samples from both locations multiple-locus variable number tandem repeat analysis (MLVA) was carried out. Clinical samples from other patients from different locations in the same high-risk area were included in the study. All genotypes showed a high degree of similarity with most genotypes differing from each other by

only a single marker, suggesting a clonal origin (J. J. H. C. Tilburg & C. H. W. Klaassen, unpublished observations) [33,34].

Eventually, 1000 human Q fever cases were notified in 2008 (Figure 2.2). The centre of the outbreak and the area with the most human cases were the same as in 2007, but there was a clear geographical spread to adjacent areas (Figure 2.3). The age distribution (range 7–87 years, average 51 years) was similar to 2007, but the hospitalization rate decreased from 50% in 2007 to 21% in 2008 [10,35]. The high hospitalization rate of 50%in 2007 might be biased by active case-finding in a retrospective survey among hospitalized cases [35]. The overall gender breakdown was the same in 2008 as in 2007: the female to male ratio was 1:1.7 [10].

In April 2009 a sharp increase in human cases was observed again resulting in a total number of 2355 cases (Figure 2.2). Again most of the human cases were from the same area as in 2007 and 2008, with yet again a wider geographical spread (Figure 2.4). Pneumonia is the predominant presentation of Q fever in The Netherlands. For patients notified in 2008 for whom clinical details were available, 545 were diagnosed with pneumonia, 33 with hepatitis and 115 with other febrile illness [31]. The age median of 50 years in 2009 did not differ from that in 2008, neither did the hospitalization rate of 20% nor the gender break down of 1:1.7 female to male ratio [35].

The overall outbreak in humans consisted of at least 10 separate clusters with multiple sources of exposure. One cluster became apparent in 2008 with a strong connection to one goat farm with abortion problems. Patients were living downwind of the goat farm. Living within 2 km of the goat farm was associated with a higher risk of Q fever infection compared to living >5 km from the farm [36]. In 2009, no abortions were notified on this farm, nor in this area, and veterinary measures such as the handling of manure, hygiene measures and a visitors ban were implemented (measures are clarified in the section 'Response in the veterinary field'), but the number of human cases still increased. To date, the source of this increase in 2009 remains unclear [31]. In general, 59% of the notified human cases in 2009 lived within a 5-km zone around a notified dairy goat or dairy sheep farm, while 12% of the Dutch population live within such zones [35].

In 2010 criteria for detection of *C. burnetii* in blood, serum or a sample from the respiratory tract were added to the notification criteria for human Q fever cases. In addition, only acute cases with the day of first illness within 90 days of notification are recorded in the national infectious disease notification databases [37].

During the period January to May 2010, 208 human cases were registered which indicates a decrease in the number of notified human cases compared to 2009 (Figure 2.2). Although weather conditions may have been unfavourable for transmission, it is hoped that the decrease in the number of human Q fever cases was caused by reduced exposure due to veterinary interventions.

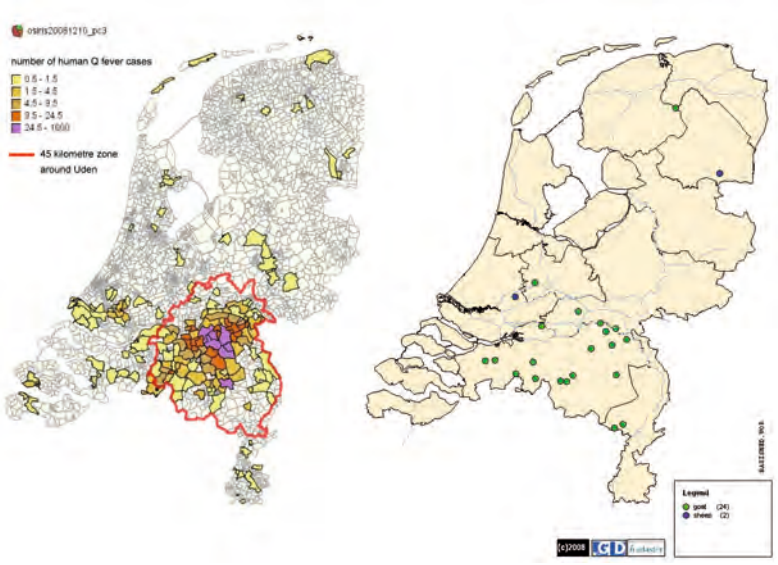

Figure 2.3. Map of The Netherlands. Left: Number of human cases in 2007 and 2008. The red line shows the dairy goat and dairy sheep voluntary vaccination area in 2008. Right: Dairy goat farms and dairy sheep farms with Q fever abortion history between 2005 and 2008. [Compiled by National Institute for Public Health and the Environment (RIVM) and Animal Health Service (GD).]

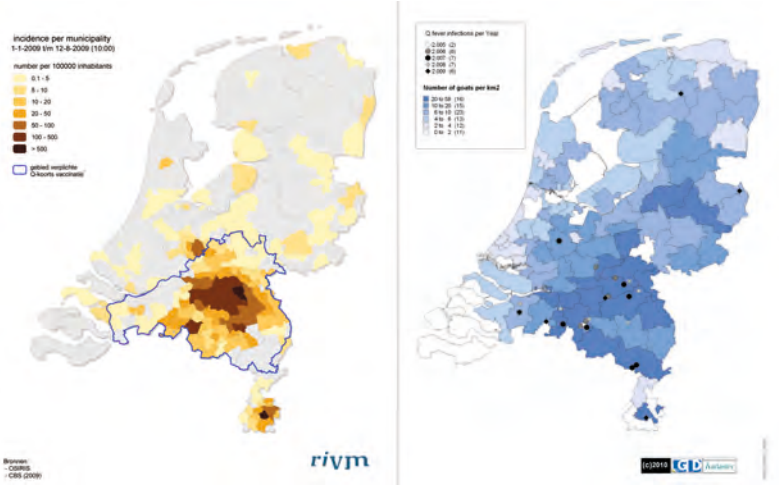

Figure 2.4. Map of The Netherlands. Left: Human Q fever incidence/100 000 inhabitants per municipality in 2009. The blue line shows the dairy goat and dairy sheep mandatory vaccination area in 2009. Right: Dairy goat farms with Q fever abortion history between 2005 and 2009. Darker blue indicates more goats/km2. [Compiled by National Institute for Public Health and the Environment (RIVM) and Animal Health Service (GD).]

Dairy goat industry and prevalence of Q fever in small ruminants

The dairy goat industry in The Netherlands is concentrated in the province of Noord-Brabant, with farm sizes ranging from 300 to 7000 goats with an average of at least 600 animals in 2007. Goat density was 38.1 goats/km2 [38]. These farms often bordered close to villages and cities. The total number of registered small ruminant farms in The Netherlands in 2008 was 52 000. The number of professional dairy goat farms with more than 200 adult goats was 350 and the professional dairy sheep industry consisted of 40 farms [39]. Dairy goat farming in The Netherlands started after the introduction of the European milk quotation system for dairy cattle in 1984 and increased after the outbreaks of classical swine fever in 1997 and foot-and-mouth disease in 2001. The total number of goats increased from 7415 in 1983 to 178 571 in 2000 and to 374 184 in 2009. The total number of dairy goats aged >1 year increased from 98 077 in 2000 to 231 090 in 2009 (Table 2.2)[40,41].

In 2008, nationwide Q fever seroprevalence in all small ruminants was low; only 7.8% of goats and 17.8%of goat farms were positive, and 2.4%of sheep and 14.5% of sheep farms were positive. In 26% of the BTM samples from 306 dairy goat and dairy sheep farms *C. burnetii* DNA could be detected. Analysis of the first 13 goat farms with abortions showed an average number of goats per farm of 900 of which 20% aborted. The average number of sheep on the two affected dairy sheep farms was 400 with an abortion rate of 5% [39].

Table 2.2. Number of goats in The Netherlands [40,41].

Year	Total no. of goats	Total no. of dairy goats aged >1 year
1983	7 415	n.r.
1995	76 063	n.r.
2000	178 571	98 077
2009	374 184	231 090

n.r., No registration

Response in the veterinary field

In June 2008, Q fever became notifiable for small ruminants kept for milk production following the advice of experts (Table 2.3). Additional measures were taken to reduce the assumed risk associated with the spread of manure and to restrict the number of visitors to infected farms. In October 2008, voluntary vaccination of goats was made possible by the Ministry of Agriculture in the high-risk Q fever area in Noord-Brabant with the so far unregistered phase-I Q fever vaccine for ruminants [Coxevac®, Ceva Santé Animale, France; Figure. 2.3 (red line), Table 2.3] [42]. A total of 36 000 goats were vaccinated in an area within a radius of 45 km around the village of Uden. This was the first time a Q fever vaccine had been used with the ultimate goal of reducing the number of human Q fever cases. In order to reduce the number of human cases the exposure of humans to *C. burnetii* should be reduced. To achieve this, excretion of *C. burnetii* from the animal host should be minimized, particularly by the prevention of abortion due to Q fever. Phase-I Q fever vaccines, contrary to phase-II vaccines, strongly reduce the number of

abortions and excretion of *C. burnetii* in challenged pregnant goats that were initially Q fever-negative [43]. In clinically Q fever-infected goat herds vaccination with a phase-I vaccine should reduce the excretion of *C. burnetii*, especially in young animals vaccinated before the breeding season [44]. In general, vaccination with a phase-I Q fever vaccine is expected to be effective in non-infected goats. The effect of vaccination in Q fever-infected goats is not clear and may imply a continuation of the risk of shedding *C. burnetii* and exposure to humans. According to the summary of product characteristics (SPC), the phase-I Q fever vaccine is not indicated for use in pregnant sheep. Assessment of the efficacy of the phase-I vaccine in pregnant cattle showed a similar probability of the animals becoming shedders when vaccinated while pregnant compared to non-vaccinated animals [45].

In February 2009, measures taken by the government were tightened with a stringent hygiene protocol made mandatory for all professional dairy goat and dairy sheep farms in The Netherlands, independent of their Q fever status. The hygiene protocol included vermin control, measures for handling manure (farmers were not allowed to remove manure from their deep litter stables for at least 1 month after the kidding season, were obligated to cover manure during storage and transport, and had to underplough manure immediately when spreading on farming land or had to store it for at least 3 months), compulsory rendering of aborted foetuses and placentas and improved general farm hygiene (such as the prevention of dust and aerosol formation, protective industrial clothing, clean delivery equipment and the use of sufficient high-quality bedding material). In addition, farmers were advised to submit aborted foetuses for pathological examination [39,46]. Vaccination became mandatory for all dairy goats and dairy sheep, and the vaccination area was extended from the 45-km zone around Uden in 2008 (red line in Figure 2.3) to the whole province of Noord-Brabant and small neighbouring areas in 2009 (indicated by the blue line in Figure 2.4).

Table 2.3. Overview of legislation concerning Q fever in small ruminants in The Netherlands [42].

Date of implementation	Document code	Measure
12 June 2008	TRCJZ/2008/1622	Q fever notifiable in dairy goats and dairy sheep; Notification when over 5% abortions within 30 days at farms with more than 100 animals and when over 3% abortions within 30 days at farms with fewer than 100 animals (abortion rates up to 5% are considered to be more or less normal)
12 June 2008	TRCJZ/2008/1645	Prohibited from removing manure from the stable for 90 days after notification
		Visitors ban for 90 days after notification
16 October 2008	TRCJZ/2008/2817	Special dispensation of Coxevac (CEVA) Q fever vaccine to be used in the Netherlands
		Voluntary vaccination in dairy sheep and dairy goats at farms with more than 50 sheep or goats, petting zoos and nursing farms in the restricted 45-km zone (Figure 2.3)
2 February 2009	TRCJZ/2009/244	Prohibited from farming more than 50 dairy goats and dairy sheep if certain hygienic measures are not implemented, such as vermin control, manure measures, rendering foetuses and placentas (see text)
20 April 2009	TRCJZ/2009/1142	Mandatory vaccination of dairy sheep and dairy goats on farms with more than 50 animals, on care farms, petting zoos and zoos in the extended area (Figure 2.4) before 1 January 2010
1 October 2009	Regulation 40823	Mandatory bulk tank milk monitoring on Q fever every two months
		Prohibited from transporting dairy sheep and dairy goats from a positive farm. Vaccinated animals may be transported to positive farms
		Visitors ban at positive farms
9 December 2009	Regulation 96744	Ban on increase of numbers of dairy goats and dairy sheep on a farm
		Ban on reproduction of goats
1 January 2010	Regulation 72246	Mandatory vaccination of dairy sheep and dairy goats, on care farms, petting zoos, zoos, on farms open to the public, mobile sheep flocks, and in natural reserves nationwide before 2011
14 December 2009	Regulation 98748	Mandatory bulk tank milk monitoring on Q fever every two weeks
16 December 2009	Regulation 99604	Prohibited from removing manure from the stable within 30 days after the ending of the lambing season
		If manure has to be removed from the stable, it should be stored on the farm for 90 days
16 December 2009	Letter to the parliament; VDC 09.2695/CPM	Culling of all pregnant goats and sheep on Q fever-positive dairy goat and dairy sheep farms
18 December 2009	Regulation 101785	Prohibited from adding sheep or goats to a farm

Figure 2.5. Map of The Netherlands with all 88 bulk tank milk-positive dairy goat and dairy sheep farms known on 17 April 2010. The red zone is the 5-km zone around a positive farm. [Compiled by Dutch Ministry of Agriculture, Nature and Food Quality (LNV).]

In response to the increasing number of human cases in 2009, additional measures were implemented in October 2009: PCR positivity of BTM on dairy goat and dairy sheep farms became a notification criterion of Q fever in small ruminants in addition to unexpectedly high abortion rates (>5%); a transport ban of animals from Q fever-positive farms and a visitors ban at Q fever-positive farms (Table 2.3). Improved monitoring of farm infection status with BTM monitoring was advised by experts. The background of this advice was that the abortion rate is difficult to measure in a flock with over 600 animals, and on infected farms *C. burnetii* can also be excreted in large quantities during normal birth. In large infected herds with many animals having normal deliveries, the total amount of excreted bacteria can also be very high with a subsequent risk for public health. BTM monitoring could give insight to the number of farms where *C. burnetii* is present. Initially, BTM monitoring was take place every 2 months, but with the increased awareness of the risk of Q fever and the possible risk that Q fever-positive farms might pose during kidding season, the frequency of monitoring was increased to every 2 weeks. Due to BTM monitoring the number of Q fever-positive dairy goat and dairy sheep farms had increased to 88 on 17 April 2010 (Figure 2.5).

With changing public and political awareness of Q fever, the Outbreak Management Team or expert panels advised the Ministries of Health and Agriculture about additional risk reduction measures. At a meeting in December 2009, experts stated that they expected vaccination would not be sufficiently effective in reducing the excretion of *C. burnetii* in the 2010 kidding season, due to the fact that not all dairy goats and dairy sheep had been vaccinated before the 2009 breeding season. A more considerable reduction in the possible excretion of *C. burnetii* and thus, environmental contamination, thereby attempting to reduce human exposure and potential risk to public health in 2010, might be achieved by preventing pregnant Q fever-positive goats on Q fever-positive farms from kidding. These animals were identified as high-risk animals. Veterinary experts from the national veterinary reference institute (the Central Veterinary Institute of Wageningen UR), taking consideration of the findings of Rousset *et al*. [47], believed it impossible to distinguish infected pregnant animals from non-infected pregnant animals by laboratory testing prior to the start of the kidding season. This was the basis of the decision to cull all pregnant animals on Q fever-positive farms. In addition, breeding dairy goats and dairy sheep was prohibited until at least June 2010. The culling started in late December 2009 and was completed in June 2010, when a number of temporary animal measures imposed by the government ended and now require revision.

The measures in the veterinary field were taken to identify risk farms and to reduce the excretion of *C. burnetii* from these farms. Although the effectiveness of certain measures is still unclear and the lag time of the measures and the contribution of environmental contamination with *C. burnetii* to the exposure of humans is unknown, the decreasing numbers of human cases in 2010 thus far might indicate that the measures taken since 2008 have been effective.

The Dutch Q fever epidemic in perspective

Q fever was present in The Netherlands long before it became a problem in public health and animal husbandry. In this period, Q fever appeared to be neither a major health problem for humans, or for domestic animals. This situation, with high seroprevalence in animal populations but few human cases, exists in most European countries [48]. In the years 2005 and 2006, Q fever became a problem in the dairy goat and dairy sheep industry. Abortion storms in small ruminants were not confirmed prior to 2005, although abortions with unknown aetiology are part of small ruminant husbandry with abortion rates up to 5%. It is not clear why Q fever became a major problem in The Netherlands but not elsewhere. Several factors could have facilitated a change in epidemiology in goats : first, an increase in goat density in specific areas of The Netherlands and second, extension of the farms over the years. These two factors could have affected in-herd and between-herd dynamics of Q fever, resulting in outbreaks. Third, there could be pathogen-related factors with circulation of a highly virulent *C. burnetii* strain. In the years 2007 and 2008, it became clear that Q fever also posed a problem to public health. The connection between Q fever problems in the dairy goat and dairy sheep industry and in the human population was made by several authors based on epidemiological and *C. burnetii* typing-based findings [28,33,35]. The increase in goat density took place in the highly populated province of Noord-Brabant (average population density in Noord-Brabant is 497 inhabitants/km2, compared to an average of 398 inhabitants/km2 for The Netherlands). 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. The relationship between the change in epidemiology of Q fever in humans and changes in animal husbandry has been shown earlier. After the collapse of the large state farms in Bulgaria in the 1990s individual farmers started to raise goats, which resulted in a more than doubling of the number of goats to 1 million in 7 years [49]. This increase, together with a change to a more extensive husbandry system, was believed responsible for the increase of human cases. The increase in human Q fever in Germany was also probably related to socio-geographical factors associated with urbanization of rural areas [50]. Despite these examples no general conclusions can be drawn, as *C. burnetii* is endemic in domestic animals throughout Europe and infection can be maintained in a wide range of husbandry systems. Although risk factors, such as an association between human infection and small ruminants, the proximity of animals (especially during parturition) and human populations, and specific weather conditions are clear, there is still an incomplete understanding of transmission pathways with regard to the maintenance of Q fever within the animal reservoir and its transmission to humans [48]. In addition, to date there are no indications of Dutch Q fever spreading to adjacent areas in Belgium and Germany [35].

The question can be raised if the Dutch outbreak is indeed the largest of its kind. A posteriori, it would be difficult to establish if the current epidemic in The Netherlands, with 3523 human cases within three consecutive years, represents a unique phenomenon. In Paragyurische

in Bulgaria, more than 2000 cases that were probably due to Q fever were diagnosed in a 6-month episode in 1993. Although confirmation of Q fever was hampered, these numbers of patients were comparable to the 2355 in 2009 in The Netherlands. The Q fever outbreak of the Balkans, named 'Balkangrippe', during the Second World War was at least comparable in size. In 1941 over 1000 cases were reported among German troops and during the years 1942–1945 comparable outbreaks were reported in general terms [51]. However, in these epidemics, no systematic investigations were performed. The importance of this in combination with the availability of diagnostic tests was also shown in the epidemic in a Swiss Alpine valley in 1983 [52]. The hospitalization of seven patients with atypical pneumonia was able to be diagnosed as due to Q fever as a result of newly available diagnostic tests. This detection was followed by a very large retrospective study in which infection was identified in nearly 15% of the inhabitants of the valley, probably correlated with migration of sheep. This showed that the proportion of patients presenting sufficiently severe symptoms to be hospitalized accounted for only 2%, and that 56% were completely asymptomatic. This means that for each patient being diagnosed, an additional 50 patients probably remain undiagnosed. This is solely based on systematic testing of the hospitalized patients with fever. Under these conditions, it is very difficult to evaluate the true incidence of Q fever, and it is highly likely that some epidemics have gone completely unnoticed. Thus, the reported incidence of Q fever depends on three distinct elements. First, it depends on the true incidence of the disease including existence of epidemics. Second, the reported incidence depends on sensitivity and specificity of the diagnostic tools used. In the Dutch epidemic the use of serological tests, which are now commercially available and the use of real-time PCR on various clinical specimens allowed a better assessment of the incidence. Finally, the interest of the clinicians and the awareness of the general public incontestably reinforces the quality of detection and the percentage of detected patients. With monitoring systems more cases might be detected, but few data exist on large-scale monitoring of Q fever in the human population. In France, preliminary data showed a regular increase in the number of diagnosed cases of acute Q fever which probably testifies to the growing interest and the diagnostic capacities for this disease. Retrospective research in The Netherlands also shows that real-time syndrome surveillance might have detected clusters of human Q fever cases up to 2 years earlier than 2007 [53].

Conclusion

The Q fever epidemic in humans in The Netherlands arose from an endemic state and was preceded by severe Q fever abortion problems in dairy goats and dairy sheep. Dairy goats and dairy sheep are considered to be the main source of human outbreaks and control measures were focused on these animal species. Although it is too early to evaluate the magnitude of the human Q fever epidemic in 2010, the decrease in the number of human cases compared to the previous year is promising. The proximity to small ruminants 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. Q fever outbreaks can easily be missed in the human field as well in the veterinary field. In general good monitoring and surveillance systems are necessary to assess the real magnitude of Q fever.

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Chapter 3

Molecular epidemiology of *Coxiella burnetii* from ruminants in Q Fever outbreak, the Netherlands

Hendrik I.J. Roest[1]
Robin C. Ruuls[1]
Jeroen J.H.C. Tilburg[2]
Marrigje H. Nabuurs-Franssen[2,3]
Corné H.W. Klaassen[2]
Piet Vellema[4]
René van den Brom[4]
Daan Dercksen[4]
Willem Wouda[4]
Marcel A.H. Spierenburg[5]
Arco N. van der Spek[5]
Rob Buijs[1]
Albert G. de Boer[1]
Peter Th.J. Willemsen[1]
Fred G. van Zijderveld[1]

[1]Central Veterinary Institute, part of Wageningen UR, Lelystad, The Netherlands
[2]Canisius Wilhelmina Hospital, Nijmegen, The Netherlands
[3]Radboud University Medical Center, Nijmegen, The Netherlands
[4]Animal Health Service, Deventer, The Netherlands
[5]Food and Consumer Product Safety Authority, The Hague, The Netherlands

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Abstract

Q fever is a zoonosis caused by the bacterium *Coxiella burnetii*. One of the largest reported outbreaks of Q fever in humans occurred in the Netherlands starting in 2007; epidemiologic investigations identified small ruminants as the source. To determine the genetic background of *C. burnetii* in domestic ruminants responsible for the human Q fever outbreak, we genotyped 126 *C. burnetii*–positive samples from ruminants by using a 10-loci multilocus variable-number tandem-repeat analyses panel and compared them with internationally known genotypes. One unique genotype predominated in dairy goat herds and 1 sheep herd in the human Q fever outbreak area in the south of the Netherlands. On the basis of 4 loci, this genotype is similar to a human genotype from the Netherlands. This finding strengthens the probability that this genotype of *C. burnetii* is responsible for the human Q fever epidemic in the Netherlands.

Introduction

Q fever is a zoonosis caused by *Coxiella burnetii*, an intracellular gram-negative bacterium that is prevalent throughout the world [1]. Domestic ruminants are considered the main reservoir for Q fever in humans [2]. However, other animal species, including pet animals, birds, and several species of arthropods, can be infected by *C. burnetii* and cause human cases of Q fever [2,3,4,5]. The main clinical manifestations of Q fever in goats and sheep are abortion and stillbirth. In cattle, Q fever has been associated with sporadic abortion, subfertility, and metritis [4,6]. With an abortion, up to 1 billion *C. burnetii* per gram of placenta can be excreted [7]. Most animal species that carry *C. burnetii* show no symptoms [4]. Transmission to humans occurs mainly through inhalation of contaminated aerosols [3,4,8,9,10].

Recently, 2 DNA-based methods for typing *C. burnetii* were reported [11,12,13]. Multispacer sequence typing is based on DNA sequence variations in 10 short intergenic regions and can be performed on isolated *C. burnetii* strains or directly on extracted DNA from clinical samples [12,14,15]. Multilocus variable-number tandem-repeat analyses (MLVA) is based on variation in repeat number in tandemly repeated DNA elements on multiple loci in the genome of *C. burnetii* and might be more discriminatory than multispacer sequence typing [13,14]. MLVA also can be performed on *C. burnetii* strains [11,14] or directly on DNA extracted from clinical samples [16]. A total of 17 different minisatellite and microsatellite repeat markers have been described [11].

Starting in 2007, the Netherlands has been confronted with one of the largest Q fever outbreaks in the world, involving 3,921 human cases in 4 successive years. On 28 dairy goat farms and 2 dairy sheep farms, abortion storms (with abortion rates up to 80%) caused by Q fever were diagnosed during 2005–2009. These small ruminants are considered the source of the human Q fever outbreak in the Netherlands [17]. The connection between Q fever abortion storms in small ruminants and human Q fever cases is based primarily on epidemiologic investigations [18,19,20,21]. A limited investigation by genotyping with MLVA recently showed that farms and humans in the Netherlands are infected by multiple different, yet closely related, genotypes of *C. burnetii* [16].

Although dairy goats and dairy sheep appear to be the source of the human Q fever outbreak in the Netherlands, no information is available about the genetic background of *C. burnetii* in these populations. This knowledge is essential for gaining insight into the molecular epidemiology of the organism and the origin of the outbreak, as well as for outbreak management purposes. Our objective was to show the genetic background of *C. burnetii* in domestic ruminants responsible for the human Q fever outbreak. This information is necessary to evaluate the epidemiologic link between the source and human cases and to compare the outbreak genotypes with internationally known genotypes. During 2008–2010, a total of 125 *C. burnetii*–positive samples from 14 dairy goat farms, 1 dairy cattle farm, and 2 sheep farms were typed by MLVA. In addition, we show the geographic distribution of these *C. burnetii* genotypes across the Netherlands and compare the genotypes with what is internationally known.

Materials and Methods

Animal Samples

Our study comprised 14 dairy goat farms (farms A–E, H, J, M, N, O, P, Q, AE, and AF), 1 dairy cattle farm (farm R), and 2 sheep farms (1 dairy sheep farm Y and 1 sheep farm Z) sampled during the Q fever outbreak in the Netherlands (Table 3.1; Figure 3.1). On 12 of the 14 dairy goat farms, multiple abortions had occurred. On 2 dairy goat farms (farms J and M) and on the dairy sheep farm (farm Y), no abortions had occurred. On 1 dairy cattle farm and on the sheep farm (farm Z), *C. burnetii* was detected in a placenta with an archived histologic section of paraffin-embedded placenta from an abortion outbreak caused by *C. burnetii* infection.

Table 3.1. Overview of *Coxiella burnetii* genotyping results for farms sampled during human Q fever outbreak, the Netherlands, 2007– 2010*.

farm ID	animal species	approximate herd size	year of sampling	approximate abortion percentage in year of sampling	sample types	number of samples tested	number of samples included in the analysis	MLVA typing results	
								MLVA ID	number of samples
A	dairy goats	617	2008	25	vaginal swabs	20	9	CbNL01	7
								CbNL05	1
								CbNL07	1
B	dairy goats	598	2008	20	vaginal swabs	20	5	CbNL01	5
C	dairy goats	546	2008	25	vaginal swabs	20	20	CbNL01	20
D	dairy goats	1498	2008	19	vaginal swabs	39	7	CbNL01	6
								CbNL04	1
E	dairy goats	1568	2008	8 (2007)	fetal tissue	3	3	CbNL01	1
								CbNL09	1
								CbNL11	1
H	dairy goats	606	2008	80	vaginal swabs	13	8	CbNL01	7
								CbNL02	1
J	dairy goats	459	2008	non	vaginal swabs	3	3	CbNL01	2
								CbNL08	1
M	dairy goats	769	2008	non	vaginal swabs	2	1	CbNL10	1
N	dairy goats	1187	2009	25	vaginal swabs	20	20	CbNL01	20
					placenta	1	1	CbNL01	1

O	dairy goats	83	2009	7	vaginal swabs	40	16	CbNL01	14
								CbNL03	1
								CbNL06	1
					milk	1	1	CbNL01	1
P	dairy goats	548	2009	10	vaginal swabs	20	6	CbNL01	6
Q	dairy goats	340	2009	10	vaginal swabs	25	19	CbNL01	19
AE	dairy goats	500	2007	> 5	placenta	1	1	CbNL12	1
AF	dairy goats	2000	2007	> 5	placenta	1	1	CbNL01	1
AG	dairy goats	590	2001	> 5	paraffin-embedded placenta	1	1		1
R	dairy cattle	70	2007	< 5	placenta	1	1	CbNL13	1
Y	dairy sheep	184	2010	non	vaginal swabs	5	1	CbNL10	1
					bulk tank milk sample	1	1	CbNL10	1
Z	sheep	2	2009	50	placenta	1	1	CbNL01	1

*ID, identification; MLVA, multilocus variable-number tandem-repeat analysis.

Vaginal swabs and milk samples from dairy goats and dairy sheep were sent to the national reference laboratory for notifiable animal diseases (the Central Veterinary Institute, part of Wageningen UR) by the Dutch Food and Consumer Product Safety Authority in accordance with the regulation in place at that time. These samples were submitted for confirmation testing of farms with clinically suspected Q fever (farms A–D, N, O, P, and Q), for tracing the source of human Q fever cases (because of proximity to human case-patients, farms H, J, and M) or for bulk tank milk monitoring (farm Y). Samples of immunohistochemically confirmed Q fever–positive goat and sheep placentas (farms N, AE, AF, and Z) and fetal tissue (farm E) were provided by the Animal Health Service, including 1 archived histologic section of paraffin-embedded placenta from a *C. burnetii* abortion outbreak in a goat farm in 2001 (farm AG), which was diagnosed retrospectively [22]. The sampled dairy goat farms represent 60% of the farms with known abortion problems during 2007–2009.

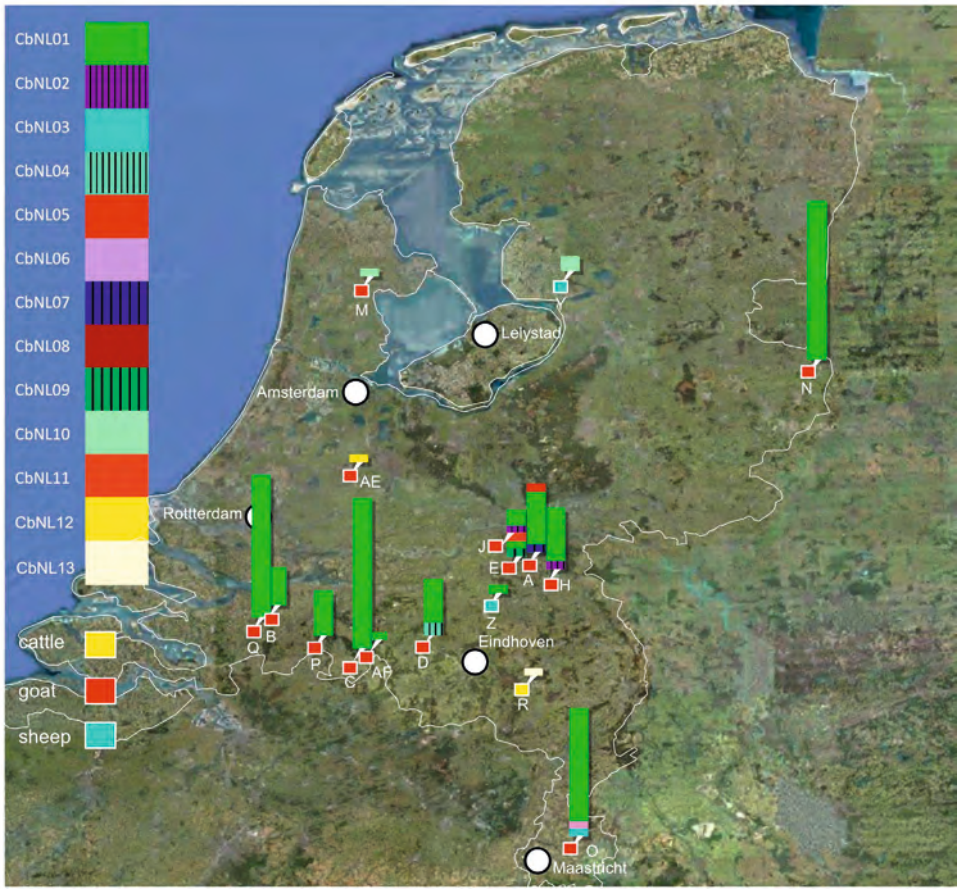


Figure 3.1. Map of the Netherlands showing locations of farms sampled during the Q fever outbreak, 2007–2010. Farms are indicated by letter and ruminant species (black squares, goats; black triangles, sheep; black star, cattle); genotypes of *Coxiella burnetii* found per farm are indicated by bars at each farm's location. The height of the bar indicates numbers of isolates per genotype.

Testing of Samples before MLVA Typing

DNA was extracted from vaginal swabs and milk by using Chelex resin (InstaGene; Bio-Rad, Hercules, CA, USA). A vaginal swab tip or 200 µL of milk was added to 400 µL of Chelex suspension and incubated and shaken for 30 min at 56°C, followed by an incubation step for 8 min at 100°C. The clarified supernatant was used for PCR and MLVA. DNA from placentas was extracted by using a DNA tissue kit (DNeasy Blood and Tissue Kit; QIAGEN, Hilden, Germany). DNA from the paraffin-embedded placenta was extracted by using MagneSil Genomic Fixed Tissue System (Promega, Madison, WI, USA).

All samples were tested by an in-house real-time PCR directed toward the *C. burnetii*–specific IS*1111a* element [23]. An inhibition control was constructed by using the primes of the

Table 3.2. Primers and probes used in the PCR for detecting *Coxiella burnetii* in clinical samples and loci and primers for MLVA of *C. burnetii*, the Netherlands, 2007–2010*.

Identification	Temp, ˚C	Primer sequence for MLVA, with label indicated, 5'à 3'	
		Forward	Reverse
Primers IS1111a	60	CATCACATTGCCGCGTTTAC	GGTTGGTCCCTCGACAACAT
Probe IS1111a	60	AATCCCCAACAACACCTCCTTATTCCCAC	
Probe Inhibition control	60	ACATAATCTCTCCGACCCCACACTTCCATAC	
Cbu0448_ms03_12bp_7U_229bp	60	6-FAM-TTGTCGATAAATCGGGAAACTT	CACTGGGAAAAGGAGAAAAAGA
Cbu1963_ms21_12bp_6U_210bp	60	NED-AGCATCTGCCTTCTCAAGTTTC	TGGGAGGTAGAAGAAAAGATGG
Cbu1980_ms22_11bp_6U_246bp	60	PET-GGGGTTTGAACATAGCAATACC	CAATATCTCTTTCTCCCGCATT
Cbu0259_ms24_7bp_27U_344bp	65	VIC-ATGAAGAAAGGATGGAGGGACT	GATAGCCTGGACAGAGGACAGT
Cbu0838_ms27_6bp_4U_320bp†	65	6-FAM-GGGTCAGGTGGCGGGTGTG	TTCTCGCAAACGTCGCACTAACTC
Cbu0839_ms28_6bp_6U_480bp†	60	VIC-TAGAAACCGATAATCCCCTTGACA	ATTCCGCCGCCATTGAG
Cbu1351_ms30_18bp_6U_306bp‡	60	NED-ATTTCCTCGACATCAACGTCTT	AGTCGATTTGGAAACGGATAAA
Cbu1418_ms31_7bp_5U_285bp‡	60	PET-GGGCATCTAATCGAGATAATGG	TTTGAGAAAATTTTGGGTGCTT
Cbu1471_ms34_6bp_5U_210bp	60	6-FAM-TGACTATCAGCGACTCGAAGAA	TCGTGCGTTAGTGTGCTTATCT
Cbu1941_ms36_9bp_4U_477bp‡	65	VIC-GAAACCAGTCTTCCCTCAACAG	ATAACCGTCATCGTCACCTTCT

*MLVA, multilocus variable-number tandem-repeat analyses; temp, annealing temperature.
† Different primer set than the proposed set by Arricau et al [11]
‡ Updated after personal communication with Ph. Le Flèche, Université Paris-Sud, Orsay cedex, France

IS*1111a* element (Table 3.2). PCR was performed on a 7500 Fast Real Time PCR system (Applied Biosystems, Foster City, CA, USA) by using 400 nmol/L of primers and 200 nmol/L of probes in 7 µL PerfeCTa Multiplex qPCR Supermix, uracil-N-glycosylase (2×) (New England Biolabs, Ipswich, MA, USA] with Low Rox dye (Quanta BioSciences, Gaithersburg, MD, USA), 1 µL of inhibition control, 5 µL of sample, and 7 µL of water. An initial uracil DNA glycosylase (UDG) incubation for 5 min at 45°C and denaturation/activation for 60 s at 95°C was followed by 40 cycles of denaturation for 10 s at 95°C, annealing for 30 s at 60°C. Results were generated with 7500 Fast System Software (Applied Biosystems).

MLVA Typing

MLVA typing was performed by using a selection of 10 of the 17 loci described by Arricau-Bouvery *et al*. [11] according to the Multiple Loci VNTR Analysis databases for genotyping (http://minisatellites.u-psud.fr/MLVAnet/ querypub1.php), except that Ms12 was omitted because of poor performance, and Ms24 was added (Table 3.2). New primers were designed for Ms27 and Ms28 to improve performance. The annotation of Ms30, Ms31, and Ms36 was updated (P. Le Flèche, pers. comm.).

The PCR amplification was performed by using an Applied Biosystems 9700 thermocycler in a total volume of 25 µL containing 1× reaction buffer, 1 U True Start *Taq* DNA polymerase (Fermentas, Glen Burnie, MD, USA), 2 mmol/L MgCl2, 0.2 mmol/L of each nucleotide (dATP, dGTP, dCTP, dUTP), 0.5 µmol/L of each primer, 0.5 U UDG (New England Biolabs), and 2–5 µL

template. An initial UDG incubation for 5 min at 37°C and denaturation/ activation for 2 min at 95°C was followed by 40 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 60/65°C, elongation for 30 s at 72°C, followed by a final extension step for 5 min at 72°C. After the amplification, 0.5 U UDG inhibitor (New England Biolabs) was added to the PCRs to prevent further UDG activity. Up to 4 different PCR products with different fluorescent dyes were diluted, depending on the PCR efficiency, and pooled. From these pooled PCR products, 4 µL was mixed with 15 µL of Hi-Di formamide (Applied Biosystems) and 0.5 µL of GeneScan 600 LIZ Size Standard (Applied Biosystems). After denaturation for 3 min at 96°C the samples were cooled on ice. The PCR products were separated on a 3130 Genetic Analyzer (Applied Biosystems) with a 36-cm array by using POP7 polymer.

The fragments were sized by using GeneMapper version 4.0 software (Applied Biosystems). The accuracy of the sizing obtained by capillary electrophoresis was determined by comparing sequencing data from the reference strain with the obtained fragment size from the capillary electrophoresis and corrected if necessary. The number of repeats for each locus was determined on the basis of the published and corrected annotation of the various loci (Table 3.2). Non–whole repeat numbers were rounded off mathematically. Reproducibility was checked with positive controls.

Data Analysis

The reference strain Nine Mile was used as reference [11]. Analyses were performed, including only genotypes of *C. burnetii* containing <2 loci with missing values. Numerical typing data were imported into BioNumerics v 6.1 (Applied Maths, Sint-Martens-Latem, Belgium) and analyzed with the multistate categorical similarity coefficient by using unweighted pair group method with arithmetic mean clustering. Missing values were imported as question marks. The genotypic diversity of the population under study was calculated by using the adapted Simpson index of diversity (Hunter-Gaston diversity index [HGDI]) [11,24].

Found MLVA patterns based on the number of repeats per locus were called MLVA types and identified as CbNLxx. We compared MLVA types with MLVA types in the publicly accessible Multiple Loci VNTR Analysis databases for genotyping: Coxiella2007 and Coxiella2009_ Netherlands (access date 2001 Jan 11). The Nine Mile strain was used as reference.

Results

The study comprised 122 samples from 15 dairy goat farms, 2 samples from 1 dairy sheep farm, and 1 sample each from 1 sheep farm and 1 dairy cattle farm were included in this study (Table 3.1). Of the farms sampled during the outbreak, 13 were situated in the southern part of the Netherlands; 3 dairy goat farms (farms M, N, and AE) and 1 dairy sheep farm (farm Y) were located outside this area (Figure 3.1). From the 238 Q fever PCR-positive samples from the farms in this study, 125 (53%) yielded a genotype with <2 missing values: 52 with a complete

genotype, 48 with 1 missing value, and 25 with 2 missing values. 113 (47%) PCR-positive samples represented partial genotypes with 3–10 missing values. From the paraffin-embedded placenta (farm AG), only a partial genotype could be shown, with 6 repeats on Ms03 and 10 repeats on Ms34.

We distinguished 13 genotypes in the 125 samples (CbNL01–CbNL13; Table 3.1; Figures 3.1, 3.2). All *C. burnetii* genotypes could be associated with abortion, except for 2 (CbNL10, farm M and Y; and CbNL08, farm J; Figure 3.1). The relationship between the genotypes in all samples is shown in Figure 3.2, including the genotype of the reference strain Nine Mile and the reference genotype of the reference strain Nine Mile from Arricau-Bouvery *et al*. [11], which were identical.

The 13 genotypes are separated in 2 clusters (Figure 3.2). One cluster containing a genotype represented by 111 (90%) of the samples (CbNL01); 1 genotype (CbNL10) represented by 3 samples (1 from a dairy goat farm and 2 from a dairy sheep farm); and 10 genotypes (CbNL02–CbNL09 and CbNL11) represented by 1 sample, all from dairy goat farms. The second cluster was distinctly separated from the other cluster, representing 2 genotypes in 1 dairy goat sample (CbNL12), in 1 dairy cattle sample (CbNL13) and the paraffin-embedded placenta. In samples from dairy goat farms with abortion problems, the same genotype (CbNL01) was present in 110 (91%) of 121 samples. One sheep sample also showed this genotype (farm Z). The geographic distribution of the genotypes according to the location of the originating farm is given in Figure 3.1. The relationship between the genotypes found in this study and the internationally known genotypes are presented in the phylogenetic trees in Figure 3.3 on the basis of 4 loci and in Figure 3.4 on the basis of 9 loci.

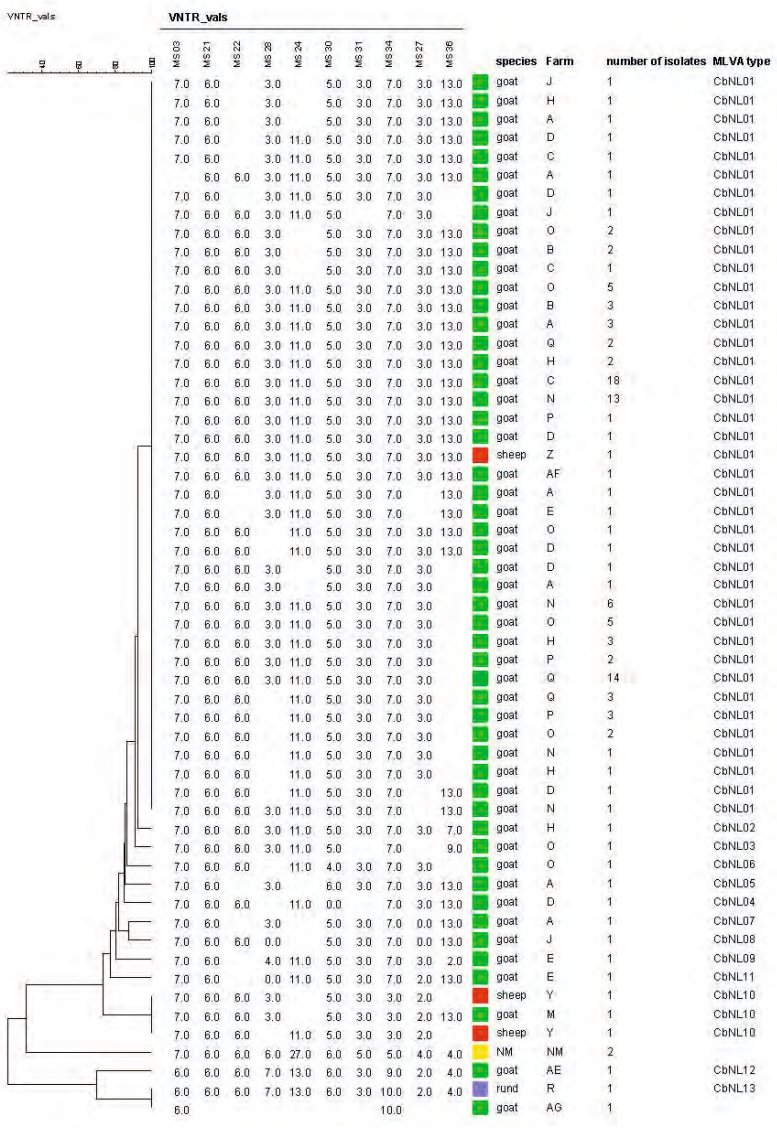
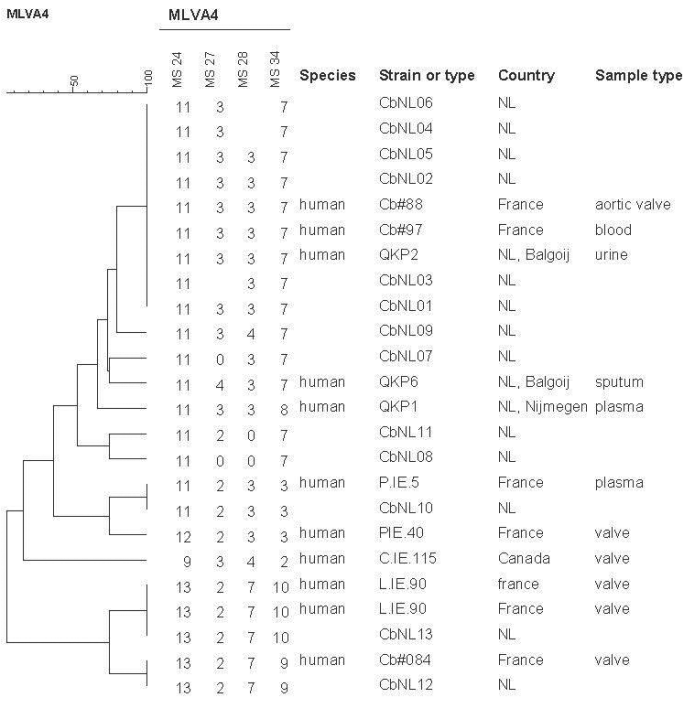


Figure 3.2. Phylogenetic tree with genotypes of *Coxiella burnetii* of all samples in the study, the Netherlands, on the basis of 10 multilocus variable-number tandem-repeat analyses (MLVA). Repeats per locus are shown; open spots indicate missing values. NM, Nine Mile reference strain.



MS 24	MS 27	MS 28	MS 34	Species	Strain or type	Country	Sample type
11	3		7		CbNL06	NL	
11	3		7		CbNL04	NL	
11	3	3	7		CbNL05	NL	
11	3	3	7		CbNL02	NL	
11	3	3	7	human	Cb#88	France	aortic valve
11	3	3	7	human	Cb#97	France	blood
11	3	3	7	human	QKP2	NL, Balgoij	urine
11		3	7		CbNL03	NL	
11	3	3	7		CbNL01	NL	
11	3	4	7		CbNL09	NL	
11	0	3	7		CbNL07	NL	
11	4	3	7	human	QKP6	NL, Balgoij	sputum
11	3	3	8	human	QKP1	NL, Nijmegen	plasma
11	2	0	7		CbNL11	NL	
11	0	0	7		CbNL08	NL	
11	2	3	3	human	P.IE.5	France	plasma
11	2	3	3		CbNL10	NL	
12	2	3	3	human	PIE.40	France	valve
9	3	4	2	human	C.IE.115	Canada	valve
13	2	7	10	human	L.IE.90	france	valve
13	2	7	10	human	L.IE.90	France	valve
13	2	7	10		CbNL13	NL	
13	2	7	9	human	Cb#084	France	valve
13	2	7	9		CbNL12	NL	

Figure 3.3. Phylogenetic tree with genotypes of *Coxiella burnetii* that are most closely related to the Dutch genotypes on the basis of 4 multilocus variable-number tandem-repeat analyses (MLVA). Genotypes are derived from the Multiple Loci VNTR Analysis databases for genotyping (http://minisatellites.u-psud.fr/MLVAnet/ querypub1.php: Coxiella2009_Netherlands [accessed 2011 Jan 11]). Repeats per locus are shown; open spots indicate missing values. NL, the Netherlands.

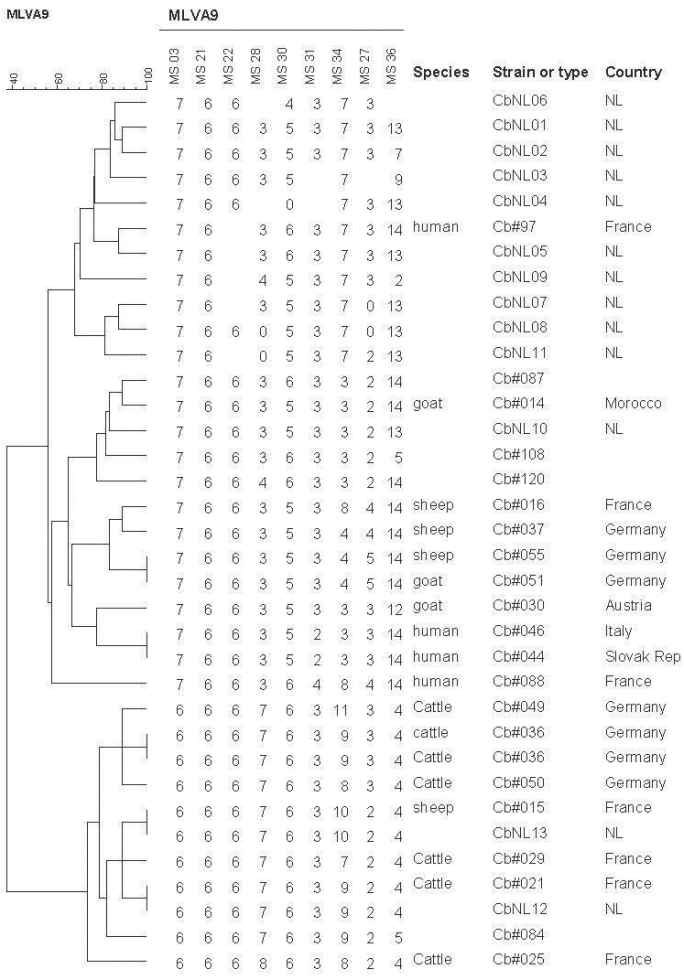


Figure 3.4. Phylogenetic tree with genotypes of *Coxiella burnetii* that are most closely related to the Dutch genotypes on the basis of 9 multilocus variable-number tandem-repeat analyses (MLVA). Genotypes are derived from the Multiple Loci VNTR Analysis databases for genotyping (http://minisatellites.u-psud.fr/MLVAnet/ querypub1.php: Coxiella2007 [accessed 2011 Jan 11]). Repeats per locus are shown; open spots indicate missing values. NL, the Netherlands; Slovak Rep, Slovak Republic.

Discussion

We performed MLVA typing of *C. burnetii* based on 10 loci on a large number of Q fever–positive samples to show the genetic background of *C. burnetii* in the domestic ruminants associated with the Q fever outbreak in humans in the Netherlands. In 125 (53%) of 237 samples, an adequate genotype for *C. burnetii* was generated. Previously, MLVA typing was performed on *C. burnetii* strains after primary isolation and cultivation [11,13,14] or, in the Netherlands, on only 11 clinical samples from humans, sheep, and goats with a selected number of 3 loci [16].

The main drawback of typing on clinical samples is the variable quality and amount of DNA. These drawbacks influence the typability of samples, resulting in partial genotypes; whether the missing values are caused by insufficient DNA concentrations and quality or by an absence of loci is unclear. If loci are absent, partial genotypes also are expected to be found in samples with high DNA loads. Such is not the case in our study. Typing of placenta material that contains high quantities of *C. burnetii*, as well as vaginal swabs with PCR cycle threshold (Ct) values <32, yielded complete genotypes. In samples with Ct values of 32–34, only partial genotypes were obtained. Samples with a Ct value >34 were poorly typable.

Arricau-Bouvery *et al*. [11] calculated diversity indices for the 17 loci used in the MLVA, which varied from 0.28 for locus Ms22 to 0.86 for locus Ms34. The HGDI for the combined panels 1 and 2 of the MLVA typing method for *C. burnetii* can be calculated on 0.99 and for panel 2 on 0.92. These HGDIs are in the upper part of the 0.438– 0.997 range reported by Hunter and Gaston [24] for typing methods for various bacteria and yeasts.

The high diversity indices for the MLVA of *C. burnetii* indicate a high discriminating power, and this capability makes MLVA typing suitable for distinguishing *C. burnetii* isolates. With this highly discriminatory typing method, we found that 1 genotype of *C. burnetii* predominated on all dairy goat farms in the southern part of the Netherlands. On 12 of 14 dairy goat farms, this genotype was found in 91% of samples, varying per farm from 33% (farm E) to 100% (farms B, C, N–Q, Table 3.1, Figures 3.1 and 3.2). Although the sample size was small compared with the number of animals on the farm (Table 3.1), these data show that 1 genotype was far more common than other genotypes found on these farms. The 9 other genotypes occurred once, each representing only 0.8% of all found genotypes on dairy goat farms. The most predominant genotype was found on all 11 dairy goat farms in the southern Netherlands and on a farm in the eastern part of the country (farm N). 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 1 genotype of *C. burnetii* could be explained by 2 phenomena. First, the dairy goat industry in the Netherlands sharply increased from almost 100,000 dairy goats in 2000 to >230,000 dairy goats on ≈350 farms in 2009 [17]. Most of these goats were bred in the Netherlands, which probably resulted in a microbial relationship between many of the dairy goat herds. In this theory, the *C. burnetii* strain with the most predominant genotype was present in the Netherlands for a long period before the abortion problems in dairy goats started in 2005. This theory is not supported by the results of the typing of the paraffin-embedded placenta from

an aborted dairy goat who in 2001. The typing result differs on 2 loci from the most predominant genotype found in this study. Second, clonal spread could have been facilitated by 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. Whether this genotype is more virulent is subject to research.

On the basis of comparison of MLVA types on 4 loci (Figure 3.3), CbNL01–06 could not be distinguished and were similar to the genotype of a person in the Netherlands (QPK2) and 2 genotypes from persons in France (Cb#88, Cb#97). The sample from a person in the Netherlands is derived from patient 2 reported by Klaassen *et al*. [16]. Patient 2 is the farmer of farm A, where genotype CbNL01 predominated, as well as CbNL05 (Table 3.1). This shows a genetic link between the *C. burnetii* DNA from the farmer and his abortive goats, which suggests that the farmer was infected by his own goats. However, this link is based on only 4 loci on 1 human sample. To further confirm the link between dairy goats and humans, more samples need to be typed with more MLVA loci to increase the discriminatory power.

The human sample with ID QKP6 is the same sample as that from patient 4 reported by Klaassen *et al*. [16] and is most closely related to CbNL07. Human sample QKP1 is the same as that of patient 1. Patient 5 fits in the genotype cluster in the Netherlands, as does patient 2. The sheep reported by Klaassen *et al*. did not abort, and their samples show a difference of 1 repeat on Ms34 compared with CbNL01. On the basis of the comparison of MLVA types on 9 loci (Figure 3.4), all genotypes in this study can be distinguished. The most predominant genotype CbNL01 clusters with other genotypes (CbNL02–CbNL09, CbNL11) and with 1 human sample (Cb#97) from France. CbNL01 differed from this human isolate on 2 loci (Ms30 and 36), which shows that the most predominant genotype in the Netherlands is unique. Whether this finding can be attributed to the small number of strains and clinical samples typed or is really a unique genotype is not yet clear. The closest relation to an isolate from France might give a clue about the origin of the genotype from the Netherlands.

The human Q fever outbreak in the Netherlands started in the southern part of the country and resulted in >3,500 human cases during 2007–2010. Dairy goats and dairy sheep are considered to be the source of this outbreak, primarily on the basis of epidemiologic findings [10,17,18,19,25,26]. In our study, samples were typed from farms suspected of being the source of the human Q fever outbreak. Results show that 1 genotype of *C. burnetii* predominated in the dairy goats and sheep in the human Q fever outbreak area in the southern part of the Netherlands, and this genotype also was present in a human case-patient in the Netherlands. This *C. burnetii* genotype is expected to have played a key role in the Q fever outbreak in small ruminants in the Netherlands and was also transmitted widely to humans, causing Q fever in the human population. If this hypothesis holds true, *C. burnetii* with the same genotype as in dairy goats should be found in most samples from human Q fever patients. To this end, a study was performed to show the genetic background of human *C. burnetii* isolates in the Netherlands by using a concordant MLVA typing method (J.J.H.C. Tilburg *et al*., unpub. data). Furthermore, the uniqueness of the predominant genotype of *C. burnetii* for the Netherlands can be part of the

explanation why the magnitude of the Q fever outbreak in the Netherlands has never been seen elsewhere.

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Chapter 4

Epidemic genotype of *Coxiella burnetii* among goats, sheep, and humans in the Netherlands

J.J.H.C. Tilburg[1]
H.I.J. Roest[2]
S. Buffet[3]
M.H. Nabuurs-Franssen[1]
A.M. Horrevorts[1]
D. Raoult[3]
C.H.W. Klaassen[1]

[1]Department of Medical Microbiology and Infectious Diseases, Canisius Wilhelmina Hospital, Nijmegen, The Netherlands
[2]Department of Bacteriology and TSE's, Central Veterinary Institute, part of Wageningen UR, Lelystad, The Netherlands
[3]Unité des Rickettsies CNRS UMR 6020, IFR 48, Faculté de Médecine de Marseille, Université de la Méditerranée, Marseille, France.

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Abstract

Genotyping shows the presence of *Coxiella burnetii* genotype MST33 in clinical samples from Dutch goats, sheep and humans. In contrast, MST genotype 20 was observed in samples originating from cattle. These observations confirm the presence of *C. burnetii* in goats/sheep as the most likely origin of the Dutch Q fever outbreak.

To the Editor: The 2007–2010 Q fever epidemic among humans in the Netherlands was among the largest reported in magnitude and duration [1]. The increase in human Q fever cases coincided with an increase in spontaneous abortions among dairy goats in the southeastern part of the Netherlands, an area that is densely populated with goat farms [1]. Genotypic analyses of the involved isolates could confirm the possible link between the human and animal Q fever cases.

In previous studies, genotypic investigations of human and animal samples in the Netherlands were performed by using a 3-locus multilocus variable-number tandem repeats analysis (MLVA) panel and single-nucleotide polymorphism genotyping, respectively [2,3]. The first study, performed on relatively few samples from a minor part of the affected area, showed that farm animals and humans in the Netherlands were infected by different but apparently closely related genotypes. More recently, genotyping by using a 10-locus MLVA panel provided additional information about the genotypic diversity of *Coxiella burnetii* among ruminants in the Netherlands: 1 dominant MLVA genotype was identifi ed among goats and sheep throughout the entire affected Q fever area [4]. A different panel of MLVA markers was applied to human samples [5]. Four markers that are shared by both panels showed identical alleles in human and animal samples, again implicating goats and sheep as possible sources of the outbreak.

MLVA, which is based on relatively unstable repetitive DNA elements, is sometimes criticized for producing results that are too discriminatory or difficult to reproduce in different settings [6]. Because of their instability, use of tandem repeats as genotyping targets can lead to problems with data interpretation and to overestimation of genotypic diversity by showing small variations in MLVA genotypes in isolates of otherwise identical background. We used a more stable, sequence based typing method, multispacer sequence typing (MST), on samples from humans and a group of ruminant animals (goats, sheep, and cattle) to establish a firmer correlation between Q fever cases in humans and animals [7]. We identified MST genotypes using a Web-based MST database (http://ifr48.timone.univmrs. fr/MST_Coxiella/mst) containing genotypes from several countries in Europe. Ultimately, this study could answer the question of whether the current outbreak situation could have been caused by a specific *C. burnetii* strain in the ruminant population in the Netherlands.

Real-time PCR-positive specimens from 10 humans and 9 Q fever–positive specimens from goats and sheep collected from various locations throughout the affected area were used [8]. We also included Q fever-positive specimens from cattle to rule out cattle as a possible source of Q fever infection. Five samples of cow's milk and 1 bovine vaginal swab sample were analyzed (Figure 4.1). MST33 was identified in 9 of 10 tested human samples and in the remaining 8 of 9 clinical samples from goats and sheep (Figure 4.1). MST33 has been isolated incidentally in nonoutbreak situations in human clinical samples obtained in France during 1996, 1998, and 1999 and from a placenta of an asymptomatic ewe in Germany during 1992. All samples from cattle in the Netherlands, 1 goat, and cow's milk contained genotype MST20. Genotype MST20 has also been identified in human clinical samples from France, in a cow's placenta from Germany isolated in 1992 and in rodents from the United States isolated in 1958. In 1 human bronchoalveolar

lavage sample, a novel (partial) MST genotype was found. This may be an incidental Q fever case unrelated to the outbreak situation. Because no historical genotyping data for the period before the outbreak of Q fever in the Netherlands are available, this explanation needs further research.

MST genotyping shows the presence of genotype MST33 in clinical samples from humans, goats and sheep. These results confirm that goats and sheep are the source of human Q fever in the Netherlands. Few worldwide genotyping studies have been conducted, and therefore information about a possible global persistence of this genotype is lacking. This study also indicates that the outbreak among humans is not linked to *C. burnetii* in cattle, although the infection is widespread among dairy herds in the Netherlands [10], exemplifying that most outbreaks are related to goats and sheep rather than to cattle. In conclusion, the increase in the number of Q fever cases in the Netherlands among humans most likely results from MST33 in the goat population in the Netherlands and could have been facilitated by intensive goat farming in the affected area and its proximity to the human population.

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Table 4.1. Overview of *Coxiella burnetii* MST genotypes from humans and ruminants sampled during the Q fever outbreak, The Netherlands*.

Sample no.	Host	Source	Location	Year	Ct value	MST genotype†	Cox2	Cox5	Cox18	Cox20	Cox22	Cox37	Cox51	Cox56	Cox57‡	Cox61
Q001	Sheep	Vaginal swab	1	2008	25.7	33	7	5	1	–§	5	9	9	4	3	2
Q002	Sheep	Vaginal swab	1	2008	16.3	33	7	5	1	6	5	9	9	4	3	2
Q003	Sheep	Vaginal swab	1	2008	18.8	33	7	5	1	6	5	9	9	4	3	2
Q004	Lamb	Throat swab	1	2008	27.9	33	7	5	1	6	5	9	9	4	3	2
Q005	Lamb	Throat swab	1	2008	29.9	33	7	5	1	–	5	9	9	4	3	2
Q006	Lamb	Throat swab	1	2008	28.9	33	7	5	1	–	5	9	9	4	3	2
Q050	Human	BAL	2	2009	22.4	33	7	5	1	–	5	9	9	4	3	2
Q052	Human	Sputum	3	2009	20.7	33	7	5	1	–	5	9	9	4	3	2
Q054	Human	Sputum	3	2009	19.4	33	7	5	1	–	5	9	9	4	3	2
Q057	Human	Sputum	3	2009	20.6	33	7	5	1	–	5	9	9	4	3	2
Q063	Human	Sputum	4	2009	29.6	33	7	5	1	–	5	9	9	4	–	2
Q066	Human	Sputum	5	2009	27.7	33	7	5	1	–	5	9	9	4	–	2
Q076	Human	Aorta valve	6	2009	17.0	33	7	5	1	–	5	9	9	4	3	2
Q084	Human	Aorta valve	7	2008	17.0	33	7	5	1	–	5	9	9	4	3	2
Q107	Human	Aorta valve	5	2010	9.0	33	7	5	1	6	5	9	9	4	3	2
Q085	Goat	Placenta	8	2009	18.0	33	7	5	1	–	5	9	9	4	3	2
Q087	Goat	Placenta	9	2009	18.1	33	7	5	1	–	5	9	9	4	3	2
Q086	Goat	Placenta	9	2009	18.0	20	3	2	6	–	5	4	4	10	6	5
Q097	Cattle	Swab	10	2009	19.0	20	3	2	6	–	5	4	4	10	6	5
Q090	Cattle	Milk	11	2010	32.0	20	3	2	6	–	5	4	4	10	–	5
Q091	Cattle	Milk	12	2010	32.6	20	3	2	6	–	5	4	4	10	–	5
Q093	Cattle	Milk	13	2010	31.7	20	3	2	6	–	5	4	4	10	–	5
Q096	Cattle	Milk	14	2010	33.4	20	3	2	6	–	5	4	4	10	–	5
Q123	Cattle	Milk	15	2010	31.6	20	3	2	6	–	5	4	4	10	–	5
Q056	Human	BAL	16	2010	28.2	New	3	3	2	–	–	9	–	–	–	–
Dugway	NA	CP000733#	NA	NA	NA	20	3	2	6	1	5	4	4	10	6	5
RSA331	NA	CP000890#	NA	NA	NA	18	3	8	1	6	3	4	7	9	6	3
RSA493	NA	AE016828#	NA	NA	NA	16	3	8	5	3	4	1	6	7	6	5
CbuG Q212	NA	CP001019#	NA	NA	NA	21	2	1	4	6	2	3	1	11	1	1
CbuK Q154	NA	CP001020#	NA	NA	NA	8	5	4	2	5	1	5	3	3	4	4

*MST, multispacer sequence typing; Ct, cycle threshold; BAL, bronchoalveolar lavage; NA, not applicable. †MST genotypes were identified by using the MST database (http://ifr48.timone.univ-mrs.fr/MST_Coxiella/mst). ‡Result obtained by using improved amplification primers for Cox57 [9]. § –, no result was obtained. The lack of results may be explained by the significantly larger PCR product that is targeted, low quantity of DNA or to overall poor performance of the PCR amplification. ¶This combination of 4 alleles has not been observed and justifies the assignment of a new MST genotype. #GenBank accession number.

Chapter 5

Coxiella burnetii infection in roe deer during Q Fever epidemic, the Netherlands

Jolianne M. Rijks[1]
Hendrik I.J. Roest[2]
Peter W. van Tulden[2]
Marja J.L. Kik[1]
Jooske IJzer[1]
Andrea Gröne[1]

[1]Dutch Wildlife Health Centre, Utrecht, The Netherlands
[2]Central Veterinary Institute, part of Wageningen UR, Lelystad, The Netherlands

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Abstract

Coxiella burnetii DNA was detected by PCR in 18 of 79 roe deer carcasses from 2008-2010. Positive cases with highest loads occurred in spring and early summer. Two MLVA-genotyped roe deer strains differed from human and domestic dairy animal strains, including the predominant strain involved in the Dutch Q-fever epidemic.

To the Editor: A Q fever epidemic among humans started in the Netherlands in 2007 and peaked in 2009 [1]. Epidemiologic evidence linked the epidemic to abortions and deliveries among *Coxiella burnetii*–infected dairy goats and dairy sheep [1,2]. However, questions arose about whether *C. burnetii* infection in freeliving wildlife might be another source of Q fever in humans. *C. burnetii* has a wide host range [3], but to our knowledge no studies had addressed its occurrence in nondomestic animals in the Netherlands [4].

The main objective of this study was to look for evidence of *C. burnetii* infection in carcasses of free-living roe deer (*Capreolus capreolus*) in the Netherlands, where *C. capreolus* is the most common species of wild ruminant. Additional objectives were to 1) analyze characteristics, location, and time of death of case-animals for more information on the infection in roe deer and 2) determine the genotype of *C. burnetii* strains from roe deer and compare them with the genotype of strains from domestic animals and humans for evidence of spillover.

The sample consisted of 79 roe deer that were euthanized or found dead in 9 of the 12 provinces in the Netherlands during January 2008–May 2010. All animals had undergone postmortem examination, and tissue samples were frozen until testing. Tissues tested were lung (n = 46), spleen (n = 50), bone marrow (n = 50), liver (n = 74), and kidney (n = 75), as available. We extracted DNA by using the DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany). A duplex quantitative PCR targeting the IS*1111a* element was used with an internal control gene, as described [2]. Tissues with cycle threshold (Ct) values <34 (1/case) were typed by using multilocus variable-number tandem-repeat analyses (MLVA) for 11 loci, as described [2,5]; results were compared with known MLVA typing data from the Netherlands.

Of the 79 roe deer examined, 18 (23%) had positive PCR results for *C. burnetii* DNA in multiple (5/18, 28%) or single (13/18, 72%) tissues. The average Ct value was 36.30 (range 32.07–39.47). Among 29 roe deer for which all 5 tissues were tested, no single tissue was more frequently positive than others for *C. burnetii* ($\chi 2 = 1.07$, df = 4, $p = 0.9$) or had lower Ct values (single factor analysis of variance, $p = 0.58$). These findings indicate that testing multiple tissues per individual enhances case detection.

No specific sex, age, or health effects were observed. Of 48 male deer, 10 (21%) had positive results, compared with 8 (27%) of 30 female deer (1 missing value; $\chi 2 = 0.35$, df = 1, $p = 0.55$). Of 50 deer >1 year of age, 15 (30%) had positive results, compared with 2 (15%) of 13 deer <1 year of age (16 missing values; 2-tailed Fisher exact test, $p = 0.49$). Postmortem findings varied for *C. burnetii*–positive deer.

C. burnetii cases occurred in most provinces studied (6/9, 66%) and in all 3 study years. Significantly more *C. burnetii*–positive deer were observed in 2010 (13/30, 43%) than in 2008 (2/18, 11%) and 2009 (3/31, 10%) ($\chi 2 = 11.62$, df = 2, $p < 0.01$). This finding might represent sample bias or indicate spatial or temporal clustering in 2010.

The *C. burnetii* genetic material found in roe deer may indicate past or ongoing infection [6]. Although positive cases occurred in all seasons, those more likely to represent ongoing infection (multiple infected tissues and Ct values <36; n = 4) occurred in March, April, and June. Clinical Q fever in roe deer might occur more frequently in late gestation and around parturition, as in

domestic ruminants [7,8]. Furthermore, Q fever in wildlife might have its own sylvatic cycle [4,9]. However, analogous to human cases in 2007–2010 [1], the pattern could also include spillover events from domestic livestock.

Tissues of 2 springtime case-animals had Ct values <34. MVLA typing of these strains yielded partial genotypes (Figure 5.1). Comparison with those of strains from domestic dairy animals or humans during 2007–2010 showed that these 2 strains from roe deer differed from the main goat- and sheep-derived strain involved in the Q fever epidemic (genotype CbNL01 [2]) and from other strains found (inconclusive for CbNL08; Figure 5.1).

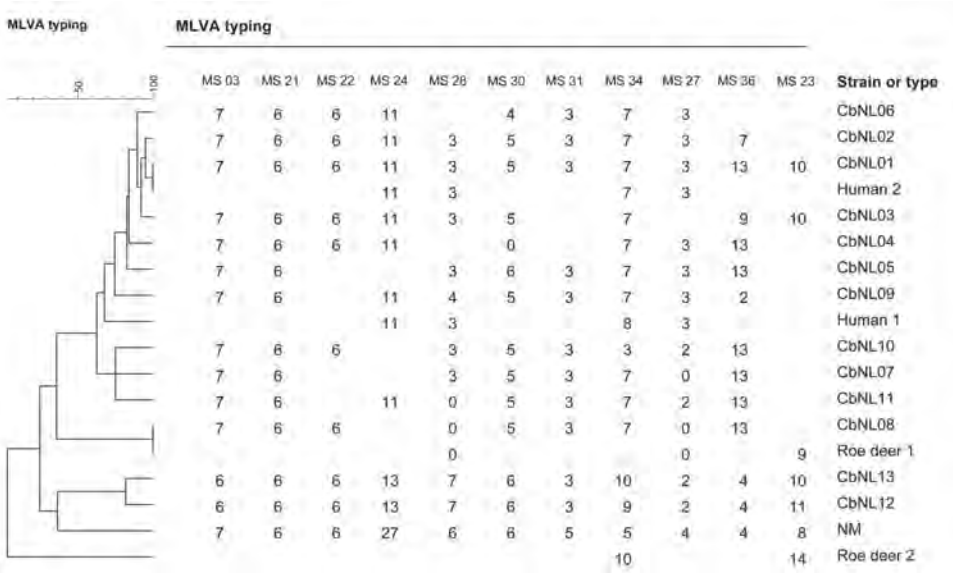

Figure 5.1. Phylogenetic tree with genotypes of *Coxiella burnetii* from goat, human, and roe deer samples from the Netherlands. Genotypes were determined on the basis of 11 multilocus variable-number tandem-repeat analyses (MLVA). The number of repeats per locus is shown; open spots indicate missing values. Roe deer 1 was an adult female found dead on March 30, 2010, in Friesland Province. Roe deer 2 was a young female deer involved in a traffic accident on April 6, 2010, in Utrecht Province. The goat and human samples have been described [2]. Scale bar indicates genetic relatedness. Human 1, QKP 1; Human 2, QKP 2; NM, Nine Mile reference strain; MS, MiniSatellite.

Our study confirmed that *C. burnetii* infection occurs in freeliving roe deer in the Netherlands. *C. burnetii* DNA was detected in roe deer of both sexes and age groups with no particular health effect, and it was detected in animals in different provinces and in all years studied; the highest *C. burnetii* DNA loads occurred in spring and early summer. Detection of genetic material by PCR does not always imply viable infective bacteria [6]. However, because the infectious dose of *C. burnetii* is low [10], our findings support the use of preventive hygiene measures [4] to

minimize zoonotic risk when handling roe deer. The 2 MLVA-typed strains provided no evidence for spillover of the predominant strain involved in the Q fever epidemic in the Netherlands. More studies are required to adequately understand Q fever cycles in wildlife and their relationship with Q fever in domestic animals and humans.

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Chapter 6

Search for possible additional reservoirs for human Q fever in the Netherlands

Hendrik I.J. Roest[1]
Conny B. van Solt[1]
Jeroen J.H.C. Tilburg[2]
Corné H.W. Klaassen[2]
Emiel K. Hovius[3]
Frank T.F. Roest[4]
Piet Vellema[5]
René van den Brom[5]
Fred G. van Zijderveld[1]

[1]Central Veterinary Institute, part of Wageningen UR, Lelystad, The Netherlands
[2]Canisius Wilhelmina Hospital, Nijmegen, The Netherlands
[3]Companion Animal Hospital 't Heike, Veldhoven, The Netherlands
[4]Veterinary Practice Tusken Diken en Feanen, Drogeham, The Netherlands
[5]Animal Health Service, Deventer, The Netherlands

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Abstract

Possible additional reservoirs of *Coxiella burnetii* for human Q fever in the Netherlands were investigated. *C. burnetii* DNA was detected in canine, equine, ovine and bovine placentas, but not in feline, porcine and caprine placentas. The Dutch *C. burnetii* outbreak genotype was identified as well as a bovine specific genotype.

To the editor: Q fever is a zoonosis caused by the bacterium *Coxiella burnetii*. The Dutch Q fever outbreak, affecting > 4000 humans over the years 2007-2010, has highlighted Q fever as an important zoonosis. Epidemiological and molecular evidence indicate dairy goats and possibly dairy sheep as the source of the human outbreak [1,2]. Knowledge about the role of possible additional sources in the Netherlands is limited. Rats, deer and dairy cattle have tested positive for *C. burnetii* [3-5], while other potential sources of human Q fever, i.e. cats, dogs and horses [6-8], have not been tested yet.

The goal of this study was to search for possible additional reservoirs of *C. burnetii* in the Netherlands. Molecular characterization was performed to show a possible correlation between the obtained genotypes and the Dutch outbreak genotype.

Placentas from 15 cats, 54 dogs and 31 horses were collected in 2011 in a veterinary practice in the north of the Netherlands and in four practices in the south of the Netherlands. Placentas were collected via targeted sampling at breeding facilities and during parturition with veterinary assistance. In addition, 27 ovine, 11 caprine, 16 porcine, 8 equine and 139 bovine placentas, originating from aborting animals from all over the Netherlands and submitted in 2011 to investigate the abortion cause, were included in the study. Samples were stored at -20°C prior to testing. DNA was extracted from the allantochorion with incorporation of the basis of the cotyledon, using the NucliSens EasyMAG extraction system (Biomerieux, Boxtel, The Netherlands). Samples were analysed using a quantitative PCR targeting the *C. burnetii* specific multi copy IS1111a element as previously described [9]. From samples with a sufficient DNA load (Ct-value < 32), DNA was extracted again using a DNA tissue kit (DNeasy Blood and Tissue Kit; Qiagen, Hilden, Germany). Samples were typed using two Multi Locus Variable Number of Tandem Repeat Analyses (MLVA) genotyping methods (MLVA-12 and MLVA-6), and the Multispacer Sequence Typing (MST) method as published before [2,10,11]. *C. burnetii* strains from the Netherlands (X09003262, 801460-2, 3345937, 8014160-001, 18430) and the Nine Mile RSA 493, Henzerling RSA 331, Herzberg, Scurry, Schperling and CbB1 strain were included as reference. To assess if the Q fever outbreak in the south of the Netherlands influenced the prevalence of *C. burnetii* DNA in the placentas, the country was divided in a southern and a northern part. The southern part included the Q fever hot spot area of human and small ruminant notified cases during the 2007-2010 epidemic [1,12]. The northern part consisted of the rest of the country.

All placentas from cats (15), goats (11) and pigs (16) tested negative for *C. burnetii* DNA. In 4 out of 54 (7.4%) placentas from dogs, *C. burnetii* DNA was detected. Positive placentas were collected in both the northern and the southern part of the country. In 3 out of 39 (7.7%) equine placentas, *C. burnetii* DNA was detected. All 3 positive placentas came from the northern part of the country. In 33 out of 139 (23.7%) placentas from cattle, *C. burnetii* DNA was detected. The number of positive placentas from the northern and southern part of the country was not significantly different. Finally, 7 out of 27 ovine placentas (25.9%) tested positive for *C. burnetii* DNA. The number of *C. burnetii* DNA positive placentas from the northern and the southern part of the country was also not significantly different (Table 6.1).

The *C. burnetii* DNA load in the placentas from dogs and horses was too low to be suitable for typing. Typing of one positive sheep sample resulted in an incomplete genotype which seemed related to the outbreak genotype from the southern part of the Netherlands (sheep 192, Figure 6.1). From the 33 *C. burnetii* DNA positive placentas from cattle 7 were suitable for typing. Results

Table 6.1. Results of the detection of *C. burnetii* DNA in the placentas of the species included in the study.

Species of origin of the placenta	Number positive/ number included	Ct-value* range positive samples	Prevalence (%)	lower CI†	upper CI†	Additional information
Cat	0/15		0.0			
Goat	0/11		0.0			
Pig	0/16		0.0			
Dog	4/54	37.4-38.0	7.4	0.4	14.4	*C. burnetii* DNA positive placentas from northern and southern part of the Netherlands
Horse	3/39	35.4-37.4	7.7	0.0	16.1	All positive placentas from the northern part of the Netherlands
Cattle	33/139	14.3-38.5	23.7	16.7	30.8	No significant difference between northern and southern part of the Netherlands
Sheep	7/27	29.9-39.7	25.9	9.4	42.5	No significant difference between northern and southern part of the Netherlands

*Ct-value, cycle threshold-value of the quantitative PCR; †, CI: 95% confidence interval

showed 1 sample with a similar genotype as found earlier in goats and humans during the Q fever outbreak in the Netherlands [9,11]. Five other samples revealed the genotype that was previously found in cattle in the Netherlands, and one sample represented a novel but bovine related genotype. MLVA-6 and MST results were consistent with the MLVA-12 results. Figure 6.1 shows the genotyping results of the samples including the results of the reference strains and previously found genotypes [9].

Results give no indications for a major reservoir of *C. burnetii* in cats, goats and pigs in the Netherlands in 2011. For cats this is an unexpected result. Worldwide several cat-related Q fever outbreaks are reported and seroprevalences of 10% have been found among cats the Netherlands in 1992 [12], indicating that cats should be considered to play a role in Q fever infections. The absence of *C. burnetii*-positive feline placentas may possible be explained by the small number of cats included in this study. Although the number of goats included in this study is relatively low, the absence of *C. burnetii* DNA in goat placentas is probably the consequence of the effectiveness of the control measures to prevent shedding of *C. burnetii* during parturition among goats [12]. Although the susceptibility of swine to *C. burnetii* was demonstrated in Uruguay[13], we found no evidence for the presence of *C. burnetii* in swine in the Netherlands. No seroprevalence data in swine in the Netherlands are available to confirm this.

Dogs and horses can be considered as a reservoir for *C. burnetii*. The detection of *C. burnetii* DNA positive placentas in dogs and horses in the northern part of the country indicate the presence of a true reservoir rather than a spillover effect from the contaminated environment during the Q fever outbreaks in the southern part of the Netherlands. Dog related outbreaks have been reported before [7] and seroprevalence in dogs in the Netherlands have been reported at 13% in 1992 [12]. Till now, horses were once discussed as risk factor in the Dutch Q fever outbreak [15].

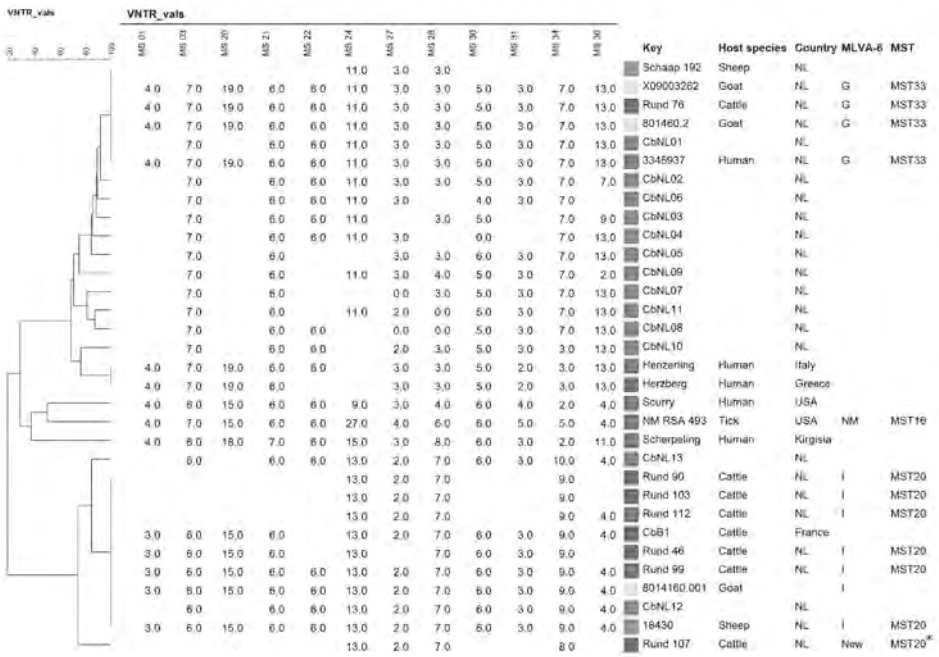

Figure 6.1. Phylogenetic tree including genotypes of *C. burnetii* of the samples from this study based on 12 multilocus variable-number tandem-repeat analyses (MLVA). Repeats per locus are shown, open spots indicate missing values. MLVA-6 results of the analysis with 6 MLVA loci [11]. MST results of the analysis with multispacer sequence typing [2]. Samples are compared with the published ruminant data indicated as CbNLxx [9] and data from *C. burnetii* reference strains (X09003262, 801460-2, 3345937, 8014160-001, 18430, Nine Mile (NM) RSA 493, Henzerling RSA 331, Herzberg, Scurry, Schperling and CbB1). NL, The Netherlands; G and I, MLVA-6 genotypes of *C. burnetii* as published earlier [11,14]; MSTxx, MST genotypes as published earlier [2]. *, based on partial genotype.

Prevalence data from sheep and cattle suggest that *C. burnetii* is present in placentas in one out of four abortion cases in these species. No indications could be found for a spill over during the Q fever outbreak in the southern part of the Netherlands. This indicates sheep and cattle as a

true reservoir for *C. burnetii.* Presence of *C. burnetii* infection in these species in the Netherlands has previously been observed [12]. This does not necessarily imply that these species pose a risk for humans. Genotyping data show a distinct genotype for 6 out of the 7 cattle samples. This may indicate circulation of a cattle specific genotype of *C. burnetii* [2,14], although the most predominant *C. burnetii* genotype in the Dutch Q fever outbreak has also been detected in one bovine and one ovine placenta.

In conclusion dogs, horses, sheep and cattle can serve as a reservoir for *C. burnetii* in the Netherlands. However, the importance of the obtained genotypes in these species for human infection needs to be further investigated.

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Chapter 7

Genotyping reveals the presence of a predominant genotype of *Coxiella burnetii* in consumer milk products

Jeroen J.H.C. Tilburg[a]
Hendrik Jan I.J. Roest[b]
Marrigje H. Nabuurs-Franssen[a]
Alphons M. Horrevorts[a]
Corné H.W. Klaassen[a]

[a]Department of Medical Microbiology and Infectious Diseases, Canisius Wilhelmina Hospital, Nijmegen, The Netherlands
[b]Department of Bacteriology and TSEs, Central Veterinary Institute, part of Wageningen UR, Lelystad, The Netherlands

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Abstract

Real-time PCR shows the widespread presence of *Coxiella burnetii* DNA in a broad range of commercially available milk and milk products. MLVA genotyping shows that this is the result of the presence of a predominant *C. burnetii* genotype in the dairy cattle population.

Q fever is a zoonosis caused by the pathogen *Coxiella burnetii*, which is prevalent throughout the world [1]. Ruminants (sheep, goats, and cattle) are often asymptomatic carriers of *C. burnetii* and are considered to be a source of infection to humans [1]. *C. burnetii* can cause abortion in small ruminants such as sheep and goats and may cause reproductive disorders in cattle [2]. Huge numbers of *C. burnetii* can be released into the environment via birth products [3]. Lower numbers are usually shed in milk, even in asymptomatic herds [4-9]. Although consumption of raw or insufficiently pasteurized milk is very rarely identified as a source of Q fever infection, asymptomatic cattle herds can be considered potential *C. burnetii* reservoirs capable of transmitting the disease to humans.

We applied real-time PCR, targeting the multicopy IS*1111a* insertion element of *C. burnetii* as described earlier [10], and a 6-locus multiple-locus variable number tandem repeat analysis (MLVA) panel [11] to a broad range of milk and milk products with the aim to determine the prevalence and genotypes of *C. burnetii* in milk (Table 7.1). The study included commercially available semi-skimmed milk samples from cows (obtained from large supermarket chains) and milk products, such as coffee creamer, obtained throughout Europe and from an additional 10 non-European countries. Samples were collected from different brands, and according to the information on the packages they were produced by the (local) dairy industry in these countries. The origin of the milk samples from Egypt, Saudi-Arabia, and Qatar could not be identified.

Eighty-eight out of 116 (76%) milk samples or milk products from 28 countries contain significant amounts of *C. burnetii* DNA (Table 7.1). No *C. burnetii* DNA was detected in milk obtained from Finland, Norway, Costa Rica, and New Zealand. MLVA genotypes I to O were identified in samples from France, Germany, The Netherlands, Portugal, Slovak Republic, Spain, Switzerland, United Kingdom, Qatar, and Saudi Arabia. MLVA genotypes P, Q, and R were identified in samples from Slovak Republic, Qatar, and Russia, respectively. A partial MLVA genotype (Table 7.1, "Part") was obtained from samples that contained insufficient DNA to obtain a full profile. In 4 samples from Slovak Republic, we observed more than one allele per locus, suggesting the presence of at least two or more different genotypes in these samples (Table 7.1). Clustering of the MLVA genotypes using the minimum spanning tree method showed a high degree of genetic similarity between the MLVA genotypes I to O (Figure 7.1). These MLVA genotypes are interconnected by repeat number changes in only one of the six markers and may represent microvariants of one founder genotype. In contrast, MLVA genotypes P and R and the genotypes of five sequenced *C. burnetii* strains all differed in at least 3 markers from the MLVA genotypes I to O.

The MLVA genotypes were compared to an in-house database containing 57 different *C. burnetii* MLVA genotypes from 197 human, caprine, ovine, and cattle clinical samples obtained from Canada, France, Germany, The Netherlands, Portugal, Spain, and the United States. MLVA genotypes I and J have also been recognized incidentally in 8 human clinical samples (placenta and heart valve) from France and in 2 animal samples (cattle and goats) from The Netherlands. However, very different MLVA genotypes (A to H) were identified in human, ovine, and caprine clinical samples from the Q fever outbreak in The Netherlands using a 6-locus and 10-locus MLVA panel [11,12], indicating that the Dutch Q fever outbreak is not related to the presence of *C. burnetii* in cattle.

The presence of highly similar *C. burnetii* genotypes in consumer milk products may indicate a widespread dissemination of a specific cattle-adapted strain. Alternatively, this genotype may have been introduced into different countries by transport of asymptomatic *C. burnetii*-positive cattle, as well as by export of milk and milk products from a restricted number of countries to other countries (e.g., Egypt, Saudi Arabia, Qatar) by the dairy industry. By testing bulk milk products instead of milk from individual animals, any positive milk specimen is likely to be diluted

Table 7.1. Prevalence of *C. burnetii* DNA in commercially available bulk tank cow milk and milk products from 18 countries throughout Europe and from 10 non-European countries[a].

Geographical source	Origin	CT value	No. of PCR pos./ total no. of samples tested	No. of samples with the same MLVA genotype	No. of repeats						MLVA type
					Ms23	Ms24	Ms27	Ms28	Ms33	Ms34	
Austria	Semi-skimmed milk	37.4	1/1	1	6	-	2	7	4	-	Part
Belgium	Semi-skimmed milk	33.6	1/1	1	6	13	-	7	4	10	Part
Croatia	Semi-skimmed milk	34.1-35.2	4/4	4	-	9	-	-	-	-	Part
Denmark	Semi-skimmed milk	34.5	1/1	1	6	13	-	-	5	-	Part
Finland	Semi-skimmed milk	-	0/1								
France	Semi-skimmed milk	30.4-34.1	6/6	1	6	13	2	7	4	9	I
				2	6	13	2	7	4	10	J
				1	6	-	2	5	-	10	Part
				1	6	-	2	7	4	-	Part
				1	6	-	-	-	4	9	Part
Germany	Low fat and semi-skimmed milk	32.0-37.3	6/6	1	6	13	2	7	4	-	Part
				1	6	13	2	6	4	10	K
				1	-	-	2	7	-	10	Part
				1	6	13	-	-	4	9	Part
				2	-	-	-	-	-	-	-
Ireland	Semi-skimmed milk	32.5	1/1	1	-	11	-	-	-	9	Part
Italy	Semi-skimmed milk	33.2	1/1	1	6	-	-	7	4	11	Part
Netherlands	Low fat and semi-skimmed milk, coffee creamer and milk powder	31.5-41.2	16 / 27	1	6	13	2	7	4	9	I
				1	6	13	2	-	4	9	Part
				1	6	13	-	7	4	9	Part
				2	6	13	2	7	4	10	J
				1	-	-	2	7	-	7	Part
				10	-	-	-	-	-	-	-
Norway	Semi-skimmed milk	-	0 / 2								
Poland	Semi-skimmed milk	35.2	1/1	1	-	13	-	-	-	-	Part
Portugal	Semi-skimmed milk	31.5-36.8	10/12	2	6	13	2	7	4	9	I
				1	6	13	2	7	4	-	Part
				1	5	13	2	-	4	-	Part
				1	6	13	-	-	4	13	Part
				1	6	-	-	7	4	9	Part
				4	-	13	2	-	-	9	Part
Slovak Republic	Semi-skimmed milk	33.0-35.5	11/11	1	6	13	2	7	5	10	L
				1	6	12	4	7	4	5	P

Geographical source	Origin	CT value	No. of PCR pos./ total no. of samples tested	No. of samples with the same MLVA genotype	No. of repeats						MLVA type
					Ms23	Ms24	Ms27	Ms28	Ms33	Ms34	
				1	9	-	4	4	-	5	Part
				1	5	13	-	-	4	-	Part
				1	6	-	-	-	4	-	Part
				2	-	-	-	-	-	-	-
				1	4/6	7	3	3/6	4	3/5	Mix
				1	4	27	3/4	3/6	4	3/5	Mix
				1	-	13	2/4	7	4	10/11	Mix
				1	6/9	8/13	-	3/6	4	3	Mix
Spain	Low fat and semi-skimmed milk	31.9-35.6	7/7	2	6	13	2	7	4	9	I
				2	6	13	2	7	4	10	J
				1	6	13	2	7	4	11	M
				1	5	13	2	7	4	9	N
				1	5	-	2	8	4	-	Part
Switzerland	Semi-skimmed milk	33.3-37.7	6/6	1	6	13	2	7	4	9	I
				1	5	13	2	7	4	-	Part
				1	5	-	-	7	4	9	Part
				3	-	-	-	-	-	-	-
Sweden	Semi-skimmed milk	36.2	1/1	1	-	-	-	2	-	-	Part
United Kingdom	Semi-skimmed milk	31.6	1/1	1	6	13	2	7	5	9	O
Australia	Semi-skimmed milk and coffee creamer	33.9-36.7	4/6	1	-	1	-	5	-	-	Part
				1	-	18	-	-	-	-	Part
				2	-	-	-	-	-	-	-
Canada	Semi-skimmed milk	32.7	1/1	1	6	13	-	7	4	11	Part
Costa Rica	Milk powder	-	0/2								
Cuba	Semi-skimmed milk	38.1	1/2	1	-	-	-	-	-	-	-
Egypt	Semi-skimmed milk	34.1-36.0	2/2	2	5	13	-	-	4	-	Part
India	Coffee creamer incl. milk powder	34.8-37.3	2/3	1	6	-	-	-	5	-	Part
				1	-	-	-	-	-	-	-
New Zealand	Semi-skimmed (powder) milk	-	0/5								
Qatar	Semi-skimmed milk	30.3-32.6	2/2	1	6	12	4	5	4	2	Q
				1	6	13	2	7	4	10	J
Russia	Semi-skimmed (powder) milk	33.3	1/2	1	4	14	2	6	3	11	R
Saudi Arabia	Semi-skimmed milk	33.5	1/1	1	6	13	2	7	4	10	J
C. burnetii Dugway	DNA				?	5	4	4	3	3	
C. burnetii RSA331					4	7	3	3	-1b	3	
C. burnetii					9	27	4	6	4	5	
C. burnetii CbuG_ Q212					?	8	3	4	2	2	
C. burnetii CbuK_ Q154					?	9	4	5	2	2	
Total			*88 / 116 (76 %)*								

[a] The number of repeats in each marker was determined by extrapolation using the sizes of the obtained fragments relative to those obtained using DNA from the Nine Mile strain. Furthermore, the genotypes of four additional *C. burnetii* strains, i.e., Dugway (Genbank accession number CP000733), RSA331 (CP000890), CbuG Q212 (CP001019) and CbuK Q154 (CP001020) were determined *in silico* using published sequences; -, no result obtained; Part, partial genotype; Mix, 2 or more genotypes; ?, the number of repeats could not be determined due to apparent sequence assembly errors.

[b] In Silico analysis resulted in a 5-repeat number difference compared to the NM strain, which by convention was assigned 4 repeats.

with negative milk specimens, leading to an average lower DNA concentration, resulting in higher threshold cycle (*CT*) values as well as partial genotypes.

This is the first report of genotypic diversity among *C. burnetii* from cow milk throughout Europe and beyond. Integration of such data in international databases can be instrumental to understand the global epidemiology of Q fever in animals.

In conclusion, this study demonstrated the presence of *C. burnetii* DNA in a broad range of commercially available cow milk and milk products, indicating a high prevalence of *C. burnetii* among the dairy cattle population worldwide and a possible clonal spread of *C. burnetii* among the European dairy cattle population. In addition, since this dominant genotype is only incidentally found in humans, the risk of obtaining Q fever via exposure to infected cattle may be much lower than via exposure to infected small ruminants. The incidental observation of mixed alleles does not exclude the possibility of the presence of other minority genotypes in cattle that may be relevant to humans after all.

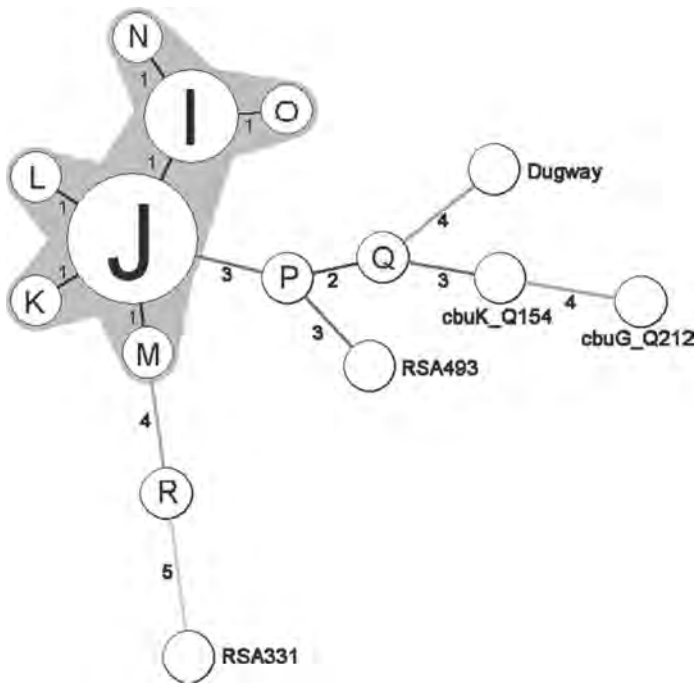

Figure 7.1. Minimum spanning tree showing the relationship between the obtained MLVA genotypes identified in this study and five sequenced *C. burnetii* strains, i.e., Dugway (GenBank accession number CP000733), RSA331 (CP000890), Nine Mile RSA493 (AE016828), CbuG Q212 (CP001019), and CbuK Q154 (CP001020). Each circle represents a unique genotype, and the size of the circle corresponds to the number of samples with that genotype. Only full MLVA genotypes were included in this analysis. Branch labels and connecting lines correspond to the number of different markers between the genotypes. Genotypes connected by a grey background differ in only one marker from each other and may represent microvariants of one founder genotype.

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Chapter 8

Q fever in pregnant goats: pathogenesis and excretion of *Coxiella burnetii*

Hendrik-Jan Roest[1]
Betty van Gelderen[1]
Annemieke Dinkla[1]
Dimitrios Frangoulidis[2]
Fred van Zijderveld[1]
Johanna Rebel[3]
Lucien van Keulen[3]

[1] Department of Bacteriology and TSEs, Central Veterinary Institute, part of Wageningen University and Research Centre, Lelystad, the Netherlands
[2] Bundeswehr Institute of Microbiology, Munich, Germany
[3] Department of Infection Biology, Central Veterinary Institute, part of Wageningen University and Research Centre, Lelystad, the Netherlands

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Abstract

Coxiella burnetii is an intracellular bacterial pathogen that causes Q fever. Infected pregnant goats are a major source of human infection. However, the tissue dissemination and excretion pathway of the pathogen in goats are still poorly understood. To better understand Q fever pathogenesis, we inoculated groups of pregnant goats via the intranasal route with a recent Dutch outbreak *C. burnetii* isolate. Tissue dissemination and excretion of the pathogen were followed for up to 95 days after parturition. Goats were successfully infected via the intranasal route. PCR and immunohistochemistry showed strong tropism of *C. burnetii* towards the placenta at two to four weeks after inoculation. Bacterial replication seemed to occur predominantly in the trophoblasts of the placenta and not in other organs of goats and kids. The amount of *C. burnetii* DNA in the organs of goats and kids increased towards parturition. After parturition it decreased to undetectable levels: after 81 days post-parturition in goats and after 28 days post-parturition in kids. Infected goats gave birth to live or dead kids. High numbers of *C. burnetii* were excreted during abortion, but also during parturition of liveborn kids. *C. burnetii* was not detected in faeces or vaginal mucus before parturition. Our results are the first to demonstrate that pregnant goats can be infected via the intranasal route. *C. burnetii* has a strong tropism for the trophoblasts of the placenta and is not excreted before parturition; pathogen excretion occurs during birth of dead as well as healthy animals. Besides abortions, normal deliveries in *C. burnetii*-infected goats should be considered as a major zoonotic risk for Q fever in humans.

Introduction

Coxiella burnetii is a Gram-negative intracellular bacterium and the causative agent of Q fever. *C. burnetii* can affect a wide range of hosts, including humans, ruminants, companion animals, birds and reptiles [1]. *C. burnetii* is present throughout the world, with the exception of New Zealand [2]. The bacterium is considered a biothreat agent in view of its very low infectious dose and high transmissibility [3]. Clinical infection in humans manifests as atypical pneumonia, hepatitis or flu-like self-limiting disease. Persistent infection may result in life-threatening endocarditis. The main infection route is via inhalation of *C. burnetii*-contaminated aerosols from the environment [4]. Excretion of *C. burnetii* by domestic ruminants is considered the source of environmental contamination and the cause of human infection [5,6,7]. The zoonotic impact of Q fever was recently underlined by the Dutch Q fever outbreak involving >4000 registered human cases in regions with high frequencies of Q fever abortions in dairy goats [8,9]. Molecular typing of *C. burnetii* isolates confirmed the epidemiological link between human and animal infections [10,11,12].

A key factor in the zoonotic transmission of the Q fever agent is the excretion of the pathogen by the animal host. Animals are assumed to become infected by inhalation or oral uptake of *C. burnetii* from the environment. *C. burnetii* infection in animals is generally asymptomatic. In pregnant animals, however, *C. burnetii* infection can become symptomatic. Metritis, abortion, stillbirth and delivery of weak offspring are the most frequent clinical signs of disease [13]. In symptomatic individuals *C. burnetii* is excreted via faeces, vaginal mucus, milk and birth products. In pregnant goats the clinical manifestation of *C. burnetii* infection is abortion and stillbirth in the final stage of gestation. Although transmission from goats to humans is assumed to occur after abortion resulting from Q fever infection, field studies suggest that *C. burnetii* may also be excreted via the placenta during normal parturition [14,15,16,17].

The excretion of *C. burnetii* in infected pregnant goats via faeces, vaginal mucus and milk is poorly understood. Excretion via these routes was confirmed in an experimental setup [18], but field observations revealed a poor correlation between the routes [17]. Excretion via faeces, vaginal mucus and milk suggest that the pathogen is disseminated towards different bodily fluids during infection. Evidence for this is limited. After subcutaneous inoculation of pregnant goats, *C. burnetii* DNA was shown to be present in the mammary glands, uterus, liver, spleen and lungs at one or both of the two investigated points in time between inoculation and abortion [19]. Whether the presence of *C. burnetii* resulted in its excretion was not investigated. The influence of the infection route on dissemination and excretion of the pathogen has not been investigated in goats either.

The goal of the present study was to systematically monitor the dissemination and excretion of *C. burnetii* in pregnant goats before and after parturition following inoculation of the agent via a natural inoculation route. Bacterial dissemination and excretion and the development of pathology were followed using a combination of microbial and DNA detection techniques, post-mortem examination and immunohistochemistry. Our results indicate that the placenta is the primary target organ and infection source of *C. burnetii* in pregnant goats. *C. burnetii* is not excreted before parturition and excretion can occur both during abortion as well as during delivery of healthy newborns.

Materials and methods

Ethics statement

All animal experiments were approved by the Animal Experiment Commission of the Central Veterinary Institute, part of Wageningen UR, in accordance with the Dutch regulations on animal experimentation (registration numbers 2009082.c, 2009079.a, 2010098.d and 2011111.c). Everything possible was done to minimise animal suffering. Humane endpoints were defined in advance. Whenever these endpoints were reached, animals were euthanised.

Inoculum

C. burnetii strain X09003262-001 was isolated from a placenta of one of the 25% of the goats that aborted on a farm during the Q fever outbreak in the Netherlands (farm N, [11]). The presence of *C. burnetii* in the placenta was confirmed by immunohistochemical (IHC) staining [20] and *C. burnetii*-specific quantitative polymerase chain reaction (qPCR) [11]. The strain was genotyped as CbNL01, the predominant *C. burnetii* genotype in the Dutch Q fever outbreak [9,12]. *C. burnetii* was isolated by crushing a part of the placenta from the allantochorion and base of the cotyledon in a ribolyser (FastPrep-24, MP Biomedicals (USA), lysing matrix D with ¼ ceramic beads, 2 times 20 sec with 6 m/sec2 and a 5 min break in between). This lysate was filtered stepwise using a cell strainer (Nunc, Denmark) and filters with pore sizes of 1.2 µm and 0.45 µm (Pall Cooperation, USA). Filtered material was inoculated onto a culture of Buffalo Green Monkey (BGM) cells (European Collection of Cell Cultures) with culture medium without antibiotics (EMEM with 10% bovine serum albumin, 1% NEAA, 1% glutamax) and incubated for 14 days at 37°C in a closed flask. Culture medium was refreshed twice a week. Growth of *C. burnetii* was monitored by vacuolisation of the BGM cells and confirmed by an immunofluorescence assay using a *C. burnetii*-specific monoclonal antibody (MAB313-oregon green, Squarix) and PCR. Cell culture was confirmed negative for *Chlamydia abortus* by qPCR targeting the OmpA gene (forward primer 5'-CTCCTTACAAGCCTTGCCTGTAG-3'; reverse primer 5'-CCTGAAGCACCTTCCCACAT-3'; probe: FAM labelled 5'-CCAGCTGAACCAAGTTTATTAATCGATGGCA-3'), *Simkania negevensis* (CVI in-house PCR: forward primer 5'-GTTACGAGCCTGGCGATGCCA-3'; reverse primer 5'-AAAGTTGCTTGGCTGCGCGG-3') and mycoplasma (forward primer 5'- GGGAGCAAACAGGATTAGATACCCT-3'; reverse primer 5'-TGCACCATCTGTCACTCTGTTAACCTC-3). A large batch of strain X09003262-001 was prepared as follows. The infected monolayer from the primary isolation was frozen at -80 °C for 30 min, thawed and then monolayer cell line debris was removed by centrifugation (10 min, 100 x *g*). The supernatant was added to BGM cells. After confirmed growth, the supernatant was collected and cell debris was removed as described above. The supernatant was stored in aliquots of 1 ml at -80 °C. The mouse infective dose (MID) of the batch was determined as described by Arricau-Bouvery *et al* [18]. In brief, four mice (Swiss random OF1, 2 month of age) were inoculated intraperitoneally with decimal dilutions of *C. burnetii* strain X09003262-001. After 9 days the mice were euthanised and *C. burnetii* was detected in the spleen by PCR. The MID was defined as the maximal decimal dilution that infected all mice in the group. Before inoculation, the inoculum was adjusted to the required MID by dilution with culture medium.

Animal experiments

Experiment I

Twelve healthy, pregnant, serologically *C. burnetii* negative Dutch dairy goats were purchased from a Dutch dairy goat farm without a known history of Q fever. All goats tested negative for antibodies against *C. burnetii* (LSIVET RUMINANT milk/serum Q-fever ELISA kit, LSI, France) and vaginal mucus tested negative for *C. burnetii* DNA and for *Chlamydia abortus* DNA by PCRs on the day of arrival. Two goats served as negative controls and were housed in animal biosafety level (aBSL)2 facilities. Five groups of two goats were housed in aBSL3 facilities. Each group of two goats was inoculated on day 90 of gestation, either orally with 10^4 or 10^6 MID, intranasally with 10^4 or 10^6 MID, or subcutaneously with 10^4 MID. Intranasal inoculation was performed during forced inhalation with a nozzle in the left nostril with the right nostril being held closed. Subcutaneous inoculation was performed just in front of the shoulder. Goats were monitored daily for general health via clinical inspection with an emphasis on behaviour, appetite and consistency of the faeces. Goats were considered infected with *C. burnetii* when *C. burnetii*-confirmed abortion occurred or the placenta was tested positive for *C. burnetii* by PCR.

Experiment II

Twenty-seven healthy pregnant serologically *C. burnetii* negative Alpine yearling goats were purchased from INRA (Institut National de la Recherche Agronomique, Domaine de Galle), France. Pregnancy and the duration of pregnancy were confirmed by echography. All goats tested serologically negative for antibodies against *C. burnetii* (LSIVET RUMINANT milk/serum Q-fever ELISA kit, LSI, France) and *Chlamydia abortus* (Chekit Chlamydophila abortus antibody test kit, IDEXX Laboratories B.V., the Netherlands) on the day of arrival. Nine negative control goats were housed in aBSL2 facilities. Two groups of eight goats (Groups A and B) were separately housed in two aBSL3 facilities. On day 76 of pregnancy, the 16 goats of Groups A and B were intranasally inoculated with 1 ml of culture medium containing 10^6 MID of *C. burnetii,* while the nine negative control animals were intranasally inoculated with 1 ml of culture medium. General health was monitored by rectal temperature and daily clinical inspection with an emphasis on behaviour, appetite and consistency of the faeces.

Sampling

Dates of sampling of biological specimens are indicated as days post inoculation (dpi). Sampling dates after parturition are indicated as days post parturition (dpp). Every 14 days (until 69 dpi) two *Coxiella*-inoculated goats and one control goat in the same stage of pregnancy were euthanised for necropsy. Goats were euthanised at one week and one, two and three months after parturition for necropsy i.e. (depending on their exact parturition date) at 77, 98, 119, 126, 140 and 141 dpi (9, 32, 57, 60, 81 and 95 dpp) for the *Coxiella*-inoculated goats, and at 84, 111 and 140 dpi (9, 32 and 63 dpp) for the control goats. Kids from the *Coxiella*-inoculated goats were euthanised on the day of parturition for necropsy; kids from the control goats were euthanised together with their does. Goats and kids were euthanised by exsanguination

following intravenous injection of sodium pentobarbital. Two tissue samples from the goat's respiratory tract (lymph nodes, mucosa, tonsils, bronchia and lung), genital tract (lymph nodes, udder, ovarium, non-placental uterus, placentome, caruncle, uterus mucus and vaginal mucus), haematopoietic system (spleen, thymus, bone marrow, blood), liver (lymph nodes, parenchyma and bile), urinary tract (kidney, bladder and urine), alimentary tract (lymph nodes, ileum and colon) and heart, glandula parotis and perirenal fat were collected with sterilised instruments. Samples were likewise connected from the kid's spleen, liver, kidney, lung and heart. One sample was stored at -20 ˚C for PCR analysis and the other was fixed in 10% phosphate buffered formalin for histopathology. For each tissue sample a new set of sterilised instruments was used to prevent cross-contamination with *C. burnetii*. Tissues with expected high numbers of *C. burnetii* (e.g. placenta) were handled at the end of the necropsy. At 119, 126, 140 and 141 dpi vaginal mucus swabs were taken from the goats via the abdomen. For this an incision was made through the peritoneum and perineum into the vagina.

Rectal faecal samples and vaginal mucus samples, obtained via the vulva, were taken aseptically from all goats starting on day 0 just before inoculation and at weekly intervals until 28 dpi, every 14 days until 56 dpi, weekly until the end of the experiment and on the day of the parturition. Jugular EDTA blood was taken on day 0 before inoculation, at 1, 2, 3, 5, 7, 9, 10, 12, 14, 16, 21, 28, 42 dpi, then at weekly intervals until the end of the experiment, and at 0, 1, 2, 4, 7, 14 and 21 dpp. After parturition, challenged goats were machine milked once a day. The control goats were not machine milked as they nursed their kids. Milk samples were taken weekly and aseptically from the day of parturition until the end of the experiment for PCR analysis.

Environmental samples were taken by swabbing clean and used wood shavings and slip mats, by sampling water and air using swabs and filters (MD 8 airscan Air Sampler, Sartorius, Germany) at 119, 126 and 140 dpi. Samples were analysed with PCR.

Experiment III

The origin and health status of the goats and the setup of this experiment were similar to that described for Experiment II except that six negative control goats and ten goats for intranasal inoculation (106 MID of *C. burnetii*) were included (Groups C and D). Goats were euthanised at 91 dpi (negative controls), at 94 dpi (Group C) or at 98 dpi (Group D). After parturition placental tissue was preserved for histopathology and vaginal mucus was collected for PCR analysis. Weak-born kids were euthanised for ethical reasons, when appropriate. Liveborn kids were kept together with their does until the end of the experiment, when they were euthanised. Kids were necropsied after death. Spleen, liver, kidney, lung and heart were collected as described in Experiment II. The air in the box of Group C was sampled by 8 min filtering at weekly intervals from 14 dpi until 84 dpi (except at 63 dpi). Samples were analysed with PCR.

Histopathology

For histology and immunohistochemistry (IHC) tissues were fixed, dehydrated, cleared, and processed into paraffin blocks. Sections of 4 µm were cut and collected on silane-coated glass

slides. Sections were routinely stained with haematoxylin/eosin and immunostained for the presence of *C. burnetii* antigen. For immunostaining, sections were first autoclaved in citrate buffer (pH 6) for 15 min and then incubated (60 min) with a *C. burnetii*-specific monoclonal antibody (MAB313, Squarix, 1/100 dilution in phosphate buffered saline containing 1% bovine serum albumin and 1% normal goat cotyledon). After washing, sections were incubated with HRP-conjugated EnvisionTM anti-Mouse-Ig and colour was developed during 5 min with DAB+chromogen (Dakopatts, Denmark). Sections were counterstained with haematoxylin for 30 sec, dehydrated and mounted in Eukitt (Kindler, Germany).

PCR for detection of Coxiella burnetii

DNA was extracted from tissues (20 mg), faeces (20 mg), vaginal mucus swabs (swab tip), EDTA blood (200 µL), milk (200 µL) and environmental samples (swab tip, filter part the size of a swab tip) using a DNA tissue kit (DNeasy Blood&Tissue Kit, Qiagen, the Netherlands) according to the manufacturer's instructions. All samples were subjected to a quantitative PCR (qPCR) targeting a single copy gene encoding a *C. burnetii*-specific hypothetical protein (gene bank number AY502846) using the forward primer 5'-ATAGCGCCAATCGAAATGGT-3', the reverse primer 5'-CTTGAATACCCATCCCGAAGTC-3', and the NED-labelled probe 5'-CCCAGTAGGGCAGAAGACGTTCCCC-3'. An inhibition control (IC) was constructed using primers for the IS1111a element and a dedicated VIC-labelled probe, as previously published [11]. PCR was performed on a 7500 Fast Real Time PCR system (Applied Biosystems, USA), using 400 nmol/L of primers and 200 nmol/L of probes in 7 µL PerfeCTa Multiplex qPCR Super mix, UNG (2X) with Low Rox dye (Quanta Biosciences, USA), 1 µL of IC, 5 µL of sample and 7 µL of water. An initial UDG incubation for 5 min at 45˚C and denaturation/activation for 60 sec at 95˚C was followed by 50 cycles of denaturation for 10 sec at 95˚C and annealing for 30 sec at 60˚C. The detection limit of the PCR was determined at 10 copies for faecal, vaginal mucus, milk and environmental samples and at 100 copies for EDTA blood and tissue samples. For tissues, PCR results were scored negative (-) when the generated PCR cycle threshold (Ct) value was 40 or more; positive (+) for Ct values between 30 and 40, (++) for Ct values between 20 and 30, and (+++) for Ct values below 20. For faecal, vaginal mucus, milk and environmental samples a negative result (Ct value of 40 or more) was scored as Ct 40, a positive result (Ct < 40) scored by the generated Ct value.

Results

Comparison of Coxiella burnetii *infection routes in goats*

To investigate natural routes of *C. burnetii* infection in goats, we compared the effectiveness of intranasal and oral routes with the subcutaneous inoculation route in pregnant Dutch dairy goats. Two goats per dose-route combination were given either an oral or intranasal dose of 10^4 or 10^6 MID, or a subcutaneous dose of 10^4 MID. The infection outcome was monitored by the occurrence of abortion and testing the placenta for the presence of *C. burnetii* DNA using PCR.

At the start of the experiment, several of the goats were in an obese condition. The goats had a poor appetite, probably due to adaptation problems from the herd diet to the diet fed in the experimental facilities. We observed three early abortions, one in the subcutaneously inoculated group (abortion at 12 dpi; placenta *C. burnetii* negative), one in the 10^6 orally challenged group (abortion at 13 dpi; placenta *C. burnetii* negative) and one in the 10^4 orally challenged group (abortion at 21 dpi; placenta *C. burnetii* positive). Two goats had to be euthanised before the end of the experiment for ethical reasons: one in the control group (at 41 dpi) and one in the 104 orally challenged group (at 62 dpi). In the intranasally inoculated group three abortions were observed and the placentas were strongly positive for *C. burnetii* DNA. In the orally inoculated group one goat gave birth to two kids and no *C. burnetii* DNA could be detected in the placenta, and one goat was euthanised at the end of pregnancy and low amounts of *C. burnetii* DNA were detected in the placenta (Ct value between 30 and 40). The results are detailed in Table 8.1. Overall, inoculation via the nasal route was most successful. It resulted in abortion at earlier time points after inoculation and a more heavily infected placenta as compared to infection via the oral and subcutaneous routes.

Intranasal infection and pregnancy outcome

In subsequent challenge experiments (II and III) we used first-parity pregnant goats raised in experimental facilities rather than Dutch goats raised in a commercial dairy farm to limit the problems associated with the adaptation of the goats to the experimental facilities. Goats were inoculated on day 76 of pregnancy via the intranasal route with 10^6 MID of the Dutch outbreak *C. burnetii* strain. Control goats were intranasally inoculated on day 76 of pregnancy with culture medium. In both experiments animals did not show any clinical signs of disease except for the occurrence of abortions in the *Coxiella*-inoculated groups. The goats' appetite was good from the start and no deviation from the normal rectal temperature of goats (38.5-40.0 °C) was measured for more than one day in either the control group or the *Coxiella*-inoculated group.

Table 8.1. Results of the comparison of the subcutaneous, oral and nasal infection route in pregnant goats with two doses of *Coxiella burnetii*.

Inoculation route	dose (MID)	goat	pregnancy outcome	dpi	PCR results placenta
None	none	1	euthanasia	41	-
		2	kidding	63	-
Oral	10^4	1	abortion	21	+
		2	euthanasia	62	+
Oral	10^6	1	abortion	13	-
		2	kidding	64	-
Nasal	10^4	1	abortion	48	+++
		2	mummification	88	+
Nasal	10^6	1	abortion	53	+++
		2	abortion	54	+++
Subcutaneous	10^4	1	abortion	12	-
		2	1 dead/1 alive	61	+++

MID: mouse infective dose; dpi: days post inoculum, "-": negative result, "+": 30 < PCR cycle threshold (Ct) < 40, "++": 20 < Ct < 30, "+++": Ct < 20.

In Experiment II, seven *Coxiella*-inoculated goats gave birth. *C. burnetii* was detected by PCR and IHC in all placentas, indicating successful intranasal inoculation in all animals. Three goats aborted at 46, 60 and 63 dpi, respectively. Aborted kids showed no gross abnormalities, although some slight autolysis was observed. This suggests that death occurred shortly before or during abortion. Four goats delivered liveborn kids; two goats delivered one weak kid each at 66 dpi, and two goats delivered a single healthy kid each at 68 and 69 dpi (Table 8.2). Four control goats delivered liveborn kids between 75 and 79 dpi, which equals a gestation of 150 to 154 days. This is a normal gestation period for goats. In Experiment III, pregnancy outcomes showed similar characteristics (Table 8.3). Again *C. burnetii* was detected in the placentas of all (ten) goats by IHC and by PCR in the vaginal mucus just after parturition. Three goats aborted (one single kid and two twins) at 52, 59 and 70 dpi; one goat delivered one stillborn and one liveborn weak kid at 69 dpi; two goats delivered one stillborn and one liveborn healthy kid each at 67 and 68 dpi; one goat delivered two weak kids at 62 dpi and three goats delivered healthy kids, all singles at 66, 67 and 68 dpi. The six control goats delivered healthy liveborn kids between 73 and 81 dpi, which equals 149 to 157 days of gestation. Together these results confirm the efficiency of the intranasal inoculation route and indicate abortion as a major manifestation of *C. burnetii* infection. However, the finding that sometimes both healthy and dead kids are delivered by the same doe indicates that the infection is not always lethal for the offspring.

Table 8.2. Pregnancy outcome and *C. burnetii*-specific PCR results using DNA isolated from the indicated tissues taken at necropsy of the kids in Experiment II. Results are measurements at the day of necropsy indicated by days post inoculation and days of gestation. Liveborn kids were euthanised on the day of parturition for necropsy. No significant difference between aborted and liveborn kids was observed.

Days post inoculation	14		28		42		46	56		60	63	66	66	68	69	69
Days of gestation	89		103		117		121	131		135	138	141	141	143	144	144
Pregnancy outcome	nec		nec		nec		abo	nec		abo	abo	kid	kid	kid	nec	kid
Number of kids	1	1	2	2	1	3*	2	2	1	2	2	1	1	1	1	1
Goat ID	25	26	27	28	31	32	36	29	30	35	40	33	38	37	34	39
Tissue																
Foetus 1																
Foetal membrane	-	-	-	-	+++	++	+++	++	+++	+++	++	+++	++	+++	+++	+++
Placentome/cotyledon	-	-	+	++	+++	+++		+	++	+++	+++	+++	+++	+++	+++	+++
Amniotic fluid	-	-	-	-	+	+		-	+						+	
Allantoic fluid	-	-	-	-	+++	+		+	-						++	
Foetal blood	-	-	-	-	++	+	-	-	-	-	-	-	-	-	+++	-
Spleen	-	-	-	-	+	+	+	+	-	+	+	-	-	+	+	++
Liver	-	-	-	-	+	++	+	-	+	+	+	-	+	+	++	++
Kidney	-	-	-	-	+	+	+	+	+	+	-	-	+	+	+	+
Lung	-	-	-	-	+	++	-	+	+	+	-	-	+	+	+	++
Heart	-	-	-	-	++	++	-	-	+	+	-	+	-	+	++	+
Foetus 2																
Foetal membrane			-	+++		-		+		+++	+++					
Placentome/cotyledon			+	++		++		+		+++	+++					
Amniotic fluid			-	-		+		-								
Allantoic fluid			-	++		+		+								
Foetal blood			-	-		+	-	-		+	++					
Spleen			-	-		+	+	-		++	+					
Liver			-	-		++	+	-		++	+					
Kidney			-	-		+	-	-		+	++					
Lung			-	-		+	+	-		++	+					
Heart			-	-		++	+	-		+	++					

nec: necropsy before kidding, abo: abortion, kid: kidding (liveborn kids), "-": negative result, "+": 30 < PCR cycle threshold (Ct) < 40, "++": 20 < Ct < 30, "+++": Ct < 20, blank: no sample available, * = results of 2 kids shown, third one like other 2.

Dissemination of Coxiella burnetii in intranasally inoculated goats as detected with PCR

To study the spread of *C. burnetii* in the goats after intranasal inoculation, two *Coxiella*-inoculated goats and one control goat were killed each 14th day until 69 dpi in Experiment II. Thereafter, one *Coxiella*-inoculated goat was killed at 77, 98, 119, 126, 140 and 141 dpi and one control goat was killed at 84, 111 and 140 dpi. Tissues of the respiratory tract, genital tract, haematopoietic system, liver, urinary tract, alimentary tract and from the heart, glandula parotis and perirenal fat were sampled for PCR analysis.

Table 8.3. Results of *C. burnetii*-specific PCR on DNA isolated from the indicated tissues taken at necropsy of the kids in Experiment III. *C. burnetii* was detected in the placentas of all *Coxiella*-inoculated goats by immunohistochemistry and by PCR in the vaginal mucus just after parturition. Healthy kids stayed alive until the end of the experiment. Results are measurements at the day of necropsy indicated by days post inoculation and days post-partum.

Days post inoculation	52	59	68	67	94	94	94	98	98
Days post-partum	0	0	0	4	26	26	28	31	31
Status kid	abortion	abortion	abortion	weak	healthy	healthy	healthy	healthy	healthy
Tissue									
Foetus 1									
Spleen	+	++	++	++	+	-	-	-	-
Liver	+	++	+	+	-	+	-	-	-
Kidney	-	++	+	+	-	-	-	-	-
Lung	+	++	+	++	-	-	-	-	-
Heart	+	++	-	+	-	-	-	-	-
Foetus 2									
Spleen		+							
Liver		++							
Kidney		++							
Lung		++							
Heart		++							

"-": negative result, "+": 30 < PCR cycle threshold (Ct) < 40, "++": 20 < Ct < 30, blank: no sample available.

At 14 dpi *C. burnetii* DNA was only detected in the upper respiratory tract of the two necropsied infected goats and in the spleen and thymus of one of these goats. At 28 dpi *C. burnetii* DNA was detected only in the uterus and placenta of the two necropsied goats. At 42 dpi higher amounts of *C. burnetii* DNA in the uterus and placenta were detected reaching Ct values <20. *C. burnetii* DNA was also detected in most other tissues, including the upper and lower respiratory tract, haematopoietic system, liver, urinary and alimentary tract and the heart. In the goats necropsied at 56 dpi comparable *C. burnetii* DNA distributions were detected as in the goats necropsied at 42 dpi, although fewer tissues were positive. At 69 dpi, one goat was necropsied just after kidding, while the other one was in parturition. The quantity of *C. burnetii* DNA in the placenta and uterus of both animals was high (Ct values 14-18). *C. burnetii* DNA was also present in the respiratory, alimentary and urinary tract, the haematopoietic system, liver and in the heart. In the days after parturition the amount of DNA in the goats necropsied decreased and at 98 dpi (32 dpp) most organs tested negative for *C. burnetii* DNA except for the tonsils and mucosa of the upper respiratory tract, the uterus and caruncles, the bladder and gut. *C. burnetii* DNA was detected in the female genital tract until 57 dpp, in the gut until 60 dpp and in the mucosa of the nostrils in the upper respiratory tract until the end of the experiment at 95 dpp. The abdominally taken vaginal mucus swabs were *C. burnetii* DNA positive at 119 and 126 dpi, but negative at 140 and 141 dpi. An overview of the amount of *C. burnetii* DNA in the sampled tissues of the *Coxiella*-inoculated goats during the experiment is presented in Table 8.4. In the control goats no *C. burnetii* DNA was detected in any of the tissues at any point in time post inoculation (data not shown). Together, our results indicate that after intranasal inoculation *C. burnetii* DNA is detectable in the upper respiratory tract up to 14 dpi and that *C. burnetii* reached the placenta between 14 and 28 dpi. Then *C. burnetii* appears to spread to different organs with increased DNA levels detectable towards parturition. After parturition, the *C. burnetii* DNA gradually decreases to undetectable levels.

Table 8.4. Results of *C. burnetii*-specific PCR using DNA isolated from the indicated tissues taken at necropsy of *Coxiella*-inoculated goats. Results are measurements at the day of necropsy indicated by days post inoculation and days post-partum. Tissues organised per organ system.

Days post inoculation	14		28		42		56		69	69	77	98	119	126	140	141
Days post-partum										0	9	32	57	60	81	95
Goat ID	25	26	27	28	31	32	29	30	34	39	37	33	40	38	35	36
Tissue																
Upper respiratory tract																
Lymph nodes	+	+	-	-	-	+	-	-	-	+	+	-	+	-	-	-
Mucosa	-	+	-	-	+	+	+	+	+	++	+	+	+	+	+	+
Tonsils	+	+	-	-	-	+	-	+	+	++	+	+	-	-	-	-
Lower respiratory tract																
Lymph nodes	-	-	-	-	-	+	-	-	-	+	+	-	-	+	-	-
Bronchi	-	-	-	-	-	+	-	+	+	++	+	-	-	-	-	-
Lung	-	-	-	-	-	-	+	-	-	++	-	-	-	-	-	-
Female genital tract																
Udder	-	-	-	-	+	-	-	-	+	+	-	-	-	-	-	-
Lymph nodes udder	-	-	-	-	+	-	-	-	+	+	+	-	-	-	-	-
Ovarium	-	-	-	-	-	+	-	-	+	+	+	-	-	-	-	-
Lymph node iliaca	-	-	-	-	-	+	-	-	+	+	+	-	-	-	-	-
Uterus, non placenta	-	-	-	+	++	++	+	+	++	+++	++	+	+	-	-	-
Placentome/caruncle 1	-	-	+	++	+++	++	+	++	+++	+++	++	+	+	-	-	-
Placentome/caruncle 2			+	++		+++	+						-			
Uterus swab													-	-	-	-
Vaginal mucus, abd. taken													+	+	-	-
Haematopoietic system																
Spleen	-	+	-	-	-	+	-	-	-	++	-	-	-	-	-	-
Thymus	-	+	-	-	-	+	-	-	-	++	-	-	-	-	-	-
Bone marrow	-	-	-	-	+	++	-	+	+	+	+	-	-	-	-	-
Blood	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Liver																
Lymph nodes	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-
Parenchyma	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-
Bile	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-
Urinary tract																
Kidney	-	-	-	-	-	+	-	+	-	+	+	-	-	-	-	-
Bladder	-	-	-	-	-	-	-	+	+	+	+	+	-	-	-	-
Urine	-	-	-	-		-		+	-	-	-			-	-	-
Alimentary tract																
Gut	-	-	-	-	+	+	-	-	+	++	+	+	-	+	-	-
Lymph nodes	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-
Miscellaneous																
Heart	-	-	-	-	-	+	-	-	-	+	+	-	-	-	-	-
Glandula parotis	-	-	-	-	-	+	-	-	+	++	+	-	-	-	-	-
Perirenal fat	-	-	-	-	+	+	-	-	-	+	+	-	-	+	-	-
Excretion products																
Faeces	-	-	-	-	-	-	-	-	++	+	-	+		+	+	+
Vaginal mucus, ext. taken	-	-	-	-	-	-	-	-	+	+++	++	+		+	+	+
Milk											+	-	-	-	-	-

abd. taken: abdominally taken, ext. taken: externally taken, "-": negative result, "+": 30 < PCR cycle threshold (Ct) < 40, "++": 20 < Ct < 30, "+++": Ct < 20, blank: no sample available.

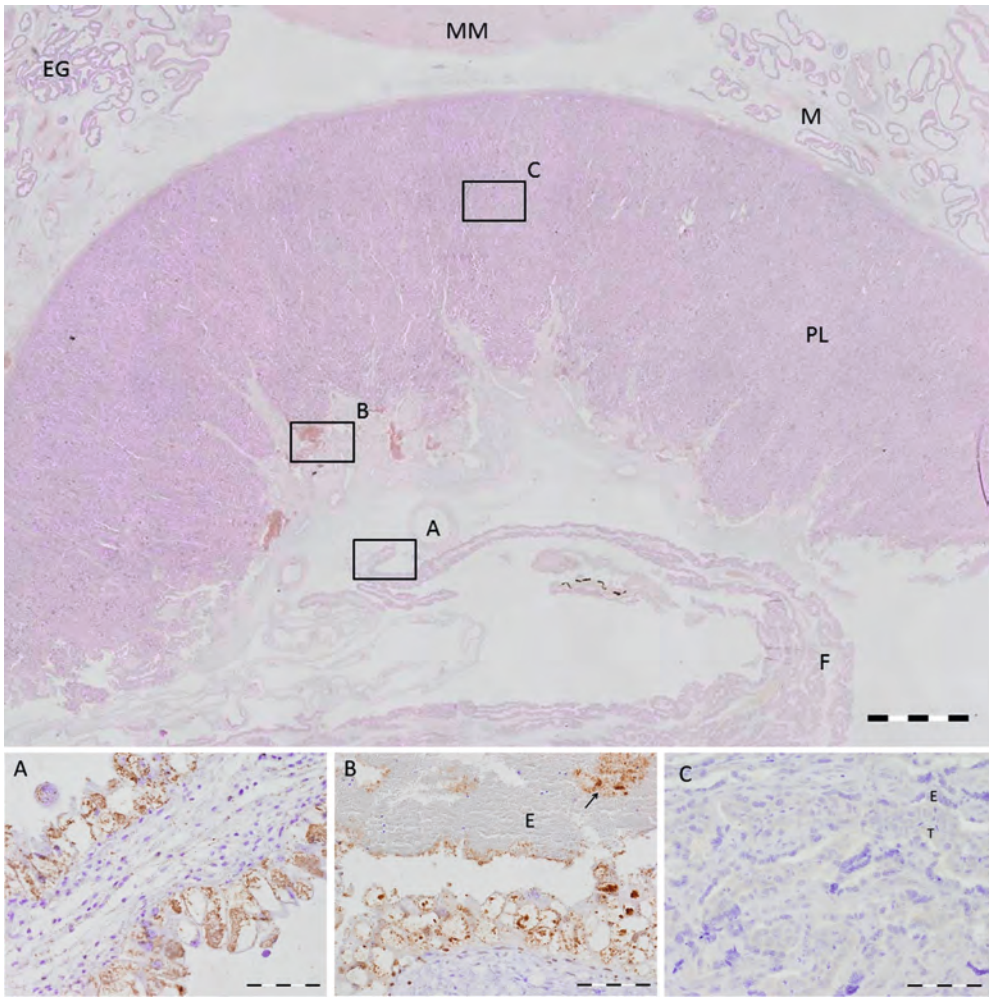

Figure 8.1. Overview of the placentome of a *Coxiella*-inoculated goat.

Top: Scanned haematoxylin and eosin stained section of the placentome of a goat necropsied at 56 dpi (day 131 of pregnancy). No inflammatory reaction in the maternal endometrium (M), the placentome (PL) or the foetal allantochorion (F). MM= myometrium, EG= endometrial glands. Bar= 1 mm.

Bottom: Higher magnification of areas A, B and C depicted by the rectangles in the overview. Serial section immunostained for the presence of *Coxiella burnetii* antigen (brownish colour).

A. Foetal allantochorion showing severe swelling of the trophoblast cells caused by the formation of large intracytoplasmic vacuoles. The vacuoles are filled with numerous *C. burnetii* bacteria. Bar = 200 µm.

B. Erythrophagous zone showing similar vacuolation and swelling of the trophoblasts at the base of the foetal villi. Numerous *C. burnetii* bacteria are seen within the vacuoles of the

trophoblasts and in the blood-filled lacuna between the foetal and maternal epithelium (arrow). E= erythrocytes. Bar = 200 µm.
C. Placentome. Absence of *C. burnetii* bacteria in the synepitheliochorial placenta. The maternal epithelium (E) and foetal trophoblasts (T) show no morphological alterations. Bar = 100 µm.

Dissemination of Coxiella burnetii *in the goats as detected by histopathology*

Tissues sampled for PCR were also investigated for histopathology. At 14 dpi neither histopathological lesions nor *C. burnetii* antigen were detected in any of the tissues of the *Coxiella*-inoculated goats. The first histopathological changes were seen in the foetal part of the placenta of one of the two goats killed at 28 dpi. The trophoblasts of the allantochorion appeared swollen and contained a large vacuole that dislocated the nucleus to the periphery of the cells, leading to a crescent cell shape. Immunohistochemistry (IHC) demonstrated numerous *C. burnetii* bacteria within the vacuoles of the trophoblasts. At this stage only the trophoblasts of the intercotyledonary allantochorion were affected, but at 42 and 56 dpi the erythrophagic trophoblasts at the base of the cotyledonary villi were gradually affected as well, starting from the periphery of the placentome. At 56 dpi all trophoblasts were loaded with large quantities of bacteria. However, *C. burnetii* antigen was never detected in the trophoblasts covering the cotyledonary villi, which form the true placenta together with the uterine epithelial cells of the maternal crypts (Figure 8.1). This indicates that the nutrient and gas exchange with the foetus was not disturbed. No inflammatory changes were seen in the allantochorion up to 56 dpi. At 69 dpi, one goat was necropsied just after kidding while the other one that was necropsied was in parturition. The allantochorion of both of these animals was severely thickened and leathery with a yellow/brownish exudate. The placentas of all *C. burnetii*-inoculated goats that aborted or delivered normally showed this appearance. These inflammatory changes were mainly confined to the intercotyledonary region of the placenta, while most cotyledons appeared normal macroscopically. Histologically, a heavy purulent to necropurulent inflammation was seen with large necrotic areas within the trophoblast layer, often accompanied by dystrophic calcification (Figure 8.2). The inflammatory response in the allantochorionic stroma consisted mainly of polymorphonuclear granulocytes and macrophages, with only a few lymphocytes or plasma cells. Occasionally, thrombosis was seen in blood vessels of the allantochorion. Although most of the trophoblast layer was ablated, numerous *Coxiella* bacteria were detected in the sloughed debris, in macrophages (Figure 8.3) and in areas where the trophoblast layer was still intact. In particular, the trophoblast layer of the former haemophagous zone at the base of the cotyledonary villi was often intact and

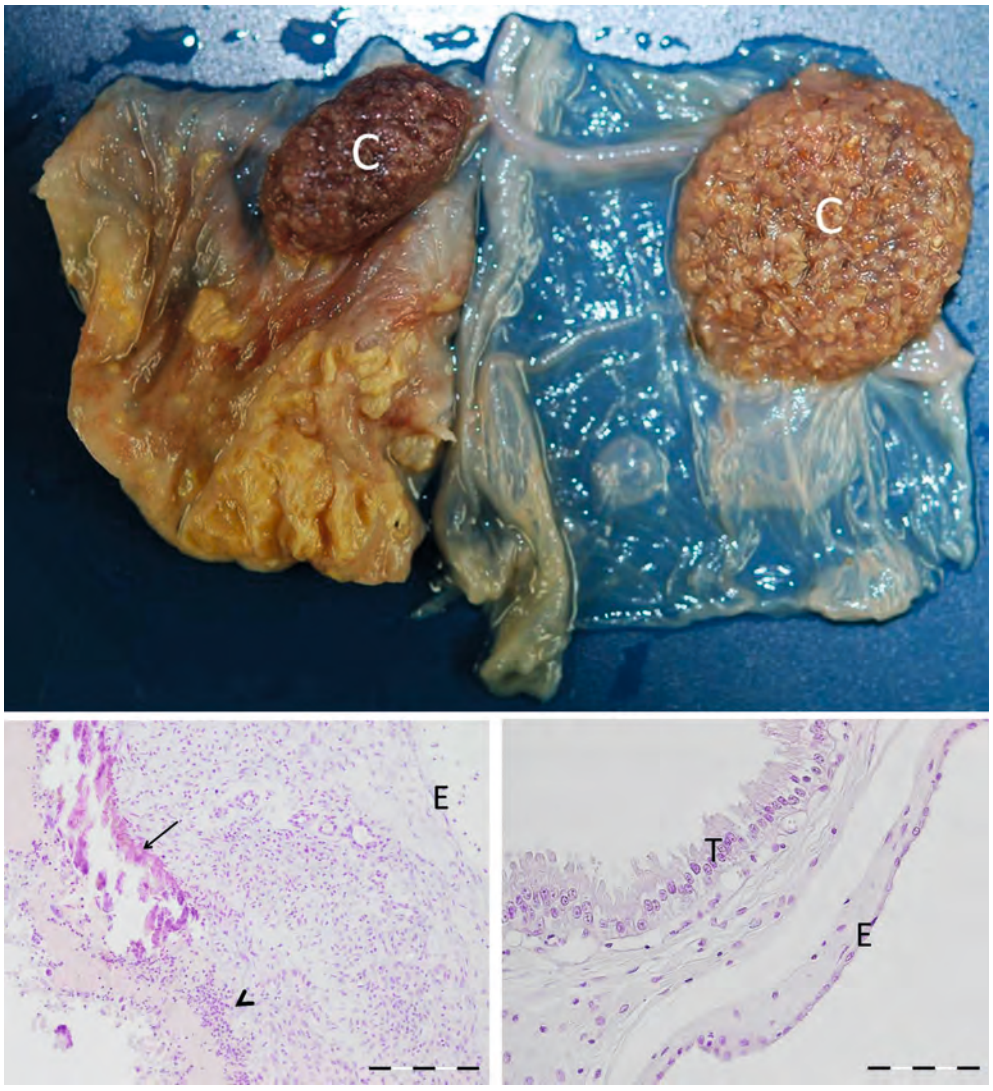

Figure 8.2. Comparison of a *C. burnetii* positive placenta and a *C. burnetii* negative placenta at parturition.

Top. Formalin-fixed allantochorion of an aborted kid (left) and the allantochorion of a kid from a control goat (right). The intercotyledonary allantochorion of the aborted kid is severely thickened and dull with a yellow-brownish exudate, while the cotyledon (C) shows no obvious macroscopic changes. The allantochorion of the control kid on the right is thin, glistening and transparent.

Bottom. Left. Haematoxylin- and eosin-stained section of the intercotyledonary allantochorion of an aborted kid. Notice the severe inflammatory changes in the stroma of the allantochorion. The trophoblast layer is lost with dystrophic calcification of necrotic tissue (arrow) and a purulent exudate (arrowhead). Bar = 200 µm. Right. Histological appearance of a normal allantochorion. Bar = 100 µm.

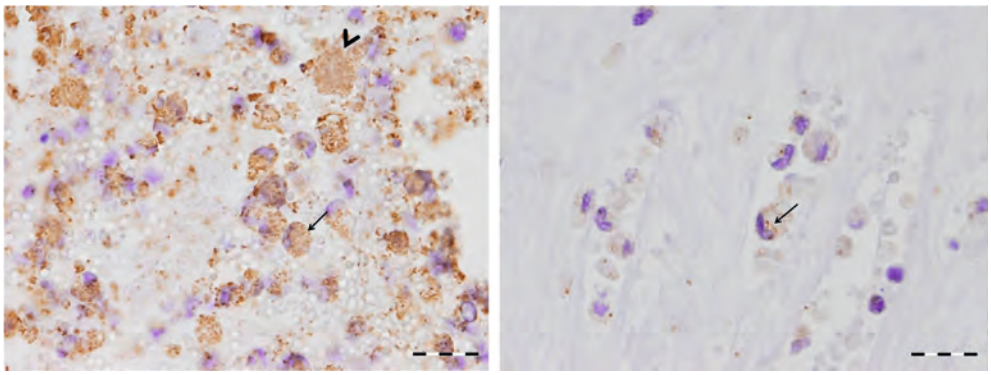

Figure 8.3. Close up of the allantochorion of an aborted kid due to *C. burnetii* infection.
The allantochorion of an aborted kid is immunostained to detect the presence of *C. burnetii* antigen. On the left: Numerous macrophages are present in the necropurulent exudate that covers the allantochorion. Both macrophages (arrow) and sloughed trophoblasts (arrow head) are filled with *C. burnetii*. On the right: macrophages in the stroma of the allantochorion have phagocytised *C. burnetii* bacteria (arrow). Bar = 20 µm.

of a kid from a control goat. The trophoblast layer is intact with a normal appearance of the trophoblast cells (T). Low cellularity in the stroma. E= endothelium of the allantochorion. Bar = 100 µm. contained large quantities of *C. burnetii*. This may therefore be the most suitable location to detect *C. burnetii* in a placenta after abortion or parturition. No *C. burnetii* antigen was detected in the endometrium or the maternal placental stroma or epithelium at any time during the infection. Compared to the control goats, there was also no significant increase in inflammatory cells in the endometrium until the time of parturition. Only at 9 dpp, was more infiltration of mononuclear cells (macrophages, lymphocytes and plasma cells) and polymorphonuclear granulocytes seen in the endometrium and caruncles as compared to the control goat euthanised at 9 dpp. However, this difference disappeared at later stages post-partum and both *Coxiella*-inoculated goats and control goats showed a similar degree of inflammatory infiltration associated with the ablation of the maternal placenta and involution of the uterus. Apart from the placenta, no histopathological lesions or *C. burnetii* antigen were detected in any of the other organs examined. Thus even in the tissues in which *C. burnetii* DNA could be detected by PCR, no *C. burnetii* antigen could be detected with immunohistochemistry. As expected, *C. burnetii* antigen was never detected in any of the tissues or placentas of the control animals. Overall, our data indicate that the trophoblasts of the allantochorion are the primary target cells of *C. burnetii* and that replication starts between 14 and 28 dpi. *C. burnetii* does not appear to infect the trophoblasts covering the cotyledonary villi and we found no evidence of *C. burnetii* replication in other tissues than the placenta.

Dissemination of Coxiella burnetii *in the foetuses and kids*

The presence of *C. burnetii* in mainly placental tissues raises the question as to whether the pathogen damages foetal tissues. To address this point, the dissemination of *C. burnetii* in aborted and euthanised liveborn kids was studied. Spleen, liver, kidney, lung and heart tissue were examined for histopathology and isolated DNA from these tissues was subjected to PCR. The investigations in Experiment II focused on the dissemination during pregnancy and at the time of parturition. In Experiment III the liveborn kids from infected does were kept alive to gain insight in the dissemination during the first weeks of their lives.

At 14 dpi, *C. burnetii* DNA was not detected in either the foetal part of the placenta or the foetal organs. At 28 dpi *C. burnetii* DNA was detected in the foetal allantochorion, the placentomes and the allantoic fluid. From 42 dpi until the end of pregnancy high quantities of *C. burnetii* DNA were detected in the foetal parts of the placenta of at least one of the kids. For most foetuses, amniotic and allantoic fluids as well as samples from the foetal spleen, liver, kidney, lung and heart tested positive for *C. burnetii* DNA. No significant difference in results was found between the placentas and foetal organs of aborted and liveborn kids. Therefore the placenta from liveborn kids also contained high amounts of *C. burnetii*. Histopathological examination of the tissues demonstrated a slight granulomatous hepatitis in some of the kids, but this was also present in kids from the control group. *C. burnetii* antigen was not detected by IHC in any of the sampled tissues. Two liveborn kids in Experiment III necropsied at 26 dpp were positive for *C. burnetii* DNA in either the spleen or liver. The three kids necropsied at 28 and 31 dpp were negative for *C. burnetii* DNA in all sampled organs. Tissues of all foetuses and kids born from the control goats tested negative for *C. burnetii* DNA. Detailed information about the amount of *C. burnetii* DNA in the sampled tissues detected by PCR of the foetuses of *C. burnetii*-inoculated goats is shown in Tables 8.2 and 3. It is important to note that the *C. burnetii* load in the placentas of aborted and liveborn kids were similar. After parturition, organs of kids initially contain high amounts of *C. burnetii*, but tested negative after 28 days.

Excretion of Coxiella burnetii *in faeces, vaginal mucous, blood, and milk*

To investigate the excretion of *C. burnetii* in faeces, vaginal mucus, blood and milk, *Coxiella*-inoculated and control goats were frequently sampled right from the start of the experiment. Milk sampling started just after parturition. Blood samples were taken to detect bacteraemia in particular after inoculation and parturition. Samples were examined by PCR for the presence of *C. burnetii* DNA.

All excreta and blood samples from the control goats remained negative for *C. burnetii* DNA for the entire duration of the experiment. For the *Coxiella*-inoculated goats, *C. burnetii* DNA was never detected in any of the faecal or vaginal mucus samples until the first abortion (goat ID 36) at 46 dpi. At the first sampling point after the first abortion (56 dpi) three of the eight goats tested positive for *C. burnetii* DNA in their faeces, while at 63 dpi (after the second and third abortions) the faeces of all eight goats tested positive. For the vaginal mucus samples this trend was even more prominent. *C. burnetii* DNA was first detected in vaginal mucus in the first

goat that aborted, while at the next sampling point (56 dpi), all eight goats already contained *C. burnetii* DNA in their vaginal mucus. This strongly suggests that *C. burnetii* is not excreted in the faeces or vaginal mucus before parturition. Monitoring of samples after parturition showed that *C. burnetii* DNA remained present in the faeces and the vaginal mucus of the goats until the end of the experiment. The results of faeces and vaginal mucus sampling of four goats that were sampled until 119 and 126 dpi and until the end of the experiment at 141 dpi, are presented in Figure 8.4 and Figure 8.5, respectively.

Analysis of the blood and milk samples only revealed *C. burnetii* DNA in the blood after the first abortion. Three goats had only one positive sample at 49 dpi, one goat had two positive samples (at 49 and 67 dpi) and goat 36 had three positive blood samples at 46, 48 and 49 dpi. *C. burnetii* DNA was detected in the milk of the *Coxiella*-inoculated goats after parturition until 38 dpp. Milk samples from later dates remained negative until the end of the experiment. The results of milk sampling of four goats that were followed until 119 and 126 dpi or until the end of the experiment at 141 dpi, are detailed in Figure 8.6.

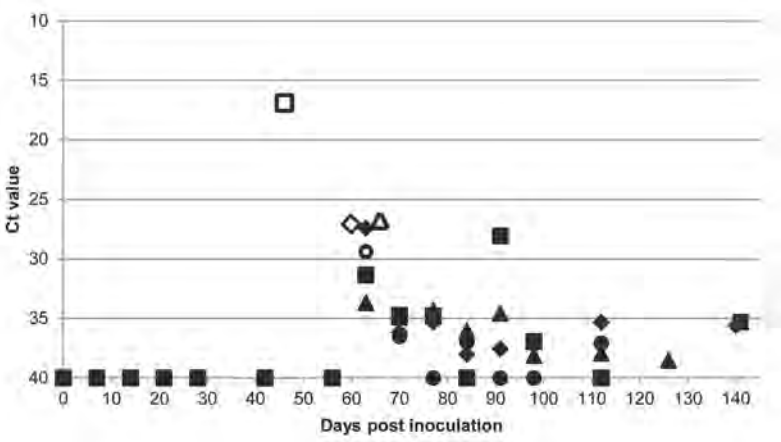

Figure 8.4. Detection of *C. burnetii* DNA in the faeces of *Coxiella*-inoculated goats over time. Detection of *C. burnetii* DNA in the faeces of four challenged goats of which a complete sampling sequence was present from inoculation until 119, 126, 140 and 141 days post inoculation (dpi). Goat ID 35: ◆, 36: ■, 38: ▲, 40: ●. Faecal samples were taken at the indicated dpi and *C. burnetii* DNA was measured by PCR. Ct value of 40 is negative, Ct value < 40 is positive. Parturition days are indicated as open symbols. Up until 42 dpi and on 56 dpi the four goats were negative. At 56 dpi three other goats of the group were positive before their parturition (data not shown). Data indicate that *C. burnetii* was detected in the faeces after parturition.

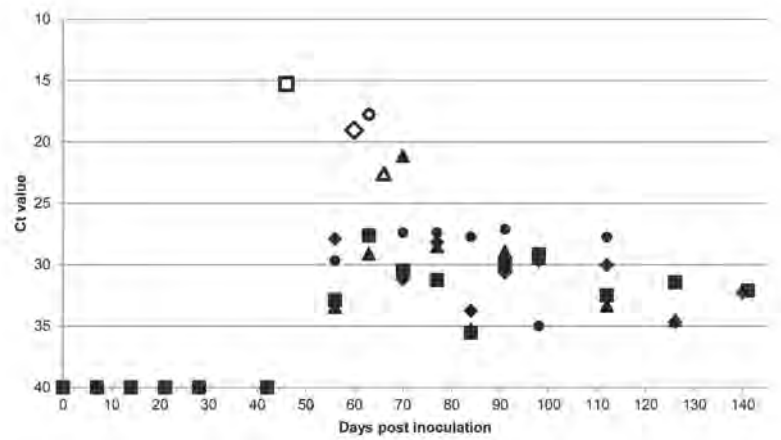

Figure 8.5. Detection of *C. burnetii* DNA in vaginal mucus of *Coxiella*-inoculated goats over time. Detection of *C. burnetii* DNA in vaginal mucus of four challenged goats of which a complete sampling sequence was present from inoculation until 119, 126, 140 and 141 days post inoculation (dpi). Goat ID 35: ◆, 36: ■, 38: ▲, 40: ●. Vaginal mucus samples were taken at the indicated dpi and *C. burnetii* DNA was measured by PCR. Ct value of 40 is negative, Ct value < 40 is positive. Parturition days are indicated as open symbols. Until 42 dpi the four goats were negative. Data indicate that *C. burnetii* was detected in the vaginal mucus after the first parturition in the group.

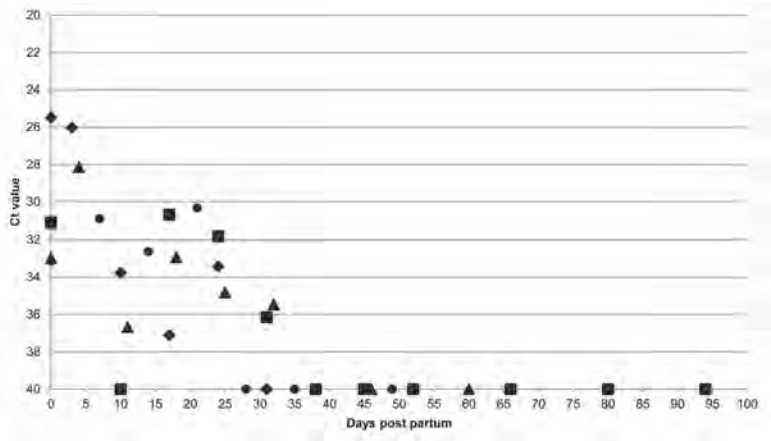

Figure 8.6. Detection of *C. burnetii* DNA in milk of *Coxiella*-inoculated goats over time.
Detection of *C. burnetii* DNA in milk of four challenged goats of which a complete sampling sequence was present from inoculation until 119, 126, 140 and 141 days post inoculation (dpi). Goat ID 35: ◆, 36: ■, 38: ▲, 40: ●. Milk samples were taken at the indicated days post-partum and *C. burnetii* DNA was measured by PCR. Ct value of 40 is negative, Ct value < 40 is positive. After parturition *C. burnetii* DNA was detected in the milk until 32 days post-partum.

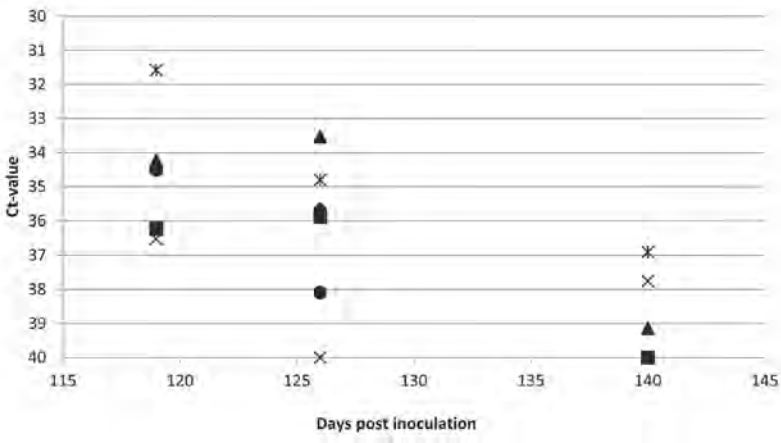

Figure 8.7. *C. burnetii*-specific PCR results of samples from the environment of *Coxiella*-inoculated goats.
C. burnetii PCR results of samples from the environment of the challenged goats measured at three time points after inoculation. ◆: clean wood shavings stored in the facility, ■: used wood shavings, ▲: swab from the slip mat, ×: swab from the air, *: swab from the water, ●: air filter. Ct value of 40 is negative, Ct value < 40 is positive. Results indicate an environmental contamination of *C. burnetii* DNA at the end of the experiment.

Detection of Coxiella burnetii *in the environment*

To investigate the possible *C. burnetii* contamination of the box environment, samples were taken from bedding material, floor, water and the air. The results of the environmental samples obtained in Experiment II are shown in Figure 8.7. Samples taken from 119 dpi onwards were positive for *C. burnetii* DNA and remained positive (albeit at lower levels) until the end of the experiment. The air samples from one of the two groups of *C. burnetii*-inoculated goats (Group C) in Experiment III were negative until 57 dpi. At 70 dpi the filter was positive for *C. burnetii* DNA (Ct value 27). This signal was lower at 77 dpi (Ct value 31) and at 84 dpi (Ct value 33). The first parturition in this group occurred at 62 dpi. These results indicate that *C. burnetii* may first spread into the environment after parturition and that the DNA persists until the end of the experiment.

Discussion

The goal of this study was to investigate the dissemination and excretion of *C. burnetii* in pregnant goats before and after parturition. To closely mimic the field situation, a natural inoculation route was preferred. In goat herds, inhalation of *C. burnetii* contaminated aerosols and oral uptake from the environment are the most likely routes of infection. As these routes of inoculation had not previously been investigated in experimental settings, we first tested the ability to infect goats via the oral and intranasal route and compared this with results from the frequently used subcutaneous route of inoculation [18,19,21]. Our results indicate that the intranasal inoculation route is most effective in infecting pregnant goats with *C. burnetii*. The high efficiency of intranasal inoculation was reproducible in two successive experiments and resembles results obtained after subcutaneous inoculation [18,19,21]. As the route of inoculation (intranasal versus subcutaneous) may influence the pathogenesis of Q fever and excretion of *C. burnetii,* we used the more natural intranasal inoculation route in our experiments.

Our results indicate that after intranasal inoculation, trophoblasts in the allantochorion of the placenta are the primary target cells for *C. burnetii*. *C. burnetii* was first detected by PCR and IHC at 28 dpi but not at 14 dpi. This suggests that it may take between 2 to 4 weeks for *C. burnetii* to multiply to detectable levels in the trophoblasts after entering the body. This time line corresponds with the results in which subcutaneous inoculation was used for challenge and in which *C. burnetii* was detected in the trophoblasts at 26 dpi [19]. A field observation suggests the progression from infection to abortion could be as short as 21 days [22]. From 42 dpi up to the time of parturition, the number of tissues that tested positive for *C. burnetii* DNA and the amount of DNA detected increased. The increase in the amount of *C. burnetii* DNA, however, does not seem to be caused by replication of *C. burnetii* in the parenchyma of the tissues. We were unable to detect *C. burnetii* antigen by IHC outside the placenta and had no histological indications for *C. burnetii* replication or inflammation. After parturition the amount of *C. burnetii* DNA in the tissues gradually decreased to zero, except for the mucosa of the nostrils. This decrease nicely reflects the trophoblast tropism of *C. burnetii*. With parturition these cells are removed from

the body, depriving *C. burnetii* of its replication niche. The observed sequence of dissemination of *C. burnetii* in goats seems to resemble those after subcutaneous inoculation [18,19]. This indicates that the inoculation route is not a major factor in the dissemination and replication of *C. burnetii* in pregnant goats. The tropism of *C. burnetii* for trophoblasts suggests that only pregnant animals are susceptible to *C. burnetii* infection. It is not clear if they should be pregnant to become infected. We cannot exclude the possibility that following infection, undetectable numbers of *C. burnetii* can hide in the body to infect trophoblasts when they become available [23].

An important finding of our study is that *C. burnetii* infection of pregnant goats does not always result in abortion. After intranasal inoculation, several infected goats gave birth to healthy liveborn kids although abortion was observed as well. Also the birth of stillborn and liveborn kids from the same doe indicates that *C. burnetii* infection is not always lethal for kids. This is the first time that the birth of liveborn kids has been confirmed under experimental conditions. Until now, only abortions and pre-term weak kids were reported in goats under experimental conditions after subcutaneous challenge with *C. burnetii* [18,19,21]. Our study outcome reflects the natural situation in *C. burnetii*-infected herds where abortions (up to 80%), weak-born kids as well as liveborn kids have been registered [9,14,16,20,24]. Remarkably, our results show no relation between pregnancy outcome and excretion of *C. burnetii* with the placentas in infected pregnant goats. Also the birth of newborn kids from infected goats was accompanied by high amounts of *C. burnetii* being excreted with the placenta. This is fully consistent with the field observation that demonstrated *C. burnetii* can be present in a placenta from a goat that delivers normally [16]. However, it challenges the suggestion that fewer bacteria are excreted when healthy kids are borne [25]. The consequence of our finding is that, besides abortions, normal deliveries in infected goats contribute to the environmental contamination and should therefore be considered as a major zoonotic risk for humans.

The birth of liveborn kids from animals with a heavily infected placenta may be explained by the histopathological changes in the placenta after inoculation. *C. burnetii* was not detected in the trophoblasts covering the cotyledonary villi involved in the exchange of gases and nutrients, which may prevent premature foetal death. Instead, foetuses may either die shortly before or during abortion, or may be born alive. This situation is different in caprine *Brucella* and *Chlamydia* infections. In these two types of infections, alterations in the foetoplacental binding lead to foetal death culminating in abortion [26,27,28,29]. The factors that determine the foetal fate in *C. burnetii* infected goats have yet to be determined.

Live kids delivered from infected goats contained *C. burnetii* DNA in several organs. However, no *C. burnetii* antigen could be detected. Therefore, like others [19], we have no indication for replication of *C. burnetii* in these organs. During the first weeks of life, the level of *C. burnetii* DNA positivity in the tested organs decreased to undetectable levels within 28 days. This clearance was also detected in the does, but finished earlier. These data suggest that *C. burnetii* is effectively cleared from the kid's tissues during the first weeks post-partum. However, the possibility of a persistent infection or carrier state with *C. burnetii* hiding in organs under the detection limit of the PCR cannot be excluded.

We found no evidence that *C. burnetii* is excreted in the faeces and vaginal mucus before parturition. Moreover, we found no indications for active replication of *C. burnetii* in the gut, liver or genital tract (besides the trophoblasts) which could lead to active excretion of *C. burnetii* in faeces and vaginal mucus before and after parturition. These results deviate from the general assumption that *C. burnetii* is excreted in faeces and vaginal mucus before parturition [6,13]. In our study faecal and vaginal mucus samples only tested positive for *C. burnetii* DNA after the first abortion in the group. After this parturition, *C. burnetii* DNA remained present in the faeces and vaginal mucus until the end of the experiment. It is possible that parturition, with the excretion of high numbers of *C. burnetii,* influences the test result either directly or via contamination of the environment. The absence of *C. burnetii* DNA in abdominally taken vaginal mucus swabs compared to the presence of *C. burnetii* DNA in vaginal mucus swabs taken externally on the same day may point to an external source of *C. burnetii*. The presence of *C. burnetii* in the environment of the infected goats after parturition may be a source of contamination as suggested by Welsh *et al* [30]. All things being considered, we favour the hypothesis that *C. burnetii* is not excreted before parturition in *Coxiella*-inoculated pregnant goats and that excretion of *C. burnetii* in faeces and vaginal mucus after parturition may be indirect following contamination by the environment.

Post-partum, all *Coxiella*-inoculated goats excreted *C. burnetii* DNA in the milk. Excretion stopped after 38 dpp. Arricau-Bouvery *et al* [18] detected *C. burnetii* DNA in the milk until at least 52 days after abortion. This difference with the present study can be explained by differences in milking pattern; Arricau-Bouvery *et al* [18] did not milk the goats, but only sampled them, so *C. burnetii* could accumulate in the milk in the mammary gland. In the present study the goats were milked daily so *C. burnetii* was discharged from the udder. Although the goats were milked in the *C. burnetii*- contaminated box environment, we were able to obtain negative results. These aseptically taken individual milk samples do not appear to have been influenced by the presence of environmental *C. burnetii*.

In summary, this study showed the intranasal inoculation route of *C. burnetii* to be effective in infecting pregnant goats. Inoculation results in strong tropism of *C. burnetii* towards the placenta and replication occurred in the trophoblasts of the placenta, but not in other tissues of goats and kids. Importantly, *C. burnetii* infection does not always result in abortion as infected goats gave birth to live and dead kids. The main excretion route of *C. burnetii* from infected pregnant goats is during abortion and during the delivery of kids. This suggests that normal parturitions in an infected herd may also form a source of infection.

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Chapter 9

Q fever in pregnant goats: humoral and cellular immune responses

H.I.J. Roest[1]
J. Post[2]
B. van Gelderen[1]
F.G. van Zijderveld[1]
J.M.J. Rebel[2]

[1] Department of Bacteriology and TSEs, Central Veterinary Institute, part of Wageningen University and Research Centre, Lelystad, the Netherlands
[2] Department of Infection Biology, Central Veterinary Institute, part of Wageningen University and Research Centre, Lelystad, the Netherlands

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Abstract

Q fever is a zoonosis caused by the intracellular bacterium *Coxiella burnetii.* Both humoral and cellular immunity are important in the host defence against intracellular bacteria. Little is known about the immune response to *C. burnetii* infections in domestic ruminants even though these species are the major source of Q fever in humans. To investigate the goat's immune response we inoculated groups of pregnant goats via inhalation with a Dutch outbreak isolate of *C. burnetii*. All animals were successfully infected. Phase 1 and Phase 2 IgM- and IgG-specific antibodies were measured. Cellular immune responses were investigated by interferon-gamma, enzyme-linked immunosorbent spot test (IFN-γ Elispot), lymphocyte proliferation test (LPT) and systemic cytokines. Two weeks post inoculation (wpi), a strong anti-*C. burnetii* Phase 2 IgM and IgG antibody response was observed while the increase in IgM anti-Phase 1 antibodies was less pronounced. IgG anti-Phase 1 antibodies started to rise at 6 wpi. Four weeks before parturition, the number of IFN-γ producing cells in peripheral blood increased and reached high levels during the first week after parturition. Our results demonstrated humoral and cellular immune responses to *C. burnetii* infection in pregnant goats. Cell-mediated immune responses seem to play a minor role in the host response of pregnant goats to *C. burnetii* infection, whereas a strong-phase specific antibody response is detected 2 wpi. This humoral immune response may be useful in the early detection of *C. burnetii*-infected pregnant goats.

Introduction

Q fever is a zoonosis caused by *Coxiella burnetii*. *C. burnetii* has a worldwide distribution except for New Zealand [1]. The bacterium has a wide host range including humans, terrestrial and marine mammals, birds and reptiles [2,3]. The zoonotic impact of the disease has recently been underlined by the Dutch Q fever outbreak in which 4029 human cases were registered during the years 2007-2010 [4,5]. More than 40,000 people are assumed to be infected [6]. In this outbreak, *C. burnetii*-infected pregnant goats and sheep were the primary source of Q fever in humans [7,8]2011). During parturition these animals excrete high numbers of *C. burnetii* into the environment. Inhalation of *C. burnetii*-contaminated aerosols is the main route of infection in humans and can result in acute or chronic Q fever [9]. In the acute phase, humans suffer from a flu-like, self-limiting disease, atypical pneumonia or hepatitis. The chronic form of Q fever may lead to life-threatening endocarditis.

C. burnetii is a Gram-negative, intracellular bacterium. As in other Gram-negative bacteria (e.g. *Brucella* spp. and *Enterobacteriaceae*), two major phenotypes (phases) of *C. burnetii* are recognised. Phase 1 of *C. burnetii* corresponds with the smooth phase of other Gram-negative bacteria and expresses full-length lipopolysaccharide (LPS) on its surface. Phase 2 corresponds with the rough phase of Gram-negative bacteria and lacks the O-antigenic region on its LPS [10]. Phase 1 is highly virulent and able to replicate in natural hosts, while Phase 2 *Coxiella* are considered avirulent and unable to replicate in immunocompetent animals [11,12]. The phase variation is interesting for the humoral immune response after *Coxiella* infection in mice, guinea pigs and humans. Following the inoculation of mice and guinea pigs with *C. burnetii* Phase 1, antibodies that recognise both Phase 1 and Phase 2 *C. burnetii* are generated [11,12]. In humans, the detection of phase-specific antibodies plays an important role in the diagnosis of acute and chronic Q fever [13]. This has not been investigated yet in goats. Tools might be found that can help in the early diagnosis of Q fever in goats and that can provide insights into the herd dynamics of Q fever infections, similar to those already anticipated in cattle herds [14].

The role of cellular immunity in the host defence against *C. burnetii* infections is not well established. In mice it is suggested that T cells are particularly important for the clearance of the bacterium after infection. Interferon-gamma (IFN-γ) and tumour necrosis factor-alpha (TNF-α) seem to be essential for the early control of *Coxiella* proliferation [12]. Furthermore, *in vitro* studies with human, peripheral blood, mononuclear cells indicate specific stimulation of T cells by human, monocyte-derived, dendritic cells (HMDCs) pulsed with *C. burnetii* outer membrane protein Com1 [15]. Recently the value of the interferon-gamma, enzyme-linked, immunosorbent spot test, a diagnostic tests based on cellular immunity, in the diagnosis of chronic Q fever in humans has also been shown [16]. In pre-vaccination screening of humans, a skin test is used to detect previously sensitised people so as to avoid adverse reactions after Q fever vaccination in these persons [17]. In cattle, the skin test as a read out for a cellular response has been used to investigate the duration of immunity after Q fever vaccination [18]. Cellular immune responses after *C. burnetii* infection of domestic ruminants have not been investigated, although this may

provide potential tools to investigate the pathogenesis of *C. burnetii* infection in ruminants and to improve its diagnosis. In addition, the vaccine efficacy in already infected hosts can probably be improved if the cell-mediated immunity can be stimulated with a new generation of vaccines.

The goal of the present study was to investigate the humoral and cellular immune response in pregnant goats after inoculation with the Dutch outbreak strain of *C. burnetii*. The humoral immune response was measured by detecting anti-Phase 1 and anti-Phase 2 IgM- and IgG-specific antibodies. Cellular immune responses were measured with the interferon-gamma, enzyme-linked, immunosorbent spot test (IFN-γ Elispot) and the lymphocyte proliferation test (LPT) as well as by measuring systemic mRNA for different cytokines. Our results indicate a strong phase-specific IgM and IgG antibody response during early infection, whereas the cell- mediated immune response seems to play a minor role in the response of pregnant goats to *Coxiella* infection.

Materials and Methods

Inoculum

C. burnetii strain X09003262-001 was isolated from a placenta of a dairy goat that aborted on a farm during the Q fever outbreak in the Netherlands. This has previously been described [19]. In short, part of an immunohistochemically confirmed, *C. burnetii*-positive placenta was crushed and filtered before inoculation with a culture of Buffalo Green Monkey (BGM) cells kept in culture medium without antibiotics (EMEM with 10% bovine serum albumin, 1% NEAA, 1% glutamax). The inoculated cells were incubated for 14 days at 37°C in a closed flask and culture medium was refreshed twice a week. The cell culture was negative for *Chlamydia abortus, Simkania negevensis* and *Mycoplasma*. A large batch of strain X09003262-001 was prepared and the mouse-infective dose (MID) of the batch was determined. Prior to inoculation, the inoculum was adjusted to the required MID by dilution with culture medium. The strain was genotyped as CbNL01, the predominant *C. burnetii* genotype in the Dutch Q fever outbreak. To ensure inoculation of Phase 1 bacteria, cell culture passage 2 of the field isolate was used. In the inoculum, no Phase 2 *C. burnetii* were detected with an immunofluorescence test set up with the serum of a goat with a high anti-Phase 2 antibody titre but no Phase 1 titre. All experiments were approved by the Animal Experiment Commission of the Central Veterinary Institute, part of Wageningen UR, in accordance with Dutch regulations on animal experimentation.

Animal experiment

The experimental set up has previously been described (Experiment III, [19]. Sixteen healthy, pregnant, serologically Q fever negative, Alpine yearling goats were purchased from INRA (Institut National de la Recherche Agronomique, Domaine de Galle), France. Pregnancy and the duration of pregnancy were confirmed using ultrasound. All goats tested serologically negative for antibodies against *C. burnetii* and *Chlamydia abortus* on the day of arrival. Six

negative control goats were housed in animal biosafety level (aBSL) 2 facilities. Two groups of 5 goats (Group A and B) were separately housed in aBSL3 facilities for inoculation with *C. burnetii.* On day 76 of pregnancy, 10 goats were intranasally inoculated with 1 ml containing 106 MID *C. burnetii* while the six negative control animals were intranasally inoculated with 1 ml of culture medium. General health was monitored by daily clinical inspection of behaviour, appetite and consistency of the faeces. All goats were kept alive until the end of the experiment at 13 weeks post inoculation (wpi) (negative controls and Group A) and 14 wpi (Group B). Weak-born kids were euthanised when necessary for ethical reasons and liveborn kids were kept together with their does until the end of the experiment.

During the experiment goats did not show any clinical signs of disease except for abortion. Three of the ten *Coxiella*-inoculated goats aborted (one single kid and two twins) at 7, 8 and 10 wpi respectively. One goat delivered one stillborn kid and one liveborn weak kid at 10 wpi. Two goats delivered one stillborn and one liveborn healthy kid each at 9 wpi; one goat delivered two weak kids at 9 wpi and three goats delivered healthy kids, all singles at 9 wpi. The six control goats delivered healthy liveborn kids at 10 and 11 wpi, which was equal to 149 to 157 days of gestation. *C. burnetii* was detected in the placentas of all ten *Coxiella*-inoculated goats by immunohistochemistry and by PCR in the vaginal mucus just after parturition. These results indicate that all *Coxiella*-inoculated goats were successfully infected with *C. burnetii*.

Sampling

Jugular blood was sampled from each of the goats weekly from 0 wpi just before the inoculation until 13 wpi, except that at 4 wpi goats were not sampled and at 13 wpi the negative control goats were not sampled for antibody detection. When results from samples were related to parturition, parturition was set at time point 0; results from samples taken in the week before parturition were indicated as Week -1 and results from samples taken in the week after parturition were indicated as Week 1. Blood was collected in coagulation tubes for antibody detection, in anticoagulation tubes (EDTA) for cell-mediated immunity and in PAXgene® Blood RNA tubes to preserve cytokine's mRNA.

Detection of phase 1 and phase 2 IgM and IgG antibodies

C. burnetii Phase 1 and Phase 2 IgM- and IgG-specific antibodies were detected in an ELISA format. *C. burnetii* Phase 1 and Phase 2 ELISA-specific plates were purchased from Virion/ Serion (Serion ELISA classic *Coxiella burnetii* Phase 1 and Phase 2, Germany). Optimal serum and conjugate dilutions were determined in advance and positive and negative controls were selected (data not shown). Plates were incubated with 100 µl 1:160 diluted serum in phosphate buffered saline (PBS), pH 7.2 with 0.5 ml 10% (v/v) tween 80 (PBS-Tw) for 1 h at 37°C. After incubation, plates were washed automatically (Schleicher, Germany), 6 times with 1400 µl of 0.5 ‰ Tween 20 in water and incubated for 1 h at 37 °C with 100 µl of diluted alkaline phosphatase-conjugated antibodies. For the detection of IgM antibodies rabbit anti-goat IgM (Bioconnect, the Netherlands) antibodies were used, 1:1000 diluted in PBS-Tw and 0.5M NaCl for the detection of

Phase 1 antibodies or 1:5000 diluted for the detection of Phase 2 antibodies. For the detection of IgG antibodies rabbit F(ab')2 anti-goat IgG (H/L) (Bioconnect, the Netherlands) were used, 1:2000 diluted for the detection of Phase 1 antibodies or 1:4000 diluted for the detection of Phase 2 antibodies. After incubation with the conjugate, plates were washed as described above and 100 µl of para-nitrophenylphosphate substrate (virion/serion, Germany) per well was added and the reaction was stopped after 30 min at 37°C with 100 µl of 1.2 N sodium hydroxide (virion/serion, Germany). The optical density (OD) was measured at 405 nm (EL 808 Ultra microplate reader, Bio-tek instruments, USA). On each plate the same negative and positive control serum was tested in duplicate per phase/Ig combination. Results of the serum were given related to the average positive control OD, both corrected for the average negative control OD.

Interferon-gamma, enzyme-linked, immunosorbent spot test (IFN-γ Elispot)

Peripheral blood mononuclear cells (PBMCs) were isolated from EDTA blood by Ficoll-Hypaque (Amersham Biosciences, Sweden) density gradient centrifugation. Interferon-gamma, enzyme-linked immunosorbent spot test (IFN-γ Elispot) assay was performed using $5 \times 10_5$ PBMCs per well (MSIPS4W10 plates, Millipore, USA) from each goat. Detection of the T cell-produced IFN-γ was performed with the Elispot kit for Bovine/Ovine/Equine IFN-γ (MabTech, Nacka Strand, Sweden). We evaluated the kit for caprine IFN-γ and found it suitable for use (data not shown). *C. burnetii* T-cell responses were examined after stimulation with culture medium (negative control), *C. burnetii* strain Nine Mile Phase 1 and Phase 2 (Virion/Serion 1227, 1:5000 diluted in culture medium) or ConA (positive control). IFN-γ spot-forming cells were counted using an ImmunoSpot analyzer (CTL, USA). We optimised counting parameters to precisely and accurately count all Elispot plates. The average results of all goats in the group are given as relative results of the positive control after correction for the medium control.

Lymphocyte proliferation test (LPT)

PBMCs suspended in medium (negative control), *C. burnetii* strain Nine Mile Phase 1 and Phase 2 (Virion/Serion 1227, 1:5000 diluted in culture medium) or ConA (positive control) were added to triplicate wells. Plates were incubated at 37°C for 72 h. For the last 18 h of incubation, alamar blue (Invitrogen, USA) was added. Supernatant was harvested and absorbance was measured at 570 nm/600 nm. Results are given as average results per group. Individual results were calculated as relative induction of the average difference of the OD at 600 nm and 570 nm of the sample in duplicate compared to the medium control.

Cytokine mRNA induction

Blood specimens (2.5 ml) collected in PAXgene® tubes were incubated at room temperature for 4 h for RNA stabilisation and then stored at −80°C. RNA was extracted from whole blood using the manufacturer›s guidelines. In brief, samples were removed from −80°C and incubated over night at 4°C to ensure complete lysis of the blood cells. Then tubes were centrifuged for 10 min at 4000 *g*, the supernatant was discarded and 5 ml of RNase-free water was added to the pellet.

The pellet was resuspended. Washing was repeated and the pellet was finally resuspended in 1 ml trizol (Invitrogen, Carlsbad, USA). Subsequently, a phase separation with chloroform was performed and RNA was precipitated using 2-propanol. Additional purification was performed with the DNA-free kit (Ambion, USA). The quality and integrity of the RNA samples were analysed using the Agilent Bioanalyzer (lab on chip, Agilent Technologies, USA). For the quantification of cytokine mRNA, cDNA was made using random hexamer primers and reverse transcriptase. Forward and reverse primers were selected to detect the cDNA of TNF-α, IL-1β, IFN-α, IFN-γ, IL-2 and IL-10 (Table 9.1). PCR was performed using Syber Green PCR Master Mix (Applied Biosystems, USA) in an ABI 7500 Real-Time PCR system (PE Applied Biosystems, USA). Results were quantified and normalised compared to the succinate dehydrogenase complex subunit A gene (SDHA) of the same sample. For the quantification, a standard curve of the plasmid with the insert of the cytokine of interest constructed in pGEM-T easy (Promega, the Netherlands) was used. For negative controls, RNA samples without reverse transcriptase in the reaction mixture were used.

Table 9.1. Sequences of the forward en reverse primers used to detect the DNA transcripts of the mRNA of TNF-α, IL-1β, IFN-α, IFN-γ, IL-2 and IL-10.

Gene of interest	NCBI code	Primer	Sequence
TNF-α	X14828	Forward	CCTTGAGAAGATCTCACCTA
		Reverse	CAAACATAAACAGAGGGAGT
IL-1β	DQ837160	Forward	TACCTGTCTTGTGTGAAAAA
		Reverse	CAAATTCAACTGTGTTCTTG
IFN-α	FJ959074	Forward	GAGGAAATACTTCCACAGAG
		Reverse	ATGACTTCTGCTCTGACAAC
IFN-γ	EF375708	Forward	GAAATTTTGAAGAATTGGAA
		Reverse	AATGACCTGGTTATCTTTGA
IL-2	AF535145	Forward	GATGTCTAGAAGCAAGGGTA
		Reverse	ACATCCAAATGAGTTCTGTT
IL-10	DQ837159	Forward	GGCAAAGTGAAGACTTTCT
		Reverse	ACTGGATCATTTCTGACAAG

Statistical analyses

QQ plots (IBM SPSS Statistics 19) were used to assess the normal distribution of the data. Results of the control group and the *C. burnetii*-inoculated group were compared using the T-test, taking into account the variances in the data. P values ≤ 0.05 correspond with a confidence interval of 95%, marked as *; P ≤ 0.01 correspond with a confidence interval of 99%, marked as **.

Results

Humoral immune response

Ten pregnant goats were intranasally inoculated with a Dutch *C. burnetii* outbreak strain and jugular blood was sampled weekly. The serum of each goat was tested for IgM and IgG *C. burnetii*-specific antibodies using *C. burnetii* Phase 1- and Phase 2-specific ELISAs. Average IgM antibody levels against Phase 2 antigen (IgMph2) started to rise significantly from two weeks post-inoculation (wpi) onwards; after 3 wpi the antibody titre decreased and stabilised until the end of the experiment (Figure 9.1A). Average anti-*C. burnetii* Phase 2 IgG antibody levels (IgGph2) rapidly increased between 2 wpi and 4 wpi and then at a slower rate till 10 wpi (Figure 9.1B).

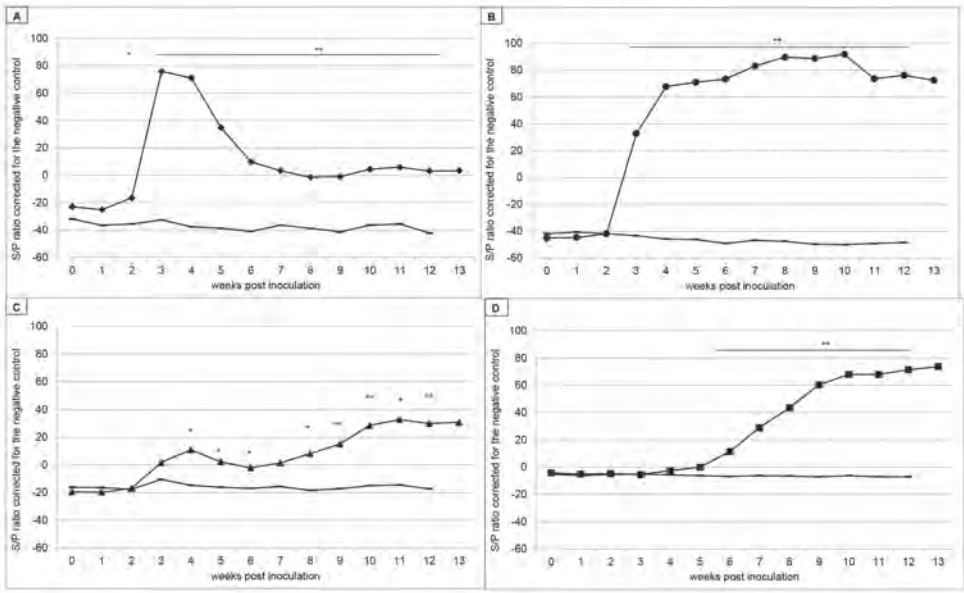

Figure 9.1 (A, B, C, D). Results of the *C. burnetii* phase 1 and phase 2 IgM and IgG specific ELISA. Average antibody titres expressed as S/P ratio corrected for the negative control for IgM Phase 2 (IgMph2, ◆, A) and Phase 1 (IgMph1, ▲, C) and IgG Phase 2 (IgGph2, ●, B) and Phase 1 (IgGph1, ■, D) as measured with an IgM- and IgG- specific conjugate in *C. burnetii* phase-specific ELISA. (–) : negative control goats. X-axis in weeks post inoculation (wpi).

Average anti-*C. burnetii* Phase 1 IgM antibody levels (IgMph1) started to rise significantly from 3 wpi onwards. Highest levels were detected at 4 wpi although the increase of antibody levels was less compared to IgMph2 and IgGph2 antibody levels. After a decline, IgM1 rose again after 6 wpi (Figure 9.1C). Anti-*C. burnetii* Phase 1 IgG antibody levels (IgGph1) started to rise at 6 wpi; at 9 wpi the average titre stabilised until the end of the experiment at 12 wpi (Figure 9.1D). IgMph2 and IgGph2 levels were not influenced by parturition. The second rise of IgMph1 started 1 week before parturition. IgGph1 started to rise 4 weeks before parturition. The data indicate

a strong IgMph2 and IgGph2 response starting two weeks after inoculation. The IgMph1 and IgGph1 response started later.

Cell-mediated immune response

To investigate the cell-mediated immune response, IFN-γ Elispot and the LPT were performed on cells isolated from the jugular blood samples. In these tests *C. burnetii* Phase 1 and Phase 2 antigens were used as stimulus. In the first four weeks after inoculation, no significant difference between the *Coxiella*-inoculated goats and the control goats was observed in the number of peripheral blood mononuclear cells (PBMCs) able to produce IFN-γ after stimulation with *C. burnetii* Phase 1 and Phase 2 antigen (Figure 9.2A). In the LPT, PBMCs did proliferate significantly more after stimulation with *C. burnetii* Phase 2 antigen at 2 wpi (Figure 9.4B). These results indicate a small but measurable cell-mediated immune response during the first four weeks of *C. burnetii* infection in pregnant goats.

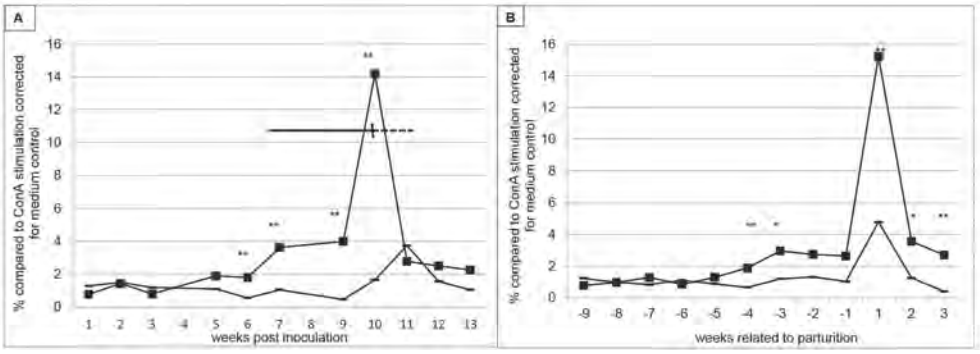

Figure 9.2 (A, B). IFN-γ Elispot results, related to inoculation and to parturition.
Average data for PBMCs stimulated with Phase 1 and Phase 2 antigen. Data were corrected for medium-incubated cells and expressed as a percentage of ConA-stimulated cells. The X-axis is in weeks post inoculation (wpi). A: data were related to inoculation. Solid bar: delivery period of the *Coxiella*-inoculated goats; dotted bar: delivery period of the control goats. B: data related to delivery. (■) *Coxiella*-inoculated goats; (–) negative control goats; * : P ≤ 0.05; ** : P ≤ 0.01.

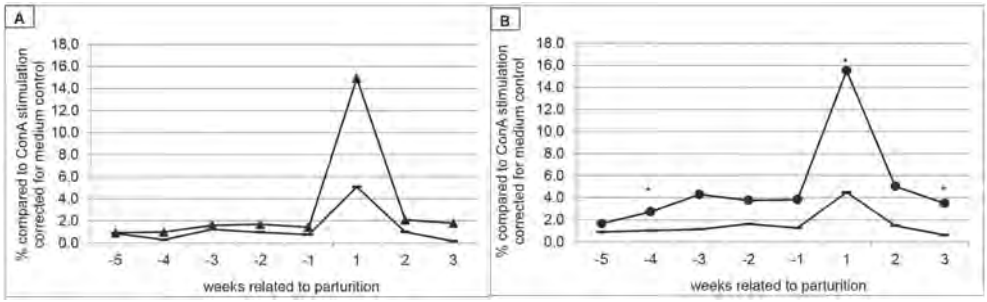

Figure 9.3 (A, B). IFN-γ Elispot results, per *C. burnetii* phase 1 and phase 2 stimulus, related to parturition.

Results of the PBMC of control goats and *Coxiella*-inoculated goats after stimulation with *C. burnetii* Phase 1 or Phase 2 antigen. Data were corrected for medium-incubated cells and expressed as percentage of ConA-stimulated cells, the X-axis is in weeks related to parturition (time point 0). A: results after stimulation with *C. burnetii* Phase 1 antigen, (▲: *C. burnetii*-inoculated goats; −: control goats). B: results after stimulation with *C. burnetii* Phase 2 antigen (●: *C. burnetii*-inoculated goats; − :control goats); * : P ≤ 0.05; ** : P ≤ 0.01.

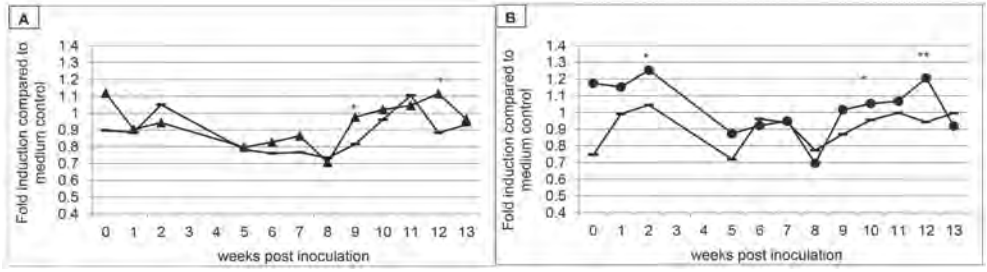

Figure 9.4 (A, B). LPT results, related to inoculation.

Lymphocyte proliferation test results of the control goats and *Coxiella*-inoculated goats. Data are expressed as average results of the group in fold induction compared to medium incubated cells. The X-axis indicates weeks post inoculation (wpi). A: results after stimulation with *C. burnetii* Phase 1 (▲: *C. burnetii*-inoculated goats; −: control goats). B: results after stimulation with *C. burnetii* Phase 2 antigen (●: *C. burnetii*-inoculated goats; −: control goats); * : P ≤ 0.05; ** : P ≤ 0.01.

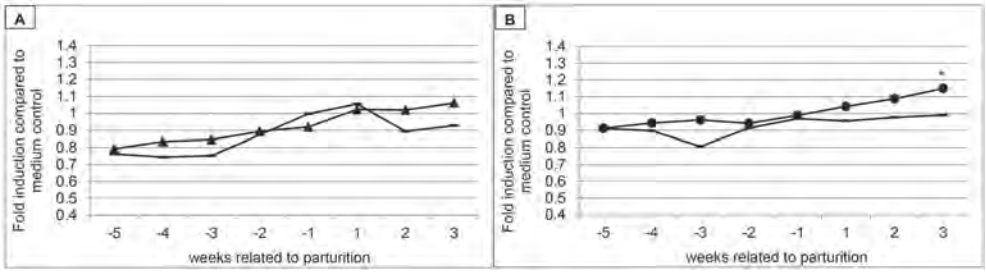

Figure 9.5 (A, B). LPT results, related to parturition.
Lymphocyte proliferation test results of the control goats and *Coxiella*-inoculated goats. Data are expressed as average results of the group in fold induction compared to medium-incubated cells. The X-axis indicates weeks related to parturition (time point 0). A: results after stimulation with *C. burnetii* Phase 1 (▲: *C. burnetii*-inoculated goats; −: control goats). B: results after stimulation with *C. burnetii* Phase 2 antigen (●:*C. burnetii*-inoculated goats; −:control goats); * : $P \leq 0.05$; ** : $P \leq 0.01$.

More detailed analysis of the IFN-γ Elispot results revealed a strong increase of the IFN-γ producing PBMCs around the date of parturition for both *Coxiella*-inoculated goats and control goats. Upon correcting for the date of parturition, the increase in IFN-γ production in both *Coxiella*-inoculated and control goats corresponded to the time of parturition (Figure 9.2B), suggesting that parturition influences IFN-γ production in goats. Further analysis of the IFN-γ Elispot and LPT results, but this time with correction for parturition, showed that about four weeks prior to parturition the number of IFN-γ producing PBMCs increased significantly after stimulation with Phase 2 *C. burnetii* antigen (Figure 9.3B). This increase continued until three weeks post parturition for the Phase 2 *C. burnetii* antigen-stimulated IFN-γ producing PBMCs. At one week after parturition, the number of IFN-γ producing PBMCs after stimulation with both Phase 1 and Phase 2 *C. burnetii* antigens increased significantly (Figure 9.3A and B). LPT results revealed an increase in proliferation ability of the PBMCs only after parturition in the *Coxiella*-inoculated goats (Figure 9.5A and B). The increase in proliferation ability after stimulation with *C. burnetii* Phase 2 antigen was stronger compared to stimulation with Phase 1 antigen (Figure 9.5B). Taken together, no clear cell-mediated immune response could be detected in the first weeks after infection. Four weeks prior to parturition, IFN-γ Elispot results showed an increase in production of IFN-γ by systemic PBMCs until the third week after parturition.

Systemic cytokine mRNA responses

Further information about the cellular and humoral immune response was obtained via weekly measurement of systemic mRNA levels of the regulation of the pro-inflammatory cytokines TNF-α and IL-1β and the regulatory cytokines IL-2 and IL-10. IFN-α and IFN-γ mRNA regulation was measured likewise. Within 4 weeks after inoculation no differential effect on cytokine mRNA levels was measured between the *Coxiella*-inoculated group compared to the control group

(data not shown). Systemic cytokine responses related to parturition are presented in Figure 6. The pro-inflammatory cytokines TNF-α and IL-1β mRNA were not differentially regulated in this period. Of the interferon's, IFN-γ mRNA was up regulated in the period before delivery, while no effect could be observed on IFN-α mRNA levels. For the regulatory cytokines IL-2 and IL-10 mRNA, no significant changes in induction of mRNA could be found in relation to delivery (Figure 9.6). Results indicate only a significant systematic up regulation of IFN-γ mRNA prior to delivery, while other measured cytokines were not differentially regulated in the *Coxiella*-inoculated goats compared to the control goats.

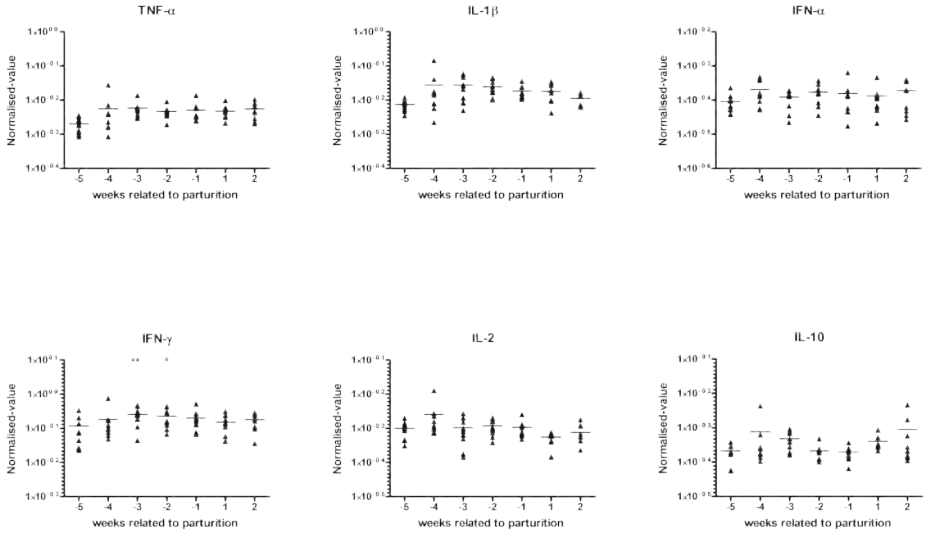

Figure 9.6. Results of the systemic cytokine mRNA response detected by qPCR.
The results show cytokine mRNA levels normalised to SDHA in the blood of *Coxiella*-inoculated goats measured by qPCR. The presence of cytokine mRNA in peripheral blood was examined in control goats and in *Coxiella*-inoculated goats every week post inoculation. Only significant differences between control goats and infected goats were indicated. Triangles represent the Ct value of individual *Coxiella*-inoculated goats. The horizontal line represents the mean of the group at that time point. * : $P \leq 0.05$; ** : $P \leq 0.01$.

Discussion

The goal of this experimental longitudinal study was to investigate the humoral and cellular immune responses in pregnant goats infected via a natural infection route with a Dutch outbreak isolate. The immune response after *C. burnetii* inoculation via inhalation in domestic ruminants had not been studied yet. This inoculation route was successful in infecting goats and resulted in *C. burnetii*-infected placentas [19]. Up until now only subcutaneous inoculations had been used to study humoral immune responses [20,21]. However, the inoculation route might be important for studying naturally occurring immune responses.

Our results indicate a strong IgM anti-Phase 2 and IgG anti-Phase 2 humoral response, which starts to rise between 2 to 3 weeks post inoculation. This time period between inoculation and the first antibody response is shorter than reported for non-phase-specific antibodies in goats after subcutaneous inoculation [20,21], but is slightly longer than reported for humans and mice [22,23]. For goats, the difference with previous studies may be due to the use of different inoculation routes. Inoculation via inhalation can generate a mucosal immune response in the lungs; in humans, the lungs have the ability to respond quickly to some pathogens, i.e. *Streptococcus pneumoniae*. In the lungs, residential antigen-specific memory B cells are present but it is also reported that infections in the lung generate a *de novo* local and systemic antibody response [24]. In goats it is not known whether *C. burnetii* can induce such a response in the lung but the fast systemic antibody response could be a result of this.

The initial humoral immune response consisted of the generation of *C. burnetii* Phase 2-specific antibodies, whereas antibodies against *C. burnetii* Phase 1 arose at a later stage. Although in line with previously published work in mice, guinea pigs and humans [22,23,25], it is not clear why an anti-Phase 2 response is generated in advance of an anti-Phase 1 response, as Phase 1 *Coxiella* bacteria were inoculated. One possible explanation is that besides Phase 1 bacteria, Phase 2 bacteria were inoculated as well. Phase 2 bacteria are efficiently internalised into phagosomes [9,26]. This results in an effective killing of the Phase 2 bacteria that gives rise to a humoral response against Phase 2 *C. burnetii*. Alternatively, it can be assumed that both LPS and surface protein antigens of Phase 1 *C. burnetii* are recognised by the immune system but that surface proteins, which Phase 1 and Phase 2 *C. burnetii* have in common [27], give an earlier and stronger humoral immune response compared to the LPS antigen. This results first in an anti-Phase 2 response to the surface antigens, followed by an anti-Phase 1 response against LPS. As we were not able to detect Phase 2 *C. burnetii* in our inoculum, we assume that both surface proteins and LPS are detected by the immune system. Therefore the initial anti-Phase 2 response may be due to an earlier and stronger response to surface proteins than to LPS.

Surprisingly both anti-Phase 2 IgM and IgG titres started to rise at almost the same time. This was not expected, as IgM is generally the first immunoglobulin class to be produced in a humoral immune response because IgM can be expressed without class switching. During human *Coxiella* infection, IgM anti-Phase 2 antibody titres also start to rise first followed by a IgG anti-Phase 2 response [22]. The phase-specific and antibody-subclass-specific humoral immune response

might help in the detection of early or more prolonged *C. burnetii* infections in goats. As pregnant goats do not excrete the bacterium [19] the detection of anti-Phase 2 IgM without anti-Phase 1 IgG will indicate an early infection. Whether phase-specific and antibody-subclass-specific antibody titres can predict or indicate chronic infections in goats should be investigated in further research, as the study period in the present study was only 13 weeks.

The cell-mediated immune response during the first weeks after inoculation was minimal, as indicated by the results of the IFN-γ Elispot and LPT and the apparent absence of a systemic cytokine mRNA response. This might indicate that the PBMCs have not been in contact with *C. burnetii.* This corresponds with our earlier results, which showed that *Coxiella* bacteria were not detectable in the blood after inoculation despite the infection of the trophoblasts of the placenta between 2 and 4 weeks after inoculation [19]. Studies in non-pregnant mice indicate that IFN-γ has a role in the early control of *C. burnetii* proliferation [12]. This is probably not true in pregnant goats because we were not able to detect an increase in IFN-γ producing cells in the weeks after inoculation. Therefore IFN-γ probably does not play a role in preventing *C. burnetii* replication in the early stages of infection in pregnant goats.

Cell-mediated immune responses were first detected at four weeks prior to parturition, and strongly increased in the first week after parturition. The mRNA levels of IFN-γ in blood cells increased one week later. Although mRNA levels and IFN-γ Elispot data cannot be compared directly, the IFN-γ response was induced before parturition due to *Coxiella* infection. The increase in the number of IFN-γ producing PBMCs after stimulation with *C. burnetii* antigen coincides with the increase in *C. burnetii* DNA in all tissues of *C. burnetii*-infected goats during pregnancy [19]. At that time in the pathogenesis of *C. burnetii* in pregnant goats, cell-mediated immune responses are probably induced. At the time of parturition, the *C. burnetii* DNA load in the tissues is maximal as previously shown [19], resulting in exposure to the systemic immune system. This probably caused the strong increase in the IFN-γ producing PBMCs at one week post parturition. However, parturition in non-infected goats also coincided with an increase in IFN-γ producing PBMCs as measured in the control goats. This could be an effect of the release of suppression of the cell-mediated immune response after parturition. During pregnancy progesterone levels are high and decrease shortly before parturition [28]. In humans it is suggested that high progesterone levels are associated with a Th-2 type immunity, resulting in an increased humoral immune response and a decreased cell-mediated immune response [29]. In pregnant goats we also assume a down regulation of the cell-mediated immune response due to progesterone during pregnancy. If the suppression is alleviated after parturition, a strong increase in IFN-γ producing cells can be expected. Overall, we assume that two mechanisms probably influence the cell-mediated immune response in *Coxiella*-infected pregnant goats, i.e. a stimulation by the release of *Coxiella* antigens and a suppression of cell-mediated immunity due to progesterone, with the overall effect that in infected goats an increased IFN-γ response was found.

The results of our study may have several implications for diagnostic applications of immunological tests for Q fever in pregnant animals. Cell-mediated immune responses did not differ enough to distinguish between *Coxiella*-infected and non-infected pregnant goats. IFN-γ

Elispot, LPT and an additional performed IFN-γ ELISA (data not shown, as results were even less discriminative) have no additional value in the diagnosis of Q fever in pregnant goats. The strong humoral response, however, is useful in the early detection of infected pregnant goats, because these goats cannot be diagnosed by the detection of *C. burnetii* [19]. The phase-specific IgM and IgG response might be useful in understanding the dynamics of Q fever in a herd, as animals in different stages of infection can be followed.

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Chapter 10

General discussion

Coxiella burnetii in pregnant goats

Coxiella burnetii is an intracellular bacterium that causes Q fever in humans as well as in animals. Q fever is regarded as an occupational disease but has a major public health impact when outbreaks occur. During the years 2007-2010 an unprecedented Q fever outbreak occurred in the Netherlands in which more than 4000 human cases were registered. During the outbreak period more than 40,000 people were assumed to be infected. Epidemiological investigations suggested abortive dairy goats and dairy sheep as the most probable source of the human outbreak. These abortions in dairy goats and dairy sheep had been occurring since 2005. However, confirmation of the epidemiological link between small ruminant Q fever abortions and human *Coxiella* infections is still lacking. This confirmation can be achieved by comparing the genomes of *C. burnetii* strains from humans and animal reservoirs.

With abortions in *Coxiella*-infected pregnant goats, high numbers of *Coxiella*-bacteria are excreted into the environment. Therefore, these abortions are considered the main risk factor for Q fever in humans. Other risk factors for human infections include the excretion of *C. burnetii* during normal parturition, as field studies suggest, and the excretion via faeces, vaginal mucus and milk. The excretion via faeces, vaginal mucus and milk is reported before and after parturition, but the correlation between these excretion routes is poor and a biological explanation for the occurrence of *C. burnetii* in these excretion products is still lacking. In addition, there is also little knowledge about humoral and cellular immune responses in pregnant goats after *C. burnetii* infection. Therefore, an accurate diagnosis of infected animals is difficult and this hampers the development of new intervention tools such as a new generation of vaccines.

The aims of the studies described in this thesis were to investigate the molecular epidemiology of *C. burnetii* in animal reservoirs during the recent large outbreak of Q fever in the Netherlands and the pathogenesis of *C. burnetii* infection in pregnant goats. Goals were to confirm the epidemiological link between goat and human infections, and to investigate possible additional reservoirs for human Q fever. This was done by characterizing *C. burnetii* outbreak isolates from infected goats and other potential animal reservoirs by genotyping. To better understand the role of goats in the outbreak, pathogenesis, excretion patterns and the immune responses to *C. burnetii* infection in pregnant goats were investigated. During three experimental infection studies in goats, inoculation routes, excretion of *C. burnetii* in relation to the pathogenesis and the humoral and cellular immune responses have been investigated. In these experimental studies a Dutch outbreak isolate of *C. burnetii* was used as the primary isolate of study.

Genotyping results of *C. burnetii* in the Netherlands

Finding the source of the Dutch Q fever outbreak

The link between human Q fever cases and abortions on dairy goat farms was already suggested in 2007, after the first outbreak of Q fever in the Netherlands [1]. Other known reservoirs for human Q fever include infected sheep, cattle, cats and dogs, but also horses and wildlife. The identification of animal species or a group of animals as outbreak source is nearly always based on epidemiological studies. These studies, however, are not able to identify causal relations. Therefore, sources identified by these studies should be regarded as 'probable sources' and need to be confirmed. Overall, the laboratory confirmation of the observed epidemiological links during Q fever outbreaks is scarce [Chapter 1]. First attempts to confirm the epidemiological sources as cause of infection have been made in the analysis of a Q fever outbreak in a psychiatric care institution in the Netherlands [2,3]. In this outbreak, MLVA typing of human and animal isolates linked infection of sheep and lambs with disease in humans. However, possible other than these ovine sources were not investigated. To address this issue in the Dutch Q fever outbreak, we performed genotyping studies on *C. burnetii* isolates from a large set of samples from dairy goats, sheep and cattle [Chapter 3, 4, 6], roe deer [Chapter 5] and dairy products [Chapter 7].

The genotyping results of dairy goats, sheep, cattle, roe deer and milk products samples clearly revealed the presence of different genotypes. Importantly, similar *C. burnetii* genotypes were observed for goats and human isolates. One MLVA type of *C. burnetii* was predominantly present on the investigated dairy goat farms, which represented 60% of all dairy goat farms with abortion problems [Chapter 3]. This MLVA type and a closely related MLVA type were also predominantly present in human patients in the outbreak area [4]. These results were confirmed with the slightly less discriminatory MST method [Chapter 4]. The results strongly indicate dairy goats as the main source of the Dutch human Q fever outbreak. Based on the genotyping results, sheep can be assumed as an additional source of human infection. Although only a few samples from sheep were genotyped, infected sheep carried the same or closely related to the genotypes in goats. In cattle and roe deer different genotypes were identified, which appeared unrelated to genotypes of *C. burnetii* in humans. Thereby it excludes these species as a possible source of the outbreak in humans (and goats) [Chapter 5, 7]. Although we obtained *C. burnetii* positive placental samples from dogs and horses, we were, unfortunately, not able to type these isolates due to the low infection rate. Therefore the contribution of these animal species to the human outbreak still remains to be evaluated [Chapter 6]. Considering the above, our results clearly confirm dairy goats and sheep as the source of the Dutch human Q fever outbreak.

Excluding C. burnetii *in cattle as zoonotic source*

C. burnetii is present in the majority of the Dutch dairy cattle herds. In samples originating from these herds and in dairy products, a distinct group of *C. burnetii* genotypes was found [Chapter 4, 6, 7]. With MLVA typing, this group clusters together and is only very distantly related

to the predominant Dutch outbreak genotype [Chapter 3, 6]. Using the MST genotyping method, this group of genotypes was indicated as MST20 [Chapter 4]. In goats and humans the cattle-related genotypes of *C. burnetii* were rarely detected [Chapter 3, 4, [5]. SNP typing confirmed the existence of a cattle-specific genotype as well as the rare presence of this genotype in other animal species, including humans. SNP types of *C. burnetii* from goats and cattle have been reported to be similar on mixed goat-cattle farms [6]. This may suggest that a farm-specific genotype may infect various animal species that are present on a farm.

The question can be raised as to whether cattle impose a potential reservoir for human Q fever. Especially because the number of dairy cattle is 10 times higher than the number of dairy goats in the Netherlands (2009: number of dairy goats 274,060; number of dairy cattle 2,734,412; http://statline.cbs.nl, access date 16 August 2012). In addition, the prevalence of *C. burnetii* on dairy cattle farms is two times higher than on dairy goat farms [Chapter 1]. So, potentially, dairy cattle are a huge reservoir of human Q fever and may pose an underestimated risk. Yet worldwide, only a few Q fever outbreaks are related to cattle [Chapter 1]. Several factors may contribute to the apparent low risk of the cattle reservoir. (i) Compared to sheep and goats, cattle seem to be less susceptible for clinical Q fever. Abortion is not a predominant clinical symptom in cattle, although we detected *C. burnetii* DNA in one out of four placentas from abortive cows [Chapter 6]. In addition, although *C. burnetii* is present in the majority of the herds, prevalence in individual animals is less than 10% [7]. (ii) Contrary to goats and sheep, cattle have no specific breeding and delivery season. This may result in an even excretion pattern of Q fever bacteria over the year in an infected herd, without peaks. The excretion level may be too low to infect humans in the surrounding areas, resulting in a low risk for public health. (iii) Cattle are normally housed on a slatted floor with a slurry pit, while dairy goats and sheep are normally housed in deep litter stables. This may result in lower dust levels and lower levels of *Coxiella*-contaminated aerosols. (iv) Finally, the cattle-specific genotype of *C. burnetii* may be less virulent for humans. Although it is still possible that humans get infected with this specific *C. burnetii* genotype and an immune response is initiated, clinical symptoms might be less severe or not present at all. Further research on this is needed to identify possible animal-specific *C. burnetii* virulence factors leading to different clinical expressions of the disease (from severe to none). Identification of the factors that influence the apparently low risk of human Q fever from the cattle reservoir may lead to a better understanding of the factors that lead to the risk small ruminants pose. This can help in a more effective Q fever risk management.

It is of interest to note that the genotype of *C. burnetii* that caused the human outbreak was surprisingly also present in one out of 97 genotyped cattle-related samples [Chapter 3, 6, 7]. It is not clear whether this is an incident or that this might be one of the first observations of the transfer of the outbreak genotype to the cattle population after the Q fever outbreak. If the latter is the case, an increase of the prevalence of the outbreak strain of *C. burnetii* in the cattle population may occur, although the appearance of cattle-specific genotypes of *C. burnetii*

suggests an adaptation of this genotype to cattle. This may imply that other genotypes have a lower ability to spread in the cattle population. However, it is not known if this coincides with a reduced virulence of the outbreak genotype of *C. burnetii* in cattle. It is also possible that the outbreak strain adapts to cattle and may spread through the cattle population. This can result in an increased risk for human Q fever. Therefore it is advisable to monitor the genotypes of *C. burnetii* that are present in the cattle population for the coming years, and thus the risk for public health.

Other possible reservoirs of C. burnetii

Dogs, horses and rats can be indicated as possible additional sources for human Q fever in the Netherlands. Surprisingly, in 4 out of 54 canine placentas *C. burnetii* DNA was detected [Chapter 6]. Additional research showed a seroprevalence in Dutch dogs of 23% and seroprevalences in the southern and western part of the country were significantly higher compared to the northern part (unpublished data). Unfortunately, we were not able to genotype canine strains [Chapter 6]. Dogs are of special interest because they live in close contact with humans and also because whelping often take place in household settings. In infected pregnant dogs, this can result in infecting owners and other household members, but also visitors. Because dogs are an important group of animals in urban areas and are normally not considered as a reservoir of human Q fever, they may be an underestimated source of human Q fever in urban areas. This may explain a part of the human cases in the western, urban part of the Netherlands (Figure 10.3). Taken together, it is advisable to further investigate the Q fever situation in dogs as these animals may be an unexpected source of human Q fever.

The predominant genotype of *C. burnetii* detected in goats was also found in six rats from three different locations [8]. Male rats can be infected with *C. burnetii* via the intranasal route [9]. In an environment where *C. burnetii* is abundantly present, for example on a farm with Q fever abortions, rats are likely to become infected with *C. burnetii*. Transmission routes within rat populations and to other hosts, including humans, have not yet been reported, but rats may play a role in maintaining Q fever on the farm level and may facilitate the transmission of *C. burnetii* between farms.

Excretion of *C. burnetii* in pregnant goats

Excretion of C. burnetii *via faeces, vaginal mucus and milk*

Up until now, the general assumption is that infected goats excrete *C. burnetii* during abortion and also via faeces, vaginal mucus and milk before and after abortion. Excretion via bodily fluids, however, is not well understood. In the studies described in this thesis we investigated the excretion of *C. burnetii* in experimentally infected goats. As described in Chapter 8, our results show that *C. burnetii* could not be detected in faeces or vaginal mucus before parturition. This is

contrary to what has been reported previously [10-13]. Excretion of *C. burnetii* via the faeces has been explained by active replication of *C. burnetii* in the liver and subsequent transport to the gut via the bile [10]. However, we found no evidence for active replication of *C. burnetii* in the gut and liver that could result in active excretion of *C. burnetii* in faeces before parturition. Neither did we find evidence for replication of *C. burnetii* in the vagina or other maternal parts of the genital tract during pregnancy. We did detect *C. burnetii* DNA in the faeces and vaginal mucus of goats before parturition, but this was only after the first kidding in the group had occurred. We believe that the parturition of the first goat in a herd is related to the positive excretion results in other goats that did not kid yet. With parturition in infected goats, high numbers of *C. burnetii* are excreted into the environment, as shown earlier for sheep [14]. This most probably causes cross-contamination of faecal and vaginal samples of other goats.

Two possible ways of contamination can be envisioned; First, especially vaginal mucus samples can become *C. burnetii* positive via direct contamination of the sample. We showed that samples taken from the box environment of the goats, including air swabs, were positive for *C. burnetii* DNA after parturition [Chapter 8]. During sampling of vaginal mucus, the swab must be transported through the air to the vagina and back. During this transport time the swab can become contaminated. The second possible route of contamination was already proposed in 1958, especially for faecal samples [14]. Welsh *et al* suggested passive contamination of the faeces due to the ingestion of contaminated food or swallowing of inhaled infectious material. In our experiments we were able to detect *C. burnetii* DNA in the air and water and on the floor of the box environment of the goats. Therefore, the ingestion and inhalation of *C. burnetii* is likely to occur once *C. burnetii* has been excreted with parturition. Based on the results described in this thesis, we assume that the detection of *C. burnetii* DNA in faecal and vaginal samples prior to parturition is due to contamination and is not an indicator of active excretion or infection.

After parturition, *C. burnetii* DNA was detected in faecal and vaginal mucus samples till the end of the experiment. This is in agreement with previous reports [Chapter 1, [10-12,15], although in these studies, the consistence in shedding via the different routes is poor and the underlying mechanism is unknown. As described in Chapter 8, we did not find any evidence for active replication of *C. burnetii* in the gut or liver after parturition which could give a biological explanation for the active excretion of *C. burnetii* in faeces. Excretion of *C. burnetii* via vaginal mucus in the first few days after parturition can be explained by the excretion of lochia. As shown in Chapter 8, *Coxiella* bacteria are present in amniotic and allantoic fluids as well as in the foetal membranes. With the clearing of the uterus in the first days after parturition these bacteria will be excreted and can be detected at high levels. However, we found no indication of replication of *C. burnetii* in the maternal parts of the placenta that could explain an active excretion of *Coxiella* bacteria after the period where lochia were excreted. However, we could still detect *C. burnetii* DNA in faeces and vaginal mucus, even though bacterial DNA had already disappeared from the genital tract, liver and gut. Therefore, we assume that the detection of *C. burnetii* DNA in

faeces and vaginal mucus after parturition is also due to contamination with *C. burnetii* from the environment. Thus, the presence of positive faecal and vaginal samples is more a reflection of the level of *C. burnetii* contamination in the sampling environment than the actual infection status of the animal.

Contrary to faecal and vaginal mucus samples, the presence of *C. burnetii* in milk samples does not seem to be directly influenced by environmental contamination [Chapter 8]. The excretion of *C. burnetii* ceased in accordance with the disappearance of *C. burnetii* DNA from the mammary gland. Milk samples that are aseptically taken can therefore be considered the best indicator of the infection status of goats.

The conclusion that the detection of *C. burnetii* DNA in faecal and vaginal swab samples does not represent the actual infection status of animals has consequences for the detection of infected animals. These samples should not be used in the diagnosis of *Coxiella* infection or in the determination of the status of infection of an individual animal because they lead to false positive results. Only aseptically taken milk samples represent the actual status of infection. Several publications focus on shedding routes of *C. burnetii* in sheep, goats and cattle [10,12,13]. In these publications an animal is considered infected when at least one of the three samples types (faeces, vaginal mucus and milk) is positive. This resulted in an overestimation of infected animals as the environmental contamination of animal samples was not taken into account. The conclusions drawn in these publications should be revised as: (i) *C. burnetii* is not actively excreted via faeces and vaginal mucus before and after parturition, (ii) bacterial shedding within herds is overestimated and (iii) parturition and milk are the shedding routes of *C. burnetii*.

Critical interpretation of results is of special importance in studies comparing shedding and serological responses [11,15,16]. In these publications a lack of serological response is shown in animals that excrete *C. burnetii* via faeces and vaginal mucus, concluding a lack of sensitivity of serological methods. However, taking into account the environmental contamination of faecal and vaginal mucus samples, this conclusion should be reconsidered.

In conclusion, the actual infection status of individual animals can best be determined via sampling birth material at the time of parturition or via sampling milk. To determine the actual infection status of a herd, several animals should be sampled at the time of parturition in addition to sampling the bulk tank milk. For an indication of the contamination status of the farm environment, faecal or vaginal mucus samples may be useful, but samples easier to obtain, e.g. dust samples, may better fit this purpose.

Excretion of C. burnetii *via parturition*

In the Dutch Q fever outbreak, abortive dairy goats and dairy sheep were considered the main risk factor for human Q fever. In our experimental studies, however, *C. burnetii* infected

goats gave birth to live or dead kids. High numbers of *C. burnetii* were excreted during abortion, but also during parturition of liveborn kids [Chapter 8]. This is a surprising result. Up until now all published results on goats experimentally infected with *C. burnetii* resulted in abortion or birth of stillborn kids. Field studies already indicated the excretion of *C. burnetii* during normal parturition, but this was never confirmed [Chapter 1].

For sheep this is different. Experimental studies already reported normal parturition after inoculation with *C. burnetii*. Also in several sheep-related outbreaks, normal lambing was indicated as a possible source [Chapter 1].

Our finding of both abortions and normal parturitions in infected goats may be explained by our use of a natural route of infection, which may influence the dose that enters the system. When the infectious dose is low only a few bacteria may be able to reach the trophoblasts of the placenta. The time needed for the bacteria to replicate to levels that abortion is induced may be longer compared to a situation when massive invasion of the trophoblasts takes place. When this time exceeds the residual pregnancy duration, liveborn kids will be delivered. Unfortunately, the factors that determine the foetal fate (i.e. abortion or normal delivery) in Q fever infected goats still remain unknown. Most importantly, our results indicate that not only abortions, but also normal parturitions from infected goats may cause excretion of *C. burnetii* in field situations. This implies that normal deliveries in infected goats also contribute to the environmental contamination and should be considered as a major zoonotic risk for humans. This risk has been underestimated up until now.

Pathogenesis of Q fever in pregnant goats

Knowledge on the pathogenesis of Q fever in goats will help to understand the disease and its epidemiology. Pathogenesis may depend on the inoculation route [17], however thus far, only the subcutaneous infection route has been used to study pathogenesis in goats. This may bias our understanding of the dissemination of the bacterium over the different organs of goats [Chapter 1]. Our studies indicate trophoblasts of the allantochorion as primary target cells of *C. burnetii* after intranasal inoculation [Chapter 8]. Bacterial replication was not detected in other organs of goats and kids. The amount of *C. burnetii* DNA in the organs of goats and kids increased towards parturition, after which it decreased to undetectable levels after 81 day post parturition in goats and after 28 days post parturition in kids [Chapter 8]. This is in agreement with earlier reports on goats infected via subcutaneous inoculation [18]. This suggests that the inoculation route has no major effect on the establishment of *Coxiella* infection in goats.

C. burnetii infection in pregnant goats results in a localized infection of the trophoblasts of the foetal part of the placenta. After parturition the goats were able to clear the *Coxiella* infection from their bodies, resulting in a gradual decrease of the *Coxiella* DNA positivity of the different organs. Even in a heavily contaminated box environment, we were not able to find indications for

a systemic infection of the non-pregnant goats. This is different in mice, guinea pigs and humans. In pregnant mice, *C. burnetii* is abundantly present in the foetal as well as in the maternal parts of the placenta [19]. Also in *Coxiella* infected non-pregnant mice, guinea pigs and humans a systemic infection occurs. In this systemic infection *C. burnetii* replicates in several organs, including the spleen [Chapter 1] (Figure 10.1). This difference in pathogenesis (i.e. localised versus systemic) may explain the lack of clinical symptoms (except for abortions) in goats and the presence of clinical symptoms of disease in mice, guinea pigs and humans. It also implies that trophoblasts in goats are needed for *C. burnetii* infection and that after parturition *C. burnetii* is eradicated from the goat's body by the shedding of the placenta. However, goats may become symptomless carriers of *Coxiella* bacteria. *C. burnetii* has been demonstrated in the oviducts and genital tissues of non-pregnant goats [20]. In addition, long-term excretion of *C. burnetii* has been demonstrated in the milk, despite the absence of *C. burnetii* antigen in the mammary gland [21]. Persisting *C. burnetii* may even be the source of trophoblast infection in subsequent pregnancies, as it has been shown that goats may experience reproductive failure and shed *C. burnetii* in successive parturitions [22]. However, he mechanisms of persistence and reinfection remain unknown. It can be speculated that the studies described in this thesis represent an acute response to *C. burnetii* infection in goats. Presumably, goats can get persistently infected, which could result in a more chronic state of infection. How this chronic state can evolve from an acute state, how it can be maintained, and if the chronic state of infection can cause reactivation of the infection, should be subject of future research.

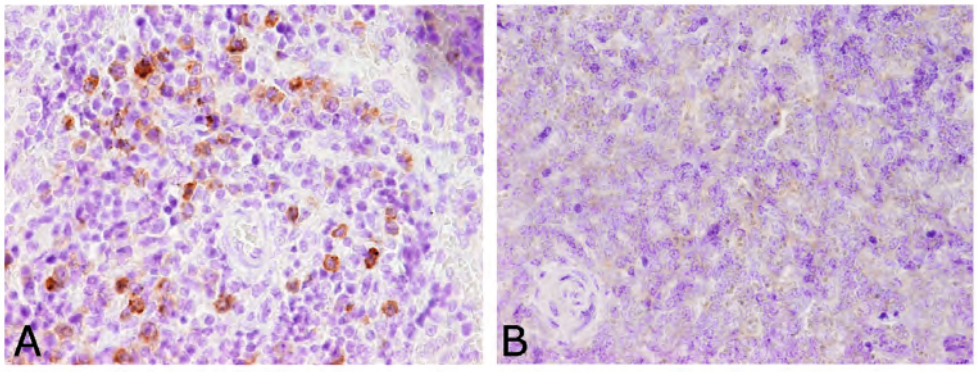

Figure 10.1. Detection of *C. burnetii* antigen in spleen sections of mice and goats after inoculation with *C. burnetii*. The immunohistochemical staining of *C. burnetii* antigen is described in Chapter 8. Presence of *C. burnetii* antigen is indicated by the brownish colouring.

A. *C. burnetii* antigen is present in a section the spleen of mice at day 9 after intraperitoneal inoculation of *C. burnetii* originating from a sheep.

B. *C. burnetii* is absent in a section of the spleen of a goat (ID 34) at day 69 after intranasal inoculation of *C. burnetii* originating from a goat.

Immune responses to *C. burnetii* after infection of pregnant goats

Immune responses to *C. burnetii* infection in domestic ruminants are poorly investigated. This is surprising, as in human medicine diagnosis of Q fever relies almost entirely upon serology [Chapter 1, [23,24]. Potentially, better understanding the immune responses to *Coxiella* infection could improve the diagnosis of Q fever in domestic animals. It could also give insight in why an infection leads to an infection of the trophoblasts but not to an infection of maternal tissues.

Our results indicate that in pregnant goats the antibody response is strong and already detectable at two weeks after inoculation. Cellular immune responses are less prominent, but measurable from 4 weeks pre-parturition [Chapter 9]. The immune responses, however, cannot prevent the replication of *C. burnetii* in the trophoblasts. An explanation is that the trophoblasts are part of the foetal placenta and as such out of reach of the maternal immune system. Also during transport from the inoculation side to the trophoblasts, *C. burnetii* is probably able to survive. This can be explained by the cells that transport the bacteria. After aerosol infection in humans and mice it is assumed that alveolar macrophages and other mononuclear phagocytes are the primary target cells of *C. burnetii* [25]. After internalisation, the bacterium can survive intracellular killing and can replicate in the acidified phagosome (Figure 1.2)[Chapter 1]. *C. burnetii* is probably transported with these mononuclear cells. In our experiments, however, we were not able to detect *C. burnetii* in the blood after inoculation [Chapter 8]. A search for *C. burnetii* in mononuclear cells in the blood was also without success (unpublished data), but we were able to detect *C. burnetii* in foetal macrophages (Figure 8.3). This suggests that in goats *C. burnetii* can also be transported by macrophages, but that the number of *Coxiella* bacteria transported by the mononuclear phagocytes is under the detection limit of the test.

Another interesting finding during our studies was the increase in *C. burnetii* DNA positivity in tissues towards parturition and the decrease after parturition in the goats, the foetuses and kids [Chapter 8]. In the tissues, *C. burnetii* was only detectable by PCR and no indication of active replication, e.g. intracellular replication vacuoles and influx of immune cells, could be detected. This may indicate that the positive signals reflect infected macrophages and other monocytes that had migrated into the different tissues rather than infection of the tissue itself, as suggested by Shannon and Heinzen Shannon [25]. Indeed, we showed that foetal macrophages in the allantochorion can contain *C. burnetii* (Figure 8.3). However, as mentioned above, we could not detect *C. burnetii* DNA in these cells in the blood.

In pregnant goats the primary target of *C. burnetii* are the trophoblasts of the placenta. It can be speculated that during the replication process the trophoblast layer ablates and cell slurry containing *Coxiella*, that borders the maternal uterus mucosa, is ingested by antigen recognizing mobile cells. These cells may disseminate and accumulate in the tissues, resulting in detectable levels of *C. burnetii* in various organs. How *C. burnetii* (antigen) disappears from the organs is not clear yet. The *C. burnetii* containing macrophages may migrate from the tissues, for example to the udder, where they may be excreted into the milk. It is also possible that the macrophages

are able to actively kill and eliminate the internalized bacteria under the influence of IFN-γ [26], when the suppression of the cell-mediated immune response is released after parturition.

Investigation of the humoral immune response to *C. burnetii* infection revealed phase-specific antibodies of IgM and IgG immunoglobulin classes [Chapter 9]. The development of antibody titres seemed to occur in two distinct intervals. The first interval was between two weeks post inoculation and six weeks post inoculation. In this period titres of IgM and IgG against phase 2 *C. burnetii* increased, while titres of IgG against phase 1 *C. burnetii* were still low. This interval can be considered as the acute response to *C. burnetii* infection. In the second interval, between six and twelve weeks post inoculation, IgG phase 2 and IgG phase 1 antibodies increased, while IgM phase 2 antibodies diminished. This second interval can be considered as the prolonged response to *C. burnetii*, although it is not known how the IgG phase 1 specific responses will develop over longer periods of time. In the laboratory diagnosis of human Q fever, the phase-specific IgM and IgG responses are important to distinguish between acute and chronic disease [23,24]. As an acute presentation and chronic presentation of Q fever infection may also occur in goats (as proposed in the text above), serology might be helpful in the identification of these presentations.

This of special importance for the detection of acute infections in pregnant animals as they represent the main risk for human Q fever. These animals do not excrete *C. burnetii* and can therefore not be identified by the detection of the agent. In this situation a strong anti-phase 2 IgM and IgG response will identify these animals, so appropriated measures can be taken, for example by containing these animals during parturition. The role in the epidemiology of Q fever of chronically infected animals can also be better assessed as these animals can now be identified by a strong anti-phase 2 IgG and anti-phase 1 IgG response. It would be interesting to see if chronically infected animals continue to shed *C. burnetii* in subsequent lambing periods.

The presence of an antibody response against *C. burnetii* during natural infection in goats is also relevant for the application of vaccines. In the control of the Dutch Q fever epidemic, goats and sheep were been vaccinated with a *C. burnetii* phase 1 inactivated vaccine. One major drawback of this vaccination is that we currently cannot distinguish the response to the vaccine from the immune response from animals that have been exposed to a natural infection. This problem might be solved by the use of novel phase-specific IgM/IgG tests.

At this time, knowledge of the phase and subclass specific immune responses after vaccination of goats with a phase 1 *C. burnetii* vaccine is insufficient and merits further investigation. In humans, the predominant response to Q fever vaccination seems to be IgM against phase 1 [27], but in mice antibodies against both phase 1 and phase 2 have been detected [28]. For ruminants, no information is available about the type of immune response after vaccination and whether this is dependent on the vaccine used and the fact whether an animal is pregnant or not.

Another solution to differentiate between infected and vaccinated animals is to develop specific vaccines that can differentiate between these two (DIVA vaccines). In this development

the main drawbacks of the currently available *C. burnetii* phase 1 whole bacterium vaccine (production under BSL3 conditions, not effective in already infected or pregnant animals) may hopefully also be solved.

Goats as experimental animals for Q fever

In the experiments described in this thesis, goats were used to study the pathogenesis of Q fever, the excretion of *C. burnetii*, and the immune response to *C. burnetii* infection [Chapters 8 and 9]. As *C. burnetii* is a biosafety level (BSL) 3 organism [Chapter 1], these studies were performed in BSL3 animal facilities. This had several practical implications. Although experimental studies on Q fever in goats have been published [10,18,29], these practical implications are poorly addressed. However, they can influence the study set-up and outcome and are therefore of major importance.

We experienced that the origin of the goats is a key factor of a successful study. Originally we used farm raised Dutch dairy goats, as these animals are considered the *C. burnetii* reservoir in the Dutch Q fever outbreak. However, farm-raised pregnant dairy goats are used to an energy-rich diet. This diet cannot be fed in the BSL3 facility due to the logistic limitations to prevent the spread of pathogens. As goats are very choosy, they easily refuse to eat after a dietary change. Especially in goats in a good condition this may result in pregnancy ketosis, which leads to a complete loss of appetite and to recumbency. Finally, the loss of the animal is inevitable. We faced this problem in our first experiment. A change to goats raised in experimental facilities and used to a specific diet, solved the problem [Chapters 8 and 9]. Apart from this, the mandatory vaccination against *C. burnetii* was another complicating factor in the use of farm raised Dutch goats, as unvaccinated animals were needed in our experiments. In 2010 and 2011 unvaccinated Dutch dairy goats were not available which made a change to another source of experimental goats necessary.

Sampling and sampling logistics under BSL3 conditions are also an important point for consideration. The samples should be processed in a BSL3 laboratory. For this, well-trained animal samplers and laboratory personnel are needed [Chapter 1] and special attention should be given to the transfer of samples from the animal facilities to the laboratory. A good example of the efforts taken to optimize sampling was the sampling of milk. In the field, goats are milked once or twice a day. To closely follow the excretion of the *Coxiella* bacterium in the milk as it occurs in the field, milking at least once a day was required. To make this feasible for several weeks, we built a dedicated milking facility in the box of the goats under BSL 3 conditions, as shown in Figure 10.2. With the goats in this dedicated milk facility we were able to machine milk the goats for more than three months. These kind of solutions resulted in valuable observations as presented in Chapters 8 and 9 and discussed above.

The studies with goats presented in this thesis differed in several ways from earlier published work. Firstly, we used the intranasal route of inoculation. Secondly, we used weekly sampling from the start until the end of the experiment. This gave us the opportunity to collect data before parturition. The frequent sampling resulted in a sampling date just before the first parturition and a next sampling date in-between the first parturition and second parturition in the group. This gave insight in the effect of parturition on the results of the samples. Thirdly, we obtained a considerable amount of tissues to closely follow the dissemination of *C. burnetii* in the goat after inoculation fortnightly. Finally, we analysed the samples during the experiment. To clarify and confirm results it was possible to take additional samples during the experiment e.g. internal swabbing of the vaginal mucus and sampling of the environment to investigate the origin of the *C. burnetii* DNA positivity of faecal and vaginal mucus samples [Chapter 8].

The challenge in future experimental infections is to cope with the environmental contamination after the first parturition of *C. burnetii* infected animals. When bacteria from the environment are likely to interfere with the experiment, ways should be found to deal with this. This is not only the case for Q fever, but is of likewise importance for other agents that can survive in the environment, such as *Chlamydia abortus* and *Chlamydia psittaci*.

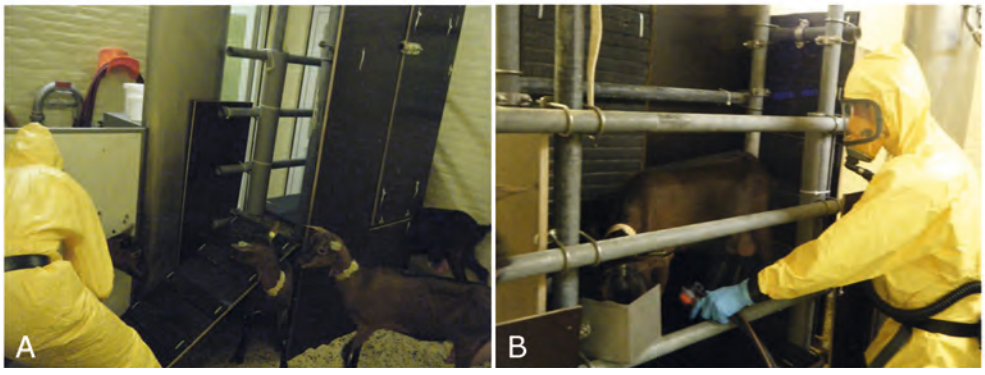

Figure 10.2. Dedicated milking facility for machine milking *C. burnetii* infected goats under BSL3 conditions.
A. A goat is driven into the milking facility by a technician in a full protection suite with a P3 filtered mechanical ventilated face mask.
B. The goat is being fed and machine-milked by the technician.

Impact of the results of this thesis

The impact on Q fever control in the Netherlands

The studies described in this thesis were initiated because of the unprecedented outbreak of human Q fever in the Netherlands [Chapter 2]. The human outbreak started in 2007. A series of veterinary precautions were taken, with the goal to reduce the human exposure to *C. burnetii* excreted by dairy goats and dairy sheep during abortions. Control measures included notification of abortions in dairy goats and dairy sheep and additional notification of *C. burnetii* DNA bulk tank milk positive farms, hygiene measures, vaccination and culling of pregnant animals on Q fever positive farms [Chapter 2]. In 2010, the number of human cases of Q fever dropped significantly compared to 2009 and this decline continued in 2011 and 2012. Also, the number of first registrations of bulk tank milk positive farms decreased over the years 2009-2012 (Figure 10.3, Table 10.1). These numbers indicate that the human Q fever outbreak in the Netherlands has almost ended [30].

An important question for policymakers is to what extent the veterinary measures contributed to the observed decline in human cases. With the results presented in this thesis several of the implemented precautions can be evaluated. Most control measures that have been taken focused on dairy goats and dairy sheep [Chapter 2]. Genotyping data confirm *C. burnetii* present in dairy goats and sheep as the primary cause of the Dutch human Q fever outbreak [Chapter 3,4,5,6]. This justifies the focus of the measures on dairy goats and sheep.

The initial notification criterion for dairy goats and sheep was an abortion rate >5%. In October 2009, *Coxiella* bulk tank milk positivity was added as an additional criterion [Chapter 2]. This addition was made to improve the identification of Q fever risk farms. The initial abortion rate criterion was set at 5% because the average annual rate of abortions in small ruminants that was assumed to be normal was approximately 5%. However, abortion rates are sometimes hard to assess, especially in large herds, making the abortion rate criterion subjective.

For that reason, experts advised to implement a more unbiased notification criterion. The bulk tank milk criterion is a more objective criterion to identify risk farms as it is an indication of the excretion of *C. burnetii* in the milk on herd level and is measured by PCR in the laboratory. The results presented in this thesis show an additional reason to add a notification criterion. In Chapter 8 we showed that infected goats not only aborted, but also gave birth to liveborn kids. During these normal parturitions the same amount of *C. burnetii* was being excreted as during abortions. This makes the initial criterion of notification on abortion rate too narrowly defined to include all animals that pose a risk to humans. Therefore, the addition of the bulk tank milk positivity as a notification criterion can be justified.

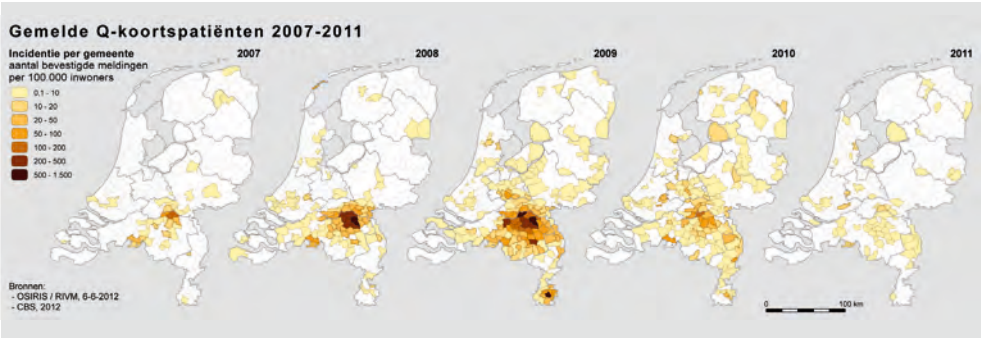

Figure 10.3. Incidence of notified Q fever patients by municipality in 2007-2011. Over the years 2007-2009 an increase in incidence over a larger area is visible followed by a decline over the years 2010 and 2011 (data kindly provided by Wim van der Hoek and Frederika Dijkstra, RIVM (http://rivm.nl/Onderwerpen/Ziekten_Aandoeningen/Q/Q_koorts).

Table 10.1. Overview of the number of human and veterinary confirmed Q fever cases over the years 2005- 2012 [Chapter 2] (additional data kindly provided by Wim van der Hoek and Frederika Dijkstra, RIVM (http://rivm.nl/Onderwerpen/Ziekten_Aandoeningen/Q/Q_koorts) and by Arco van der Spek and Dennis Bol, NVWA).

	Human		Veterinary			
year	number of notified cases	number of deaths (RIVM data)	confirmed Q fever abortion farms		confirmed BTM Coxiella burnetii positive farms[1]	
			dairy goat	dairy sheep	dairy goat	dairy sheep
2005	5	n.r.	2	-	n.r.	n.r.
2006	10	n.r.	6	1	n.r.	n.r.
2007	168	0	7	-	n.r.	n.r.
2008	1000	1	7[2]	1	n.r.	n.r.
2009	2354	7	6	-	62	-
2010	504	11	-	-	29	3
2011	81	5	-	-	8[3]	-
2012[5]	53	1	-	-	3[4]	-

1 : first registrations, n.r.: no registration, - : no cases, 2 : including one farm with 2 locations, 3 : 4 first registrations and 4 farms that became negative and then positive again, 4 : 2 first registrations and 1 farm that became negative and then positive again, 5 : till July 2012.

The most drastic measure to prevent the excretion of *C. burnetii* from infected pregnant goats to humans was the culling of all pregnant animals on bulk tank milk positive farms. Veterinary experts debated on the issue if infected pregnant goats could be distinguished from non-infected pregnant goats [Chapter 2]. When the non-infected goats could be distinguished from the infected goats, targeted culling of the infected pregnant goats (which are the high risk animals for human Q fever) could be considered, saving the lives of non-infected pregnant goats. However, at the time of the discussion in 2009, results obtained from published studies were not clear

about the epidemiological sensitivity of the PCR on faeces and vaginal mucus samples, although it was indicated that *C. burnetii* was possibly excreted before parturition [10].

The results obtained in this thesis clearly show that infected pregnant goats cannot be distinguished from non-infected goats by the detection of the agent, as *C. burnetii* is not excreted prior to parturition [Chapter 8] making the detection impossible. Serological investigations could have solved this problem, but the majority of the goats were vaccinated at that time and sero-negative shedders had been suggested [31]. However, based on the current knowledge, goats could not have been saved from culling based on the detection of *C. burnetii* in excretion products. What might have helped were serological investigations to selected pregnant goats that were sero-negative, assuming that the detection of sero-negative shedders is an artifact due to environmental contamination of the samples.

Impact for other diseases

An disease that closely resembles the clinical presentation of Q fever in small ruminants is chlamydiosis. The etiological agent is *Chlamydia abortus*. Bacteria belonging to the genus *Chlamydia* (*C. psittaci*, *C. abortus*, *C. pecorum*, *C. felis*, *C. caviae*, *C. pneumoniae*, and *C. suis*, *C. trachomatis* and *C. muridarum* [32] have much in common with *C. burnetii*: (i) an intracellular lifecycle, (ii) a spore-like form (elementary bodies) and a replicative form (reticulate bodies), (iii) the resistance to environmental stress and (iv) the zoonotic potential, especially of *C. abortus* and *C. psittaci*.

As it is for *C. burnetii*, it is also important for the zoonotic Chlamydia to have a applicable genotyping system that can be used in human diagnosis as well as in veterinary diagnosis. Such a system can be used in source identification and source confirmation. Although several genotyping tools for *C. psittaci* have been developed [33,34 ,35] none of these is widely used at the moment. Also the lack of knowledge about the genetic background of *C. psittaci* and *C. abortus* present in the Netherlands hampers the evaluation of the discriminatory power of the available typing systems and hampers a focussed outbreak management when it is needed. It is advisable to implement a typing system that can be used both in the human and in the veterinary field and to investigate the genetic background of the bacteria present in ruminants for *C. abortus* and in birds for *C. psittaci*.

As we showed in this thesis, the contaminated environment can influence the results of samples taken from individual animals. The elementary bodies of Chlamydia species can also survive for some time in the environment and can be aerosolized. Therefore, they can potentially contaminate diagnostic samples. This should be taken into account in the setup of animal experiments and when samples are taken for diagnostic purposes.

Abortions in small ruminants can be caused by *C. burnetii* and *C. abortus,* but also by several other zoonotic agents, suc as *Brucella* spp, *Campylobacter* spp, *Listeria* spp and *Toxoplasma*

gondii. Except for *Brucella*, they all are present in the Netherlands [36]. It is not known to what extent these micro-organisms are excreted during normal parturition and if normal parturitions pose a human health risk. This should be taken into account when the need for a monitoring system for these agents is evaluated, because this influences the choice between a passive (based on abortions) or an active surveillance system.

Future scientific perspectives

Results presented in this thesis have several future perspectives:

(i) Normal parturitions from infected pregnant goats should be taken into account as an important excretion route of *C. burnetii*. These normal parturitions may imply a risk for humans as well. In outbreak investigations and outbreak management, these normal parturitions should be taken into account to reduce the risk for the human population.

(ii) The available genotyping tools for *C. burnetii* are already useful in outbreak investigations and molecular epidemiology, although development and improvement of techniques are still ongoing. Especially for agents causing airborne diseases that survive in the environment and that can be transported over significant distances, confirmation of epidemiological links is necessary. For example, recently the number of human Q fever cases in Friesland, in the North of the Netherlands, increased (http://www.ggdfryslan.nl/sjablonen/2/infotype/news/item/view.asp?objectID=7083, access date 13 August 2012). A common source for this increase has not yet been identified. Genotyping of the involved *C. burnetii* may have provided this insight.

(iii) Results clearly identify the allantochorion at the basis of the cotyledon as the part of the placenta to detect *C. burnetii* by IHC after abortion or parturition [Chapter 8]. It should be stressed that *C. burnetii* is not present in the centre of the cotyledon, not even in abortion cases. Therefore, samples from the centre of the cotyledon should not be used for Q fever laboratory diagnosis on placentas. Although *C. burnetii* can be detected in the trophoblasts in every part of the allantochorion at certain time points, the trophoblasts ablate from the allantochorion in more progressed stages of infection. Therefore, infections can be missed. So, to improve the diagnosis of Q fever by IHC, samples from the allantochorion at the basis of the cotyledon should be investigated.

Several questions about *C. burnetii* in goats remain to be addressed. For the epidemiology and transmission of *C. burnetii* to humans it should be clarified if parturition, with the excretion of high numbers of *C. burnetii*, can occur in absence of abortion in a *C. burnetii* infected herd. Although monitoring of *C. burnetii* on herd level, for example in the BTM, may be an indication for this, it is not known how many normal parturitions with the excretion of *C. burnetii* occur. Knowledge of the mechanism as to how *C. burnetii* evades the immune system and reaches the trophoblasts of the allantochorion of the foetal placenta can help to prevent Q fever infections in goats, for instance by the development of improved DIVA vaccines. With this knowledge

intervention strategies can be designed that help prevent *Coxiella*-infections in the future. Also, the factors that determine the foetal fate in *C. burnetii* infected pregnant goats, as well as how goats can become persistently infected with *C. burnetii*, are still unexplained. Finally, the role of infected non-pregnant goats in the epidemiology of Q fever and other animals as potential source of infection needs to be investigated to eradicate Q fever in goat herds and prevent unexpected outbreaks from other animal sources.

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Summary

Coxiella burnetii is the causative agent of Q fever. Since it was first recognised as a disease in the 1930s, knowledge about the agent and the disease itself has increased, although knowledge gaps are still present. Therefore the name Q(uery) fever still holds true.

Chapter 1 introduces the agent and the disease as background information to provide a better understanding of the studies presented in this thesis. *C. burnetii* is a bacterium which naturally replicates inside human or animal host cells. On the surface of the bacterium lipopolysaccharide (LPS) is present. The LPS has two phenotypic expressions. The Phase 1 phenotype expresses full length LPS, while the Phase 2 phenotype expresses a truncated form, lacking the O-antigenic region. This difference is of interest, as only Phase 1 is highly virulent and specific antibodies can be generated against these two phases.

The clinical presentation of Q fever varies per host species. *C. burnetii* infection in animals is mainly asymptomatic but in pregnant ruminants abortions can occur. In humans the disease is also mainly asymptomatic. Clinical presentations include acute and chronic Q fever and the post-Q fever fatigue syndrome.

Q fever is a zoonotic disease with animal reservoirs, especially domestic ruminants, which are the main source for human Q fever. Q fever has a major public health impact when outbreaks occur. In outbreaks, source identification nearly always relies on epidemiological studies and laboratory confirmation was scarce until the start of this research.

Similarities and differences between *C. burnetii* strains can best be assessed via their genetic heterogeneity. Several techniques are described for this, of which multiple locus variable number tandem repeats analysis (MLVA) and multispacer sequence typing (MST) are widely used. These two techniques can be applied to clinical samples, so an initial isolation and cultivation step is not necessary. This increases the usability in outbreak investigations.

Knowledge of the pathogenesis in and excretion of *C. burnetii* from infected animals is crucial in understanding the transmission routes and risks of human infection. Abortion is considered the main excretion route of *C. burnetii* in goats, although excretion of the bacterium via faeces, vaginal mucus and milk has also been reported. Excretion in goats, however, is not well understood and has not yet been investigated in relation to a natural infection route. Also, knowledge on the humoral- and cellular immune responses in goats upon *Coxiella* infection is limited. Knowledge about the pathogenesis, excretion and immune response will help to improve surveillance systems, control outbreaks better and improve the efficacy of vaccines and vaccination strategies.

The aim of this thesis is to investigate the epidemiology and pathogenesis of *C. burnetii* infection in goats during the recent large outbreak of Q fever in the Netherlands. The main objectives are to characterise *C. burnetii* outbreak isolates from infected goats and other animals using geno-

typing and to understand the pathogenesis, excretion patterns, and immune responses during experimental infection of pregnant goats.

In **Chapter 2** the Dutch Q fever history is described. This provides the context in which the studies presented in this thesis are performed. Seroprevalence studies suggest that Q fever was endemic in the Netherlands decades before the first Q-fever-induced abortions in dairy goats and dairy sheep. During this period no human or animal Q fever outbreaks were noticed. In 2005, two years before the first human Q fever outbreak, clinical Q fever in animals was diagnosed for the first time in two dairy goat herds. Between 2005 and 2009, Q fever abortions were registered on 30 dairy goat and dairy sheep farms. In 2007 the first human Q fever outbreak in the Netherlands was registered with 168 human cases. In this outbreak a relation with abortive goats in the surrounding area was assumed. A total of 3,523 human cases were notified between 2007 and 2009, making the Dutch Q fever outbreak the largest laboratory-confirmed Q fever outbreak reported worldwide. Implemented control measures aimed to identify potential risk farms, to prevent excretion of *C. burnetii* from dairy goats and sheep and to prevent transmission of the bacterium from these farms to the human population. Overall, it is important to note that the Dutch Q fever outbreak emerged from an endemic state.

Although all epidemiological evidence pointed towards small ruminants as the source of the Dutch human Q fever outbreak, laboratory confirmation was still lacking. In **Chapter 3** we determined the genetic background of *C. burnetii* in domestic ruminants deemed responsible for the human outbreak by MLVA genotyping. One unique MLVA genotype predominated in dairy goat herds, representing 60% of all dairy goats herds with abortion problems, and in one sheep herd in the human Q fever outbreak area in the South of the Netherlands. On the basis of 4 loci, this genotype was similar to a human genotype from the Netherlands. We hypothesised that this uniform and worldwide unique genotype played a key role in the Dutch Q fever outbreak. In **Chapter 4** this hypothesis was confirmed using MST. In outbreak samples from humans, goats and sheep the same genotype (MST33) was present. By contrast, a different genotype (MST20) was present in samples from cattle.

To further assess the role of different potential reservoirs for human Q fever, roe deer as well as cats, dogs, pigs and horses were investigated. Roe deer were investigated as representative for Dutch wildlife. Results in **Chapter 5** show that *C. burnetii* DNA was detected in 23% of the investigated roe deer carcasses from 2008-2010, indicating a sylvatic cycle for Q fever. Two MLVA-genotyped roe deer strains differed from human and domestic dairy animal strains, including the predominant strain involved in the Dutch Q fever outbreak. In **Chapter 6** placentas originating from cats, dogs, horses, pigs, goats, sheep and cattle and collected in 2011, were investigated for the presence of *C. burnetii* DNA. Molecular characterisation was performed to show a possible correlation between the genotypes obtained and the Dutch outbreak genotype. *C. burnetii* DNA was detected in canine, equine, ovine and bovine placentas, but not in feline, porcine and

caprine placentas. The negative results in the caprine placentas most probably indicate the effectiveness of the control measures, especially vaccination, to prevent the shedding of *C. burnetii* during parturition. The control measures had been implemented since 2008. Unfortunately, the *Coxiella* DNA load in placentas from dogs and horses was very low and insufficient for genotyping. In sheep, one isolate was found having the predominant Dutch genotype related to the major outbreak. In cattle, one outbreak genotype as well as six bovine-related genotypes were detected. These findings suggest that roe deer, dogs, horses, sheep and cattle can be reservoirs for *C. burnetii*. Genotyping results suggest species-specific genotypes for roe deer and cattle, which are unrelated to the Dutch outbreak genotype. However, the outbreak genotype was detected in one bovine placenta.

To address the lack of knowledge about the genetic background of *C. burnetii* present in dairy cattle, consumer milk products were analysed for the presence of *C. burnetii* DNA in **Chapter 7**. *C. burnetii* DNA was detected in a broad range of milk products from European and non-European countries. Genotyping results confirmed the presence of a specific cluster of bovine-related genotypes of *C. burnetii* that are only found incidentally in humans.

Although *C. burnetii*-infected dairy goats were the major source of the Dutch human Q fever outbreak, tissue dissemination and excretion pathways of the pathogen in goats were poorly understood. **Chapter 8** describes the studies to better understand this. Pregnant goats were infected via the intranasal route and a strong tropism of *C. burnetii* towards the trophoblasts of the placenta was demonstrated. *C. burnetii* was not excreted before parturition. Infected goats gave birth to live and dead kids. Massive excretion of *C. burnetii* occurred during abortion, but also during parturition of liveborn kids. Therefore, apart from abortions, normal deliveries in *C. burnetii*-infected goats should also be considered as a major zoonotic risk for human Q fever.

In **Chapter 9** the humoral and cellular immune response upon *C. burnetii* infection of pregnant goats is described. After intranasal infection, a strong anti-*C. burnetii* Phase 2 IgM and IgG antibody response was observed. Anti-*C. burnetii* Phase 1 IgM responses were less pronounced, succeeded by an anti-*C. burnetii* Phase 1 IgG response. Cell-mediated immune responses were measured around parturition, but seemed to play a minor role in the host response of pregnant goats.

In the general discussion (**Chapter 10**), the major findings presented in this thesis are discussed in relation to the Dutch Q fever outbreak within the scope of the international literature. One of the important outcomes highlighted in the discussion is the confirmation via genotyping that dairy goats and sheep were the primary source of the Dutch human Q fever outbreak. No evidence for additional sources for human Q fever was found. In cattle, a specific cluster of bovine-related genotypes was detected which is only distantly related to the predominant Dutch outbreak genotype and only incidentally found in humans. However, it is advisable to monitor the genotypes

of *C. burnetii* present in cattle for the coming years, as the outbreak genotype was detected in one of the cattle-related samples. A second important outcome is the confirmation of what has been suggested in field observations: *C. burnetii*-infected goats are able to give birth to live kids. High numbers of *C. burnetii* were excreted during parturition of liveborn kids, comparable to the excretion during abortion. This implies that normal deliveries in infected goats also contribute to the environmental contamination and should be considered as a major zoonotic risk for humans. Thirdly, excretion of *C. burnetii* during parturition is the most important excretion route. Faecal and vaginal mucus excretion before parturition was not observed. Excretion via faeces and vaginal mucus after parturition can be questioned, as active replication of *C. burnetii* was not observed and environmental cross-contamination was more plausible. As a consequence of this, the conclusions of several published studies on the excretion of *C. burnetii* should be reconsidered. Finally, the detection of *C. burnetii* phase-specific IgM and IgG antibodies may be useful in the early detection of *C. burnetii*-infected pregnant goats and may contribute to the understanding of the herd dynamics of Q fever.

Samenvatting

Coxiella burnetii is een bacterie die de ziekte Q-koorts veroorzaakt. Q-koorts werd voor het eerst herkend als ziekte in Australië in de jaren 30 van de vorige eeuw. Sindsdien is de kennis over de bacterie en de ziekte toegenomen, hoewel veel vragen nog onbeantwoord zijn. Daarom doet de ziekte haar naam als vraagteken-ziekte (Q staat voor Query, vraagteken) nog steeds eer aan.

In **hoofdstuk 1** wordt achtergrondinformatie gegeven over de bacterie en de ziekte om het onderzoek dat in dit proefschrift wordt beschreven te introduceren. *C. burnetii* is een bacterie die zich onder natuurlijke omstandigheden vermenigvuldigt binnenin cellen van mensen en dieren. Deze intracellulaire vermenigvuldiging komt ook voor bij virussen, parasieten en enkele andere bacteriesoorten.

Een kenmerk dat *C. burnetii* met veel andere bacteriën gemeen heeft is de aanwezigheid van lipopolysacharide (LPS) aan de buitenkant. Het LPS kan bij *C. burnetii* twee verschillende vormen aannemen: fase 1 en fase 2. Deze twee vormen zijn van belang omdat zowel tegen fase 1 als tegen fase 2 afweerstoffen kunnen worden gemaakt door geïnfecteerde mensen of dieren.

De ziekte Q-koorts kan op verschillende manieren tot uiting komen. Bij gezonde, nietdrachtige dieren verloopt Q-koorts meestal zonder symptomen, de dieren zijn dan niet ziek. Bij herkauwers (koe, geit en schaap) kan voortijdige vruchtafdrijving (abortus) of vroeggeboorte optreden, maar dit is niet altijd het geval. Bij mensen verloopt een infectie met *C. burnetii* meestal ook zonder symptomen. Als mensen ziek worden, wordt onderscheid gemaakt tussen een acuut of chronisch ziektebeeld of het post-Q-koortsvermoeidheidssyndroom.

Q-koorts is een zoönose (een ziekte die van dieren op mensen kan overgaan) waarbij meestal schapen of geiten de bron zijn voor ziekte bij mensen. Hoewel individuele ziektegevallen bij mensen voorkomen, zijn het vooral uitbraken van Q-koorts die de aandacht trekken. Wereldwijd komen uitbraken van Q-koorts regelmatig voor. Het aantal patiënten kan variëren van een tiental tot enkele honderden. De bron van Q-koortsuitbraken bij mensen wordt meestal gevonden via epidemiologisch onderzoek, maar dit werd tot nu toe slechts zelden bevestigd door laboratoriumonderzoek.

C. burnetii-stammen kunnen van elkaar worden onderscheiden op basis van verschillen in genetisch materiaal (DNA). Om onderscheid tussen stammen te maken zijn verschillende technieken beschreven, waaronder de multiple locus variable number tandem repeats analysis (MLVA) en multispacer sequence typing (MST). Doordat deze twee technieken kunnen worden toegepast op monsters van dieren of mensen zijn ze geschikt om in uitbraaksituaties de bron van een besmetting vast te kunnen stellen. Op deze manier kan het zogenaamde genotype van een *C. burnetii*-stam worden bepaald.

Kennis over de verbreiding van *C. burnetii* in dieren en over de uitscheiding van *C. burnetii* door dieren is van groot belang om te begrijpen hoe de overdracht van de bacterie naar mensen

plaatsvindt en om de risico's van overdracht in te schatten. Bij schapen wordt de bacterie uitgescheiden tijdens abortus maar ook tijdens de geboorte van normale lammeren. Bij geiten wordt verondersteld dat na een infectie met de Q-koortsbacterie altijd abortus optreedt. Uitscheiding van de bacterie zou ook kunnen plaatsvinden via de mest of melk. Het mechanisme van uitscheiding na natuurlijke infectie bij geiten is echter niet duidelijk. Tevens bestaat er weinig kennis over de afweerreactie bij geiten na een infectie met de Q-koortsbacterie. Met de kennis over de verbreiding, uitscheiding en afweerreactie van dieren kunnen bewakingssystemen voor Q-koorts beter worden ingericht. Ook zal de kennis leiden tot verbetering van de bestrijding van uitbraken van Q-koorts en aanpassing van vaccins en vaccinatiestrategieën.

Dit proefschrift richt zich op het bestuderen van de verspreiding van *C. burnetii* tijdens en na de grote Q-koortsuitbraak in Nederland. Hiertoe werd het genotype bepaald van *C. burnetii* afkomstig van besmette geiten en andere mogelijke besmette diersoorten.

Daarnaast werd onderzocht hoe *C. burnetii* zich in de geit verbreidt na infectie, in welke cellen de bacterie zich precies vermenigvuldigt en hoe het geitenlichaam daarop reageert. Ook werden de uitscheidingsroutes bestudeerd omdat op die manier de bacterie uiteindelijk bij mensen terecht kan komen. Het ziekteproces in de geit en de uitscheiding van de bacterie werden bestudeerd tijdens proeven met drachtige geiten besmet met een Nederlandse *Coxiella*-uitbraakstam.

In **hoofdstuk 2** wordt een overzicht gegeven van de geschiedenis van Q-koorts in Nederland. Dit geeft tevens de context aan waarin het onderzoek heeft plaatsgevonden. De eerste beschrijvingen van Q-koorts bij mensen in Nederland dateren uit de jaren 50. Studies naar het voorkomen van afweerstoffen tegen *C. burnetii* bij mensen en dieren laten zien dat Q-koorts sinds die tijd in Nederland voorkomt, hoewel er geen uitbraken van Q-koorts zijn geregistreerd tot 2005.

In 2005, twee jaar voor het begin van de recente Q-koortsuitbraken bij mensen, werd Q-koorts in de vorm van abortussen bij geiten waargenomen op twee melkgeitenbedrijven. In totaal werden tussen 2005 en 2009 op 30 melkgeiten- en melkschapenbedrijven abortussen als gevolg van Qkoorts geregistreerd. In 2007 vond de eerste Q-koortsuitbraak bij mensen plaats in Nederland, waarbij 168 mensen ziek werden. Van 2007 tot en met 2009 werden in totaal 3523 ziektegevallen bij mensen geregistreerd. Hiermee is de Nederlandse uitbraak de grootste geregistreerde, goed onderbouwde uitbraak van Q-koorts wereldwijd.

Maatregelen ter preventie en bestrijding van Q-koorts waren en zijn nog steeds voornamelijk gericht op het aantonen van bedrijven die risico voor de volksgezondheid zouden kunnen vormen, op het voorkómen van uitscheiding van de bacterie door melkgeiten en schapen, en op het voorkómen van overdracht vanaf deze bedrijven naar mensen. Het is belangrijk om te benadrukken dat Q-koorts voor Nederland geen nieuwe ziekte was; de bovengenoemde Q-koortsuitbraken ontstonden vanuit een situatie waarin Q-koorts reeds lang in Nederland aanwezig was.

Op basis van de verspreiding van Q-koorts onder mensen en dieren werden geiten en schapen gezien als primaire bron van de Q-koortsuitbraak bij mensen in Nederland. Dit was echter moeilijk onafhankelijk te bevestigen. In **hoofdstuk 3** hebben we door middel van MLVA de genotypen van *C. burnetii* bepaald die voorkwamen bij kleine herkauwers met abortusproblemen. In totaal werden 60% van alle melkgeitenbedrijven en één schapenbedrijf met abortusproblemen als gevolg van Q-koorts onderzocht. Uit de resultaten bleek dat in verreweg de meeste monsters één uniform maar wereldwijd uniek genotype van *C. burnetii* werd aangetroffen. Dit genotype kwam overeen met het genotype gevonden bij een Nederlandse Q-koortspatiënt. Wij veronderstellen dat dit genotype een sleutelrol heeft gespeeld in de Nederlandse Q-koortsuitbraak.

In **hoofdstuk 4** werd deze veronderstelling bevestigd door gebruik te maken van MST. In monsters afkomstig van mensen, geiten en schapen, afgenomen tijdens de uitbraak, werd hetzelfde MST-genotype gevonden (MST33). In monsters afkomstig van koeien werd een ander genotype aangetroffen (MST20). Het lijkt er dus inderdaad op dat de bacterie van geit naar mens is overgegaan en dat de bacterie in koeien geen effecten heeft gehad op de uitbraak bij de mens tussen 2007 en 2010.

Om informatie te verkrijgen over mogelijke andere bronnen van Q-koorts bij mensen zijn monsters van reeën, katten, honden, varkens en paarden onderzocht op aanwezigheid van DNA van de Q-koortsbacterie. De resultaten in **hoofdstuk 5** laten zien dat in 23% van de onderzochte reeën DNA van *C. burnetii* kon worden aangetoond. Het genotype in twee verder onderzochte positieve monsters van reeën verschilde van de typen gevonden bij mensen en landbouwhuisdieren, en er lijkt dus geen verband met het uitbraaktype van *C. burnetii*.

In **hoofdstuk 6** werden placenta's (moederkoeken) van katten, honden, paarden, varkens, geiten, schapen en runderen, verzameld in 2011, onderzocht op aanwezigheid van DNA van de Q-koortsbacterie. Het is namelijk bekend dat ook bij deze diersoorten Q-koorts kan voorkomen, waardoor mensen ziek zouden kunnen worden. Aan de hand van genotypering werd onderzocht of er een verband was met het genotype van de Nederlandse uitbraak.

In placenta's van katten, varkens en geiten werd geen DNA van *C. burnetii* aangetroffen, dit in tegenstelling tot placenta's van honden, paarden, schapen en runderen. De afwezigheid van DNA van *C. burnetii* in geitenplacenta's is een indicatie dat de maatregelen, genomen om de epidemie onder controle te krijgen, zoals vaccinatie van melkgeiten, effectief zijn geweest. Bij enkele monsters afkomstig van één schaap en één koe werd het uitbraakgenotype van *C. burnetii* vastgesteld, terwijl bij zes koeien andere genotypen gevonden werden. De bevindingen geven aan dat *C. burnetii* voorkomt bij reeën, honden, paarden, schapen en runderen. Bij reeën en koeien lijken echter specifieke genotypen van de Q-koortsbacterie voor te komen die anders zijn dan die bij mensen en geiten voorkomen.

Om de genetische achtergrond van Q-koortsbacteriën die voorkomen bij runderen verder te onderzoeken, werden melk en melkproducten onderzocht op aanwezigheid van *C. burnetii*-DNA (**hoofdstuk 7**). *C. burnetii*-DNA werd aangetoond in een groot aantal melkproducten afkomstig uit zowel Europese als niet-Europese landen. De resultaten van de genotypering bevestigden het voorkomen van een specifiek cluster van koe-gerelateerde *C. burnetii*-typen, die slechts zelden bij mensen gevonden worden.

Hoewel geiten de belangrijkste bron van de Nederlandse Q-koortsuitbraak zijn, is er weinig bekend over de manier van verbreiding van *C. burnetii* in de geit zelf na infectie en over de manier waarop de bacterie vervolgens wordt uitgescheiden. In **hoofdstuk 8** worden studies beschreven die hierop ingaan.

Tijdens proeven werden geiten besmet met *C. burnetii* na toediening via inademing. Op deze manier werd de natuurlijke infectieroute nagebootst. In het geitenlichaam bleken de Q-koortsbacteriën een sterke voorkeur te hebben voor bepaalde cellen in de placenta van geiten: de trofoblasten. In deze cellen was *C. burnetii* in staat om zich te vermenigvuldigen. Hoewel *C. burnetii* DNA ook in andere lichaamscellen kon worden aangetoond, zijn er geen aanwijzingen dat daarin vermenigvuldiging plaatsvindt. Voor de geboorte kon dan ook geen uitscheiding van bacteriën worden aangetoond.

Tijdens het lammeren werden naast dode ook levende lammeren geboren uit geïnfecteerde geiten. In beide gevallen werden grote hoeveelheden Q-koortsbacteriën uitgescheiden. Daarom vormen niet alleen abortussen bij geiten een risico voor Q-koorts bij mensen, maar blijken ook normale geboortes uit geïnfecteerde geiten risicovol.

In **hoofdstuk 9** wordt de afweerreactie van drachtige geiten beschreven na infectie met *C. burnetii*. Zowel de afweer door middel van het produceren van afweerstoffen (de humorale immuniteit) als door middel van witte bloedlichaampjes (de cellulaire immuniteit) werd onderzocht. Na infectie worden veel afweerstoffen gericht tegen fase 2 van *C. burnetii* geproduceerd. Afweerstoffen gericht tegen fase 1 volgen pas in een later stadium. Een toename van de cellulaire immuniteit vindt voornamelijk plaats rondom het moment van lammeren, maar is veel minder duidelijk waarneembaar.

In **hoofdstuk 10** worden de bevindingen van de studies in dit proefschrift bediscussieerd en in perspectief van de internationale wetenschap geplaatst. Een van de belangrijkste resultaten is de bevestiging, via genotypering, dat melkgeiten en schapen de bron waren van de Nederlandse Q-koortsuitbraak bij mensen. Er zijn geen aanwijzingen gevonden voor andere bronnen van waaruit mensen besmet hadden kunnen worden. Bij runderen werd een specifiek cluster van genotypen gevonden dat niet direct gerelateerd is aan het Nederlandse uitbraakgenotype en dat slechts incidenteel bij mensen is vastgesteld. Het is echter aan te raden om toch ook de genoty-

pen die bij runderen voorkomen te monitoren omdat het uitbraakgenotype ook is gevonden in tenminste één runderplacenta.

Een tweede belangrijke conclusie is dat geïnfecteerde geiten levende lammeren ter wereld kunnen brengen. Bij deze normale geboorten worden echter ook grote hoeveelheden Q-koortsbacteriën uitgescheiden, gelijk aan de hoeveelheden die tijdens abortus worden uitgescheiden. Het gevolg hiervan is dat ook geïnfecteerde geiten die normaal lammeren bijdragen aan de besmettingsgraad van de omgeving en daarmee een risico vormen voor Q-koorts bij mensen.

Ten derde is de uitscheiding van *C. burnetii* tijdens het lammeren de belangrijkste uitscheidingsroute. Uitscheiding via de mest en vaginaal slijm vóór het lammeren werd niet waargenomen. Ook zijn er geen aanwijzingen voor actieve uitscheiding van *C. burnetii* na het lammeren. Mogelijk dat kruisbesmetting vanuit de besmette omgeving invloed heeft op de uitkomst van diagnostische monsters. Met dit gegeven moet rekening worden gehouden bij de interpretatie van eerdere publicaties over de uitscheiding van *C. burnetii* bij dieren.

Een laatste belangrijke conclusie is dat het aantonen van antilichamen tegen fase 1 en fase 2 van *C. burnetii* een geschikt hulpmiddel kan zijn in de vroege opsporing van *C. burnetii*-besmette geiten en voor het inzicht in de dynamiek van Q-koorts in een koppel dieren.

Dankwoord

Science is we

De Franse arts Claude Bernard (1813-1878) zei ooit “Art is I; science is we”. Nu heb ik niet zo veel verstand van kunst, maar inmiddels iets meer van wetenschap. Ook dit proefschrift is het bewijs dat je wetenschappelijk onderzoek nooit alleen kunt doen; het vraagt een gemeenschappelijke inspanning om tot het beste resultaat te komen. Zonder de hulp van velen zou dit proefschrift niet tot stand zijn gekomen. Ik ben daarom ook iedereen die een bijdrage aan dit proefschrift heeft geleverd zeer dankbaar voor de hulp en ondersteuning.

Annemarie Rebel en Alex Bossers, mijn co-promotoren. Jullie stonden aan het begin van dit project. Ik zie ons nog zitten op mijn kamer op vleugel 26 ergens begin 2008. Jullie kwamen min of meer zeggen dat ik een Q-koortsprojectvoorstel moest indienen voor de vrijvalgelden voor WOT-O. Ik had het toen inmiddels wel gehad met Q-koortsvoorstellen; de twee voorgaande jaren had ik Q-koortsvoorstellen ingediend die niet werden gehonoreerd (is onderzocht door de commissie van Dijk in de evaluatie van het Q-koortsbeleid “Van verwerping tot verheffing”). Ik wilde me richten op het clusterleiderschap en botulisme, ook een interessant onderwerp om op te promoveren. We zaten te praten en kwamen uiteindelijk tot de conclusie dat ik toch de aangewezen persoon was om het projectvoorstel in te dienen, en zo geschiedde dus. Het projectvoorstel “Q fever in goats” werd gehonoreerd en in januari 2009 gingen we van start. Q-koorts leek ook een ‘makkelijker’ onderwerp voor promotieonderzoek dan botulisme en zo kon een lang gekoesterde wens om te promoveren in vervulling gaan. Annemarie en Alex, zeer veel dank voor dat begin en de rest van de ondersteuning en begeleiding. Jullie zijn een enorme stimulans geweest en zijn dat nog steeds. Alex, we hebben het Q-koortsonderzoek samen verder gestalte gegeven binnen het CVI, we hebben nu een prachtige groep en ik kijk uit naar de verdere samenwerking en mooie publicaties. Annemarie, ook in de laatste fase heb je een doorslaggevende bijdrage geleverd aan dit boekje. Ik vind het knap dat je daar ook nog tijd voor had, naast je andere drukke werkzaamheden. Zeer veel dank. Ik kijk uit naar onze samenwerking, we gaan er wat van maken!

Fred van Zijderveld, mijn afdelingshoofd. Zeer veel dank voor de vrijheid en feedback die je me gegeven hebt, en ook voor de mogelijkheid voor het nemen van een sabbatical om het boekje af te schrijven. Je vormde een niet te onderschatten klankbord voor ideeën en uitwerkingen. Dat heb ik zeer gewaardeerd. Ik ken niemand van zo’n fenomenale, encyclopedische veterinair-microbiologische kennis. Ik heb daar dankbaar uit geput en hoop daar nog uit te putten in de toekomst, ook als je minder gaat werken. Je vormde een vertrouwde aanwezigheid. Zeer veel dank daarvoor.

Jos van Putten, mijn promotor . Wij hadden onze eerste afspraak op het hoogtepunt van de Q-koortscrisis in december 2009. Je hebt me met open armen ontvangen en stemde direct in om promotor te zijn. Ik kijk met zeer veel genoegen terug op de zeer leerzame discussies over resultaten, papers, schrijven en publiceren. De verdieping en de ‘wetenschappelijke scholing’ werden

daardoor gegarandeerd. Je nam er de tijd voor, geweldig. Ook in de laatste fase, in de zomer van 2012, heb je vele correcties aangebracht. Je maakte het daarmee mogelijk om het grootste deel van het schrijfwerk af te ronden tijdens mijn sabbatical. Veel dank daarvoor.

Annemieke Dinkla, enorm bedankt voor je tomeloze enthousiasme, inzet en werklust. Niets was te veel en als je het niet wist of kon, zorgde je dat je het leerde. Ik denk met veel genoegen terug aan het begin van het project in 2009. We hadden een dierproef gepland ergens in oktober, maar de isolatie en kweek van de Q-koortsbacterie moest nog wel 'even' gebeuren. We hadden daarvoor 6 maanden de tijd. We begonnen met een doel, celkweekervaring, een Q-koortspositieve geitenplacenta, protocollen en enige ervaring vanuit Duitsland; pionieren dus. En het is ons gelukt, met dank aan jou! Hulde.

Betty van Gelderen, je vormde een steun en toeverlaat voor de dierproeven en voor alle testen die we gedaan hebben. Afspraken met DB, planning voor de testen, uitvoeren, opzetten, verwerken van de resultaten tot en met de archivering: het was altijd accuraat en tot in de puntjes geregeld. Je haalde me erbij als ik iets wat te makkelijk geregeld had, en dan kwam het altijd weer goed. Heel veel dank daarvoor!

Dear Dimitrios, thank you very much for all your help on Q fever and the culture of *C. burnetii*. Thanks for your hospitality to host us for the Q fever culture workshop in Munich back in 2009. This workshop was fundamental for the work in this thesis. We discussed a lot, on typing, Q fever and the bug, about publishing and all the duties we both had during the last years. I hope we can work together for many more years!

Twee mannen van het eerste uur, Robin Ruuls en Peter Willemsen, hartelijk dank voor het vele werk en de nuttige discussies over typering en PCR-detectie. We begonnen eind 2007 met het opzetten van de Q-koorts-PCR en de MLVA-typering. Het heeft ons en het CVI geen windeieren gelegd en zorgde ervoor dat we in juni 2008 klaar waren toen er een beroep op het CVI werd gedaan bij de start van de meldplicht. Tevens vormde het een eerste aanzet tot dit proefschrift. Zeer veel dank voor jullie inzet en vooral ook Robin voor het typeren van enorme aantallen *C. burnetii*-monsters.

Op de achtergrond altijd 'gewoon' aan het werk, maar gaandeweg ontzettend veel werk verricht in het BSL-3-lab met het verwerken van de monsters: Arie Hoogendoorn. Het is bewonderenswaardig hoeveel werk je verzet hebt, heel veel dank daarvoor. Ook Jacob Post, je vormde een aangename toevoeging aan de Q-koortsgroep met een goede inbreng, open voor discussie. Dank voor het opzetten van de testen voor de celgebonden immuniteit en het uitvoeren daarvan, ook vele uren in het BSL-3-lab. Ik heb je inbreng en inzet zeer gewaardeerd. Lucien van Keulen, ik ken je al vanaf begin jaren '90 toen we in hetzelfde studentenhuis woonden. Ik heb genoten van je inbreng en onze samenwerking. Je dook in de Q-koorts, in de pathologie en de fysiologie van de

dracht bij geiten. Het leverde een prachtig artikel op! Dank je wel. Ook Conny van Solt hartelijk dank voor het testen en typeren van de placenta's.

Dan velen die meer of minder bij het Q-koortsonderzoek betrokken waren en zijn: Michiel Kroeze, Frank Harders, Hilde Smith, Daniel Bontje, Aline de Koeijer, André Bianchi, Bregtje Smit, Rineke de Jong, Albert de Boer, Jeanet van der Goot, Peter van Tulden, Erwin Claassen en Marieke van Es (voor het BSL-3-lab), Fred van Welie (voor het maken van de vele plaatjes en de voorkant!) en niet te vergeten onze aiossen Runa Kuley en Anne Ammerdorffer. Het is een voorrecht om met zo'n groep gemotiveerde mensen te werken.

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Buiten het CVI hebben ook vele collega's hun bijdrage geleverd aan de totstandkoming van dit boekje. Enerzijds natuurlijk collega's uit het veterinaire werkveld, maar minstens zo belangrijk bij een zoönose als Q-koorts zijn de collega's uit het humane werkveld.

Piet Vellema, Daan Dercksen, René van den Brom, Lammert Moll en Willem Wouda, allen werkzaam bij de GD. Dank voor de goede samenwerking en de vele inhoudelijke discussies die we hebben gehad.

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Jolianne Rijks en Andrea Gröne van het DWHC, dank voor de fijne samenwerking in het reeënproject, het heeft een mooie publicatie opgeleverd. Collega's Arco van der Spek, Marcel Spierenburg en Dennis Bol van de NVWA, dank voor jullie inbreng en het beschikbaar stellen van data. Miel Hovius en Frank Roest, hartelijk dank voor het meedenken en het verzamelen van placenta's van

verschillende diersoorten. En natuurlijk Wim van der Hoek van het RIVM, dank voor je inbreng en fijne samenwerking. Ik hoop dat we die samenwerking verder kunnen versterken, we hebben gelukkig al een begin gemaakt!

The work package 'Development and application of improved diagnostics for Q fever' of the MedVetNet network of excellence was very beneficial for the research performed in this thesis. Special thanks to Elodie Rousset, Karim Sidi-Boumedine and Richard Thiéry from ANSES, France, for all the work you have done to make this work package successful.

In de verschillende Q-koortsprojecten en tijdens de uitbraak heb ik met vele mensen samengewerkt. De Q-koortsuitbraak heeft ervoor gezorgd dat de samenwerking tussen het humane en veterinaire werkveld sterk verbeterd is. Ik hoop dat we dat in de toekomst verder kunnen uitbreiden. Al die mensen die belangstelling hebben getoond in de voortgang het mijn onderzoek: hartelijk dank daarvoor. Het gaf een stimulans om door te gaan!

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Curriculum vitae

About the author

Hendrik Ido Jan (Hendrik-Jan) Roest was born on 23 January 1969 in Leiden, the Netherlands. In 1986 he graduated from senior general secondary education (HAVO) and in 1988 from pre-university education (VWO) at the 'Christelijk lyceum dr. W.A. Visser 't Hooft' in Leiden. In 1988 he started his degree in Veterinary Medicine at Utrecht University. During his study he was a student member of the faculty's Educational Board and Faculty Board. He participated in a curriculum renewal as student-assistant at the Department of Education. In 1996 he graduated with a clear pass in the differentiation large animal medicine and animal production.

In 1996 Hendrik-Jan started to work as a veterinarian at 'Dierenartsenpraktijk Ravenstein-Schayk e.o.'. At the start of the classical swine fever outbreak in 1997 he switched to the 'Dierenartsenassociatie Tielerwaard' in Waardenburg. He became partner in the association in 1999. After two years the association merged with 'Dierenartsenpraktijk Geldermalsen e.o.' into 'Dierenartsenpraktijk West-Betuwe' located in Meteren. During these years he worked as an all-round veterinarian with the emphasis on farm animals.

In 2003, after six-and-a-half years, Hendrik-Jan left the veterinary practice to start a specialisation in veterinary microbiology at the Laboratory for Antimicrobial Resistance of the former Central Institute for Animal Disease Control (CIDC) Lelystad. In 2005, he became head of the Laboratory for General Bacteriology and Serology and in 2006 he became OIE expert on Contagious Equine Metritis (CEM). Between 2008 and 2010 he was head of the cluster General Bacteriology and Fish Diseases of the newly formed Central Veterinary Institute, part of Wageningen UR (CVI). He finished his specialisation as a veterinary microbiologist in 2012. Since September 2012 he has been head of the Department of Bacteriology and TSEs.

Hendrik-Jan became involved in the Q fever outbreak in the Netherlands with the start of the mandatory notification of Q fever in small ruminants in June 2008. The laboratory tests to confirm Q fever in small ruminants at CVI were performed under his supervision. From then on he was closely involved in the Q fever outbreak as member of the outbreak management team and advisor to the government. He was a member of two panels of the European Food Safety Authority (EFSA) for the scientific opinion on Q fever and the development of harmonised schemes for the monitoring and reporting of Q fever in animals in the European Union. At CVI he initiated the Q fever research and was leading or involved in numerous projects on Q fever, several of which form the basis of this thesis. He is still closely involved in Q fever research and is co-supervising two PhD projects.

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List of abbreviations

Abbreviation	English explanation	Dutch explanation (where applicable)
°C	degrees Celsius	
Abbr.	abbreviation	
abd. taken	abdominally taken	
abo	abortion	
aBSL	animal biosafety level	
ACCM	acidified citrate cysteine medium	
ANSES	French Agency for Food, Environmental and Occupational Health & Safety	
BAL	bronchoalveolar lavage	
BGM	Buffalo Green Monkey	
BTM	bulk tank milk	
C	cotyledon	
CbNLxx	Dutch C. burnetii genotype with number xx	
CDC	Centres of Disease Control and Prevention	
CEM	Contagious Equine Metritis	
CFT	complement fixation test	
CI	confidence interval	
CIDC-Lelystad	(former) Central Institute for Animal Disease Control-Lelystad	(voormalig) Centraal Instituut voor DierziekteControle-Lelystad
Com1	Coxiella outer membrane protein 1	
ConA	concanavaline A	
Ct/CT	PCR cycle threshold	
CVI	Central Veterinary Institute, part of Wageningen UR	
CWZ	Canisius Wilhelmina Hospital	Canisius Wilhelmina Ziekenhuis
dATP	deoxyadenosine triphosphate	
DB	(department) Animal technology	(afdeling) Dierverzorging en Biotechniek
dCTP	deoxycytidine triphosphate	
DG Sanco	Directorate General for Health and Consumers (Santé et Consommateurs)	
dGTP	deoxyguanosine triphosphate	
DIVA	Differentiating Infected from Vaccinated Animals	
DNA	deoxyribonucleic acid	
dpi	days post inoculation	
dpp	days post parturition	
dTTP	deoxythymidine triphosphate	
dUTP	deoxyuridine triphosphate	
DWHC	Dutch Wildlife Health Centre	
E	(maternal) epithelium	
e.g.	exempli gratia; for example	
EC	European Commission	
EDTA	ethylenediaminetetraacetic acid	
EFSA	European Food Safety Authority	
EL&I	(former) Dutch Ministry of Economic Affairs, Agriculture and Innovation	(voormalig) Ministerie van Economische Zaken, Landbouw en Innovatie
ELISA	enzyme-linked immunosorbent assay	

Abbreviation	English explanation	Dutch explanation (where applicable)
EMEM	Eagle's minimal essential medium	
e.o.	and environs	en omstreken
EU	European Union	
ext. taken	externally taken	
F	foetal allantochorion	
F(ab')2	fragment antigen-binding as derived after pepsin cleavage of the heavy chain of IgG antibodies	
GD	Animal Health Service	Gezondheidsdienst voor Dieren
h	hour	
H/L	heavy/light chain combination of IgG antibodies	
HAVO	senior general secondary education	hoger algemeen voortgezet onderwijs
HGDI	Hunter-Gaston diversity index	
HMDCs	human monocyte-derived dendritic cells	
i.e.	id est; in other words; that is	
IAP	integrin associated protein	
IC	inhibition control	
ID	identification	
IFN	interferon	
IFN-α	interferon-alpha	
IFN-γ	interferon-gamma	
IFN-γ Elispot	interferon-gamma enzyme-linked immunosorbent spot test	
IFT/IFA	indirect immunofluorescence test/assay	
Ig	immunoglobulin	
IgGph1	anti-C. burnetii phase 1 IgG antibody	
IgGph2	anti-C. burnetii phase 2 IgG antibody	
IgMph1	anti-C. burnetii phase 1 IgM antibody	
IgMph2	anti-C. burnetii phase 2 IgM antibody	
IHC	immunohistochemical/immunohistochemistry	
IL	interleukin	
INRA	Institut National de la Recherche Agronomique	
IS	insertion sequence	
kb	kilo base	
kid	kidding (liveborn kids)	
L	litre	
LCV	large cell variant	
LNV	(former) Dutch Ministry of Agriculture, Nature and Food Quality	(voormalig) Ministerie van Landbouw, Natuurbeheer en Voedselkwaliteit
LPS	lipopolysaccharide	
LPT	lymphocyte proliferation test	
M	maternal endometrium	
MAB	monoclonal antibody	
mg	milligram	
MHS	Municipal Health Service	
MID	mouse infective dose	
min	minute	
mL	millilitre	

Abbreviation	English explanation	Dutch explanation (where applicable)
MLVA	multi(ple)-locus variable number tandem repeat analysis	
MLVA type “part"	partial MLVA genotype	
MLVA-12	MLVA with 12 loci included in the analysis	
MLVA-6	MLVA with 6 loci included in the analysis	
mmol	millimol	
mRNA	messenger ribonucleic acid (RNA)	
MS	MiniSatellite	
MST	multispacer sequence typing	
MucZ	gene of C. burnetii able to induce mucoidy	
(N)VWA	the Netherlands Food and Consumer Product Safety Authority	(Nederlandse) Voedsel -en Warenautoriteit
n.r.	no registration	
NA	not applicable	
NaCl	sodium chloride	
nec	necropsy	
NL	The Netherlands	
NM	Nine Mile reference strain of Coxiella burnetii	
nmol	nanomol	
OD	optical density	
OIE	World Organisation for Animal Health (former Office International des Epizooties)	
P3 filter	filter penetration limit 3: Filters at least 99.95% of airborne particles	
PBMCs	Peripheral blood mononuclear cells	
PBS	phosphate buffered saline	
PBS-Tw	PBS (pH 7.2) with 0.5 ml 10% (v/v) tween 80	
PCR	polymerase chain reaction	
pers. comm.	personal communication	
PL	placentome	
Publ. year	publication year	
Q fever	Query fever	
QC	quality control	
QFS	Q fever fatigue syndrome	
qPCR	quantitative polymerase chain reaction	
RAPD	randomly amplified polymorphic DNA	
Ref.	reference	
RFLP	restriction fragment length polymorphism	
RIVM	National Institute for Public Health and the Environment	Rijksinstituut voor Volksgezondheid en Milieu
RNA	ribonucleic acid	
rRNA	ribosomal ribonucleic acid (RNA)	
SCV	small cell variant	
Slovak Rep	Slovak Republic	
SNP	single nucleotide polymorphism	
SPC	summary of product characteristics	
spp.	species	
strep	dihydrohydroxystreptose	

Abbreviation	English explanation	Dutch explanation (where applicable)
T	(foetal) trophoblasts	
temp	(annealing) temperature	
TLR	Toll-like receptor	
TNF	tumor necrosis factor	
TNF-α	tumor necrosis factor-alpha	
U	unit	
UDG	uracil DNA glycosylase	
unpub. data	unpublished data	
v/v	volume/volume	
vir	virenose	
VWO	pre-university education	voorbereidend wetenschappelijk onderwijs
WOT-O	Regulatory research tasks-Research part	Wettelijke Onderzoekstaken (01-Dierziekten)-Onderzoeks deel
wpi	weeks post inoculation	
µL	microlitre	