Veterinary aspects of a Q fever outbreak in the Netherlands between 2005 and 2012

The research described in this thesis was carried out at GD Animal Health (Deventer), Faculty of Veterinary Medicine, Utrecht University (Utrecht), Central Veterinary Institute, part of Wageningen University and Research centre (Lelystad), National Institute for Public Health and the Environment (Bilthoven) and Jeroen Bosch Hospital (´s-Hertogenbosch).

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Nomenclature Q fever
In his later recollection, characteristically blunt, Macfarlane Burnet told how the disease got its name: "Problems of the nomenclature arose. The local authorities objected to "abattoir's fever", which was the usual name amongst the doctors in the early period. In one of my annual reports I referred to "Queensland rickettsial fever", which seemed appropriate to me, but not to people concerned with the good name of Queensland. Derrick, more or less in desperation, since "X-disease" was preoccupied by [sic, meaning "already applied to"] what is now Murray Valley encephalitis, then came out for "Q" fever (Q for "query"). For a long time, however, the world equated Q with Queensland, and it was only when the disease was found to be widespread around the world that "Q fever" came to stand firmly in its own right as the name of the disease." (source: Spillover: Animal Infections and the Next Human Pandemic by David Quammen)

Veterinary aspects of a Q fever outbreak in the Netherlands between 2005 and 2012

Diergeneeskundige aspecten van een Q-koorts uitbraak in Nederland tussen 2005 en 2012
(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

donderdag 5 februari 2015 des middags te 2.30 uur

door

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geboren op 25 februari 1977 te Amsterdam

Promotor: Prof.dr. J.A. Stegeman

Copromotor: Dr. P. Vellema

Contents

Chapter 1

General introduction

Introduction

In a six week period from May 2007 onwards, almost one hundred patients from Herpen, a small village in the province of Noord-Brabant, were diagnosed with a lower respiratory tract infection, and ultimately, *Coxiella burnetii* was found to be the causal agent (Van Steenbergen et al., 2007; Van der Hoek, 2012a). This finding made up the start of the largest human Q fever outbreak ever recorded.

Q fever is a zoonosis caused by *C. burnetii*, an aerobic Gram-negative highly resistant bacterium which is able to infect several animal species (Arricau-Bouvery and Rodolakis, 2005). The existence of *C. burnetii* has been described worldwide, except in New Zealand (Woldehiwet, 2004). Domestic ruminants are the primary reservoir of *C. burnetii*, but infections are also found in rodents, birds and arthropods (Babudieri and Moscovici, 1952), and in addition to ruminants, cats and dogs are also able to shed the organism, causing infection in humans (Marrie et al., 1988; Buhariwalla et al., 1996). The main symptom in infected sheep and goats is abortion, mainly during late pregnancy, and placental material of infected small ruminants may contain up to millions of *C. burnetii* per gram of tissue (Babudieri, 1959; Roest et al., 2012). During abortion or parturition, billions of bacteria are excreted which can easily be aerosolised, and infection in humans mainly occurs through inhalation of airborne *C. burnetii* (Marrie, 1990a; Maurin and Raoult, 1999; Schimmer et al., 2010).

Q or query fever was described as a human febrile illness which was first observed early 1933 in abattoir workers in Brisbane, Queensland, Australia (Derrick, 1937). The manifestation of Q fever in humans is highly variable, and the clinical presentation can differ from asymptomatic to fatal chronic infections (Arricau-Bouvery and Rodolakis, 2005; Muskens et al., 2007; Nabuurs-Franssen et al., 2008). Historically, human Q fever was mainly described as a disease in occupationally exposed people like farmers, sheep shearers, veterinarians and slaughterhouse personnel (Van den Brom and Vellema, 2009). In descriptions of individual or small clusters of human Q fever patients, infections were also mainly related to direct contact with *C. burnetii* shedding animals or their excretion products (Langley et al., 1988; Marrie et al., 1988; Buhariwalla et al., 1996; Stein and Raoult, 1999; Berri et al., 2003). Clusters of human Q fever patients in the community have been described before and were often related to indirect contact with infected small ruminants on a single event e.g. a farmers' market (Dupuis et al., 1987; Porten et al., 2006; Gilsdorf et al., 2008; Panaitov et al., 2009).

In a Dutch survey in 1954, no evidence was found for the existence of Q fever in man and in cattle (Wolff and Kouwenaar, 1954). In the 1980's, the seroprevalence in sheep and goats in the Netherlands was considered to be low (Houwers and Richardus, 1987). In 2005, it was for the first time in the Netherlands that *C.*

burnetii was detected as causal agent of abortion waves on dairy goat farms (Rapportage Monitoring Dierziekten Kleine Herkauwers, second half of 2005; Van den Brom and Vellema, 2009), where up to 50% of the pregnant goats aborted (Wouda and Dercksen, 2007). In the period 2005-2009, in total, 28 *C. burnetii* related abortion waves on dairy goat farms were confirmed (Van den Brom et al., 2012b; Vellema and Van den Brom, 2014). These dairy goat farms were mainly located in the southern part of the Netherlands. In the same period, cases of abortion caused by *C. burnetii* were also confirmed at two dairy sheep farms.

In humans, Q fever has been diagnosed for the first time in the Netherlands in 1956 (Westra et al., 1958), and became a notifiable disease in 1978. Between 1978 and 2006, 1-32 human patients were diagnosed annually, with an average of 17 (Schimmer et al., 2009). After the increase in human Q fever patients in 2007, dairy goats were mentioned as the suspected source of *C. burnetii* (Van Steenbergen et al., 2007), and in that year, a total of 168 human Q fever patients was notified (Schimmer et al., 2008). In 2008, Q fever returned, and at the end of 2008 1,000 human patients were notified. This outbreak stimulated the start of a large multidisciplinary research portfolio, aiming at generating better knowledge about the background and transmission of *C. burnetii* to be able to take adequate control and preventive measures.

Outline and aim of this thesis

The aim of this thesis is to describe veterinary aspects of a Q fever outbreak in the Netherlands between 2005 and 2012 to be able to improve control and preventive measures aiming at reducing the shedding of *C. burnetii* and thus environmental contamination, in order to reduce human exposure. As no recent information was available on professionally exposed persons, the research described in this thesis additionally aims to determine consequences of exposure for livestock veterinarians.

In **chapter 2**, a description is given of Q fever outbreaks in small ruminants and people in the Netherlands, including historical background, clinical presentation in small ruminants as well as in people, ongoing research and implemented measures. **Chapter 3** describes *C. burnetii* seroprevalences and associated risk factors in sheep and goats in the Netherlands in 2008. In the same year, dairy sheep and dairy goat farms were given the opportunity to voluntarily submit a bulk tank milk (BTM) sample. **Chapter 4** describes BTM *C. burnetii* PCR and ELISA results of 308 dairy goat and dairy sheep farms. Agreement of both tests was compared with the serological status of thirteen individual animals per herd, and correlations with a history of *C. burnetii* abortion were determined. After an increase in the number of human Q fever patients from 168 in 2007 to 1,000 and 2,342 in 2008 and 2009, respectively, among other measures, mandatory *C. burnetii* BTM surveillance was implemented for all dairy sheep and dairy goat farms with more than fifty animals,

using the above mentioned PCR and ELISA. At the end of 2009, the Dutch government decided to cull all pregnant sheep and goats on *C. burnetii* BTM PCR positive dairy farms. **Chapter 5** describes the results of the mandatory BTM surveillance program (2009-2014) and discusses BTM surveillance as a method to detect *C. burnetii* shedding on small ruminant dairy farms. Effect of culling and vaccination on shedding of *C. burnetii* was analysed. The role of individually shedding dairy goats on *C. burnetii* BTM PCR results was unknown. **Chapter 6** describes the results of pooling individual milk samples and detection and removal of individual shedders on a single *C. burnetii* BTM PCR positive farm. In 2009, the actual role of manure in the spread of *C. burnetii* in the Q fever outbreak was not clear. On two farms with a recent history of abortion caused by *C. burnetii*, temperatures in the dunghill were measured and compared with the heat resistance of *C. burnetii*. The unlikeliness of the role of goat manure, after proper composting, in the human Q fever outbreak is discussed in **chapter 7**. In 2009, mandatory vaccination of all animals on dairy sheep and dairy goat farms was implemented, starting in the affected southern part of the Netherlands. **Chapter 8** describes the efficacy of vaccination (Coxevac®, CEVA Santé Animale) on bacterial shedding in vaginal swabs, milk samples and uterine content from vaccinated and unvaccinated culled animals. In 1987, a high seroprevalence of *C. burnetii* in Dutch veterinarians was found. Consequences of the human Q fever outbreaks for occupationally exposed veterinarians were unknown. **Chapter 9** describes the seroprevalence and associated risk factors of *C. burnetii* in 189 livestock veterinarians. In **chapter 10,** *C. burnetii* infections in small ruminants are reviewed. The rise and control of the 2007-2012 human Q fever outbreak in the Netherlands is described in **chapter 11**. A summarizing discussion of the findings of the present thesis in relation to scientific literature is given in **chapter 12**.

Chapter 2

Q fever outbreaks in small ruminants and people in the Netherlands

René van den Brom
Piet Vellema

Small Ruminant Research 2009; 86(1-3), 74-79

Abstract

Q fever is an almost ubiquitous zoonosis caused by the highly resistant aerobic Gram-negative bacterium *Coxiella burnetii*. Cattle, sheep and goats are the primary animal reservoirs, but infection with this organism may occur in several animal species. Infected sheep and goats may abort, mainly in late pregnancy. The causative agent is shed in urine, vaginal fluids, faeces, milk and, in high concentrations, in birth fluids and placentas of infected small ruminants. Transmission to humans mainly occurs through the aerosol route. In the Netherlands, Q fever is not a newly recognized human disease; between 1978 and 2006, the average number of notifications per annum was 17. In 2007, 182 human cases were confirmed, mainly in the southern part of the country, in an area with a high density of large dairy goat farms. Q fever recurred in 2008, mainly in the same area and at the end of the year exactly 1000 human cases had been registered, making it the largest human outbreak ever recorded. In 2005, Q fever was diagnosed for the first time as a cause of abortion at two dairy goat farms. In 2006, 2007 and 2008, six, seven and seven new cases at dairy goat farms were confirmed, respectively. The infected dairy goat farms were mainly located in the same area where human cases occurred and they are considered the most plausible source of human infection although evidence is still inconclusive. In the same period, two cases of abortion caused by *C. burnetii* were confirmed at two dairy sheep farms, one in the southern and one in the northern part of the country however these two cases do not appear to be related to human cases. This article aims to describe the Q fever situation in the Netherlands in 2007 and 2008. It starts with an overview of the causal agent, the disease and its history and focuses on the sheep and goat industry in the Netherlands and the Q fever problems. Research has started and measures have been taken aimed at reducing the shedding of *C. burnetii* and thus environmental contamination, trying to reduce human exposure in 2010.

Historical background

Q fever is a zoonosis caused by *Coxiella burnetii*, an aerobic Gram-negative highly resistant bacterium, which is able to infect several animal species, as well as people. Cattle, sheep and goats are the primary animal reservoirs; infected sheep and goats may abort, mainly in late pregnancy (Zeman et al., 1989; Damoser et al., 1993; Maurin and Raoult, 1999; Hatchette et al., 2001; Wouda and Dercksen, 2007). In some areas, cats are a major source of infection (Marrie et al., 1988). The organism is shed in the urine, the faeces, the milk and abounds in foetal membranes and foetal fluids of infected animals. The placenta of infected small ruminants may contain over 10^9 hamster infective doses or organisms per gram of tissue (Babudieri, 1959; Fournier et al., 1998). The organism is transmitted to humans, mainly through aerosols (Marrie, 1990b; Maurin and Raoult, 1999; Schimmer et al., 2009).

Q fever was described as a febrile illness, which had started to occur in 1933 in abattoir workers in Brisbane, Queensland, Australia, but attempts to isolate the etiological agent by inoculating guinea pigs with the blood or urine of infected patients were unsuccessful (Derrick, 1937). Burnet and Freeman (1937) reproduced the disease in guinea pigs, mice, monkeys and albino rats with an emulsion of infectious guinea pig liver received from Derrick and demonstrated rickettsial organisms in spleen sections from infected mice. In the same period, Davis and Cox (1938), working on the possible vectors of Rocky Mountain spotted fever at the Rocky Mountain Laboratory in Hamilton, Montana, USA, allowed *Dermacentor andersoni* ticks collected near Nine Mile Creek, Montana, to feed on guinea pigs and found that some animals developed a febrile illness with enlarged spleens. They further characterized the "Nine Mile agent" and showed that it had filterable properties. The organism was observed intravacuolarly in infected tissue cultures (Cox, 1938; Cox, 1939) and was found to cause an infection in people (Dyer, 1938). Both groups in Brisbane and Montana demonstrated that the aetiological agent displayed properties of both viruses and rickettsiae (Burnet and Freeman, 1937; Cox, 1938; Davis and Cox, 1938); in 1938, *Rickettsia diaporica*, the proposed name (Cox, 1939) for the organism, which incorporated both rickettsial features and the ability of the organism to pass through a bacteriological filter, was propagated in tissue cultures and in developing chicken embryos (Cox, 1939; Cox and Bell, 1939). Derrick (1937) proposed the name Q fever or query fever for this disease, with a wink to Queensland where he first described this disease in detail.

In the era when modern means of communication, like telephone, radio and television, were scarcely out of the egg and other means, like computers and Internet, were not yet available at all, the American and Australian groups started exchanging information and infected materials after a laboratory-acquired Q fever infection occurred in the Rocky Mountain Laboratory in 1938 (Dyer, 1938). They demonstrated that the Australian Q fever agent, the zoonotic agent, and the Nine

Mile agent were in fact isolates of the same microorganism, *Rickettsia burneti* (Derrick, 1939; Maurin and Raoult, 1999), later renamed as *C. burnetii* (Philip, 1948), a name which honours both Cox and Burnet as pioneers in this field.

Since the first documented Australian and American outbreaks, Q fever has been described in many other countries all over the world. Kaplan and Bertagna (1955) reported its existence in 51 countries on five continents. In the Netherlands, the first description dates from 1956 (Westra et al., 1958).

This article describes the human and small ruminant situation of Q fever in the Netherlands in recent years. The sheep and goat industry and the recently found abortion waves in small ruminants caused by *C. burnetii* will be discussed, followed by a picture of the recent increase in human Q fever cases and the possible links between both. Finally, the article presents results of finished research and elaborates on preliminary findings of ongoing research in the following pages.

Q fever in small ruminants

C. burnetii can infect several animal species, as well as humans. Infections have been described in cattle, goats, sheep, dogs, cats, horses, rabbits, buffaloes, small rodents, swine, camels, water buffaloes, rats, mice, birds like pigeons, turkeys, chickens, ducks and geese and in several species of ticks (Babudieri and Moscovici, 1952; Babudieri, 1959; Marrie, 1990a; Maurin and Raoult, 1999; Arricau-Bouvery and Rodolakis, 2005; Muskens et al., 2007). Cattle, sheep and goats are the primary animal reservoirs (Zeman et al., 1989; Damoser et al., 1993; Maurin and Raoult, 1999; Hatchette et al., 2001; Wouda and Dercksen, 2007), although in some areas, cats are also a major source of infection (Marrie et al., 1988).

Infection in animals is generally asymptomatic. In the acute phase of the infection the presence of *C. burnetii* can be demonstrated in lungs, liver, spleen and blood. No symptoms have been described in the chronic phase of infection (Maurin and Raoult, 1999). In cattle, an infection is usually asymptomatic, but may result in abortion, subfertility and metritis (Arricau-Bouvery and Rodolakis, 2005). In small ruminants, an infection may result in abortion, stillbirth, retention of foetal membranes, endometritis and infertility. Abortion usually takes place at the end of pregnancy (Arricau-Bouvery and Rodolakis, 2005; Muskens et al., 2007; Wouda and Dercksen, 2007); occasionally, prior to the abortion, the animal may be slow and have a reduced appetite (Wouda and Dercksen, 2007), but in most cases no preceding symptoms are present (Arricau-Bouvery and Rodolakis, 2005). In some infected flocks/herds, concomitant pneumonia may be seen (Arricau-Bouvery and Rodolakis, 2005; Muskens et al., 2007) and newborn lambs may suffer from diarrhoea and respiratory problems (Wouda and Dercksen, 2007).

High abortion rates are rare, but may occur in goat herds, where up to 90% of the pregnant animals may abort (Palmer et al., 1983; Hatchette et al., 2003; Arricau-Bouvery and Rodolakis, 2005; Wouda and Dercksen, 2007). In the lambing season following an abortion wave, the reproductive problems are often much less prominent (Berri et al., 2007; Wouda and Dercksen, 2007).

Infected animals can shed the organism in birth products, urine, faeces and milk. Shedding can last for months, is longer in goats than in sheep (Arricau-Bouvery and Rodolakis, 2005) and differs between ruminant species (Rodolakis et al., 2007). Goats can be chronically infected and may shed *C. burnetii* for up to two pregnancies after being infected (Hatchette et al., 2003).

In *in vitro* tests, *C. burnetii* is susceptible to several antibiotics, including tetracyclines and macrolides. The efficacy of both groups of antibiotics is very difficult to examine in practice (Muskens et al., 2007). Guatteo et al. (2008) and Matthews (1990) found that in goat herds with abortion problems, tetracyclines can be able to control the number of abortions, but do not prevent the animals from shedding the organism. They suggested that the first injection of oxytetracycline should be administered on day 105 of gestation and the second two weeks later. However, Wouda and Dercksen (2007) did not record a reduction in the number of abortions in an infected goat herd after treatment with oxytetracycline.

Clinical presentation of Q fever in people

Q fever is often an occupational hazard. People working with farm animals, such as livestock handlers, farmers, veterinarians, slaughterhouse and laboratory personnel, are at higher risk of infection. Nevertheless, urban outbreaks have also been described (Derrick, 1937; Tselentis et al., 1995; Armengaud et al., 1997; Lyytikäinen et al., 1998; Nabuurs-Franssen et al., 2008).

The manifestation of Q fever in humans is highly variable. In the first described cases, fever and headache were the most prominent symptoms. The onset of the disease was acute and the course and duration of the fever varied. Headache was often severe and persistent and in many cases the chief complaint. In comparison with the high fever, the pulse rate of the patients was slow (Derrick, 1937). Nowadays, it is clear that the clinical presentation can differ from asymptomatic to fatal chronic infections (Arricau-Bouvery and Rodolakis, 2005; Muskens et al., 2007; Nabuurs-Franssen et al., 2008). The incubation period for acute Q fever varies from one to four weeks and in some cases even up to six weeks or longer (Maurin and Raoult, 1999; Steenbergen et al., 2007; Delsing and Kullberg, 2008) and depends in part on the infective dose of *C. burnetii* (Maurin and Raoult, 1999).

Acute Q fever may present itself as a non-specific flu-like illness, atypical pneumonia or hepatitis. The symptoms of the non-specific flu-like illness are characterized by sudden onset, high and sometimes biphasic fever (Maurin and Raoult, 1999), headache, non-productive coughing, vomiting, myalgia, diarrhoea and weight loss. Because of the atypical symptoms, the disease often remains undiagnosed (Richardus et al., 1987; Maurin and Raoult, 1999; Arricau-Bouvery and Rodolakis, 2005; Steenbergen et al., 2007; Delsing and Kullberg, 2008).

In many cases, atypical pneumonia is the major clinical presentation of Q fever (Maurin and Raoult, 1999). This is characterized by non-productive cough and sometimes by chest pain and inspiratory crackles. However, minimal changes in auscultation are usually perceptible (Maurin and Raoult, 1999; Arricau-Bouvery and Rodolakis, 2005).

Hepatitis may be a symptom of Q fever. The hepatitis can be asymptomatic and characterized only by an increase in liver enzymes, but it can also be associated with fever (Maurin and Raoult, 1999; Arricau-Bouvery and Rodolakis, 2005). Hepatomegaly is common, whereas jaundice can only be seen in rare cases. A liver biopsy may reveal granulomatous hepatitis (Richardus et al., 1987).

Acute Q fever can also present itself by skin rash, meningo-encephalitis, pericarditis, thrombophlebitis, uveitis, myocarditis, arthritis, pleuritis, abortion and pancreatitis (Maurin and Raoult, 1999; Arricau-Bouvery and Rodolakis, 2005; Steenbergen et al., 2007; Nabuurs-Franssen et al., 2008). The mortality rate in the acute phase is 1% to 2% (Delsing and Kullberg, 2008).

In about 1% to 5% of the cases, an acute infection may lead to a chronic infection (Nabuurs-Franssen et al., 2008), although there is some debate on the definition of this state of infection (Maurin and Raoult, 1999; Nabuurs-Franssen et al., 2009). Chronic infection can manifest as endocarditis, chronic fatigue syndrome and problems related to pregnancy. Of these, endocarditis is the main presentation (Maurin and Raoult, 1999; Nabuurs-Franssen et al., 2008; Schimmer et al., 2008). People with valvular abnormalities are more susceptible. The male/female ratio is 75% and most patients are older than forty years (Maurin and Raoult, 1999). Endocarditis can occur months, even years, after an acute infection (Arricau-Bouvery and Rodolakis, 2005).

Chronic fatigue syndrome is characterized by inappropriate fatigue. Other symptoms are night sweats, myalgia, arthralgia, mood swings and changes in sleeping pattern. The syndrome can occur after an acute infection and can last for months or years (Arricau-Bouvery and Rodolakis, 2005; Delsing and Kullberg, 2008). About 1% to 5 % of the chronic cases result in fatal complications (Arricau-Bouvery and Rodolakis, 2005).

Q fever infections during pregnancy are almost always asymptomatic (Tissot-Dupont et al., 2007; Nabuurs-Franssen et al., 2008), but serious obstetric complications may occur, such as placentitis, spontaneous abortion, intrauterine growth retardation, intrauterine foetal death, premature delivery and subnormal birth weight (Jover-Diaz et al., 2001; Raoult et al., 2002; Langley et al., 2003; Carcopino et al., 2007; Delsing and Kullberg, 2008). Infections during pregnancy may lead to repeated abortions in following pregnancies (Arricau-Bouvery and Rodolakis, 2005), caused by the fact that latent infections in women can be reactivated during following pregnancies (Delsing and Kullberg, 2008; Nabuurs-Franssen et al., 2008). Breast feeding after *C. burnetii* infection during the preceding pregnancy, is contraindicated (Delsing and Kullberg, 2008).

Several laboratory tests are available to confirm a diagnosis of Q fever: complement fixation, indirect immunofluorescence, immunosorbent assay and microagglutination (Maurin and Raoult, 1999; Arricau-Bouvery and Rodolakis, 2005; Delsing and Kullberg, 2008; Nabuurs-Franssen et al., 2008). Tests used differ per laboratory. Polymerase chain reaction is also highly specific, can be used in an early stage of the disease and can detect *C. burnetii* in several specimens (Maurin and Raoult, 1999; Delsing and Kullberg, 2008; Nabuurs-Franssen et al., 2008). Two to three weeks after the onset of the symptoms, serology-based tests become positive and PCR negative (Nabuurs-Franssen et al., 2008).

In involved human organs, typical histological lesions occur in acute and chronic Q fever. *C. burnetii* can be detected in involved organs by immunodetection techniques. Culture of *C. burnetii* is not easy and special precautions must be taken, because of the high infectivity of the organism. Therefore, isolation and culture is only carried out in biosafety level 3 laboratories (Maurin and Raoult, 1999).

Most of the Q fever infections pass unnoticed and are not treated. If treatment would be required, different possibilities exist which have been described extensively in the literature (Stein and Raoult, 1998; Wagner-Wiening et al., 2009; Steenbergen et al., 2007; Carcopino et al., 2007; Delsing and Kullberg, 2008; Nabuurs-Franssen et al., 2008).

Q fever in small ruminants in the Netherlands

The Dutch sheep and goat industry

The sheep and goat industry in the Netherlands is relatively small with less than one million breeding ewes and a quarter of a million breeding goats (I&R-database, 2009). The total number of registered small ruminant farms is a bit over 50,000, of which 360 are professional dairy goat farms with over 200 adult goats and 40 are professional dairy sheep farms. Bar approximately 17,000 goats kept on organic farms, dairy goats are housed throughout the year. The dairy goat industry started after the introduction

of the milk quota system in the dairy cattle industry in 1984. In 25 years, total production has grown from almost zero to over 150,000 tons of milk annually.

There have always been dairy sheep in the Netherlands (Jansen, 1985). In the past, these were kept in small numbers in many places all over the country to supply the family or the local community with dairy products. Nowadays, number of dairy sheep per farm differs widely from less than fifty to almost a thousand. Most of them are not housed, at least for part of the year. Unfortunately, detailed information on production is not available.

Cases of Q fever in small ruminants in the Netherlands

In 2005, Q fever was diagnosed for the first time in the Netherlands, as a cause of abortion on a dairy goat farm; diagnosis was confirmed by using immunohistochemistry on sections of placenta (Wouda and Dercksen, 2007). A second case was diagnosed later in 2005. In 2006, 2007 and 2008, six, seven and seven, respectively, new cases were confirmed on dairy goat farms, mainly in the southern part of the country. In the same period, two cases of abortion caused by *C. burnetii* were found on dairy sheep farms, one in the southern and one in the northern part of the country. The average number of goats per infected farm was 900, of which 20% on average (10% to 60%) had aborted. The average number of sheep for the two infected sheep farms was 400 and the abortion rate was 5%.

Q fever in people in the Netherlands

Q fever has been described in many countries all over the world. Based on a survey of the World Health Organization and an analysis of published reports, Kaplan and Bertagna (1955) reported its existence in 51 countries on five continents, mainly in cattle, sheep, goats and people. In New Zealand, Poland, the Scandinavian countries and the Netherlands no infections were found at that time. In a survey held in 1954, no evidence was found for the occurrence of Q fever in the Netherlands (Wolff and Kouwenaar, 1954) and between 1954 and 1956, the investigation of 6000 blood samples from people with an atypical pneumonia found no antibodies against Q fever (Dekking and Zanen, 1958).

In the Netherlands, Q fever has been diagnosed for the first time in 1956 (Westra et al., 1958). In a sero-epidemiological study performed between 1968 and 1983, seroprevalences in high-risk groups of veterinarians, taxidermists and female wool spinners were found to be on average 76%. 186 of 222 (84%) farm animal veterinary practitioners blood-sampled in 1982, were seropositive compared to 86 of 359 (24%) blood donors sampled in 1983. The seropositive results of the veterinarians were equally distributed over all age groups, suggesting most infections had occurred in early childhood. Males were more often infected than females (Richardus et al., 1984; Houwers and Richardus, 1987; Richardus et al., 1987).

In 1978, Q fever became a notifiable disease in humans. The number of notifications between 1978 and 2006, ranged between 1 and 32 cases annually, with an average of 17 cases per year. These cases predominantly involved patients with occupational risk. The total number of hospitalised persons in the period 1994–2001 was 49 (Delsing and Kullberg, 2008; Schimmer et al., 2009).

In May 2007, several cases of atypical pneumonia were reported to the municipal health service in the province of Noord-Brabant by a medical microbiologist. In the same month, an alert physician in Herpen reported an increase in cases of atypical pneumonia in his practice. The patients did not react as expected to the antibiotic treatment. A few weeks later, another physician in the same region also reported an increase of atypical pneumonias in his practice (Steenbergen et al., 2007). Retrospective investigation proved that *C. burnetii* was the causal agent and in 2007, a total of 182 confirmed human cases were reported. The onset of the majority of cases was between week 18 and 24. Age of the patients ranged from 7 to 87 years, female to male ratio was 1:1.7 and the preliminary hospitalisation rate was 43% (Schimmer et al., 2008). Many patients suffered from persisting fatigue for several months after the onset of the disease (Nabuurs-Franssen et al., 2009).

In 2008, Q fever returned and at the end of the year 1,000 human cases had been registered, making it the largest community outbreak of Q fever ever recorded in the world. The main symptoms were fever, fatigue, night sweating, headache and general malaise. In 65% of the cases, pneumonia was reported (Delsing and Kullberg, 2008; Schimmer et al., 2008; Schimmer et al., 2009). Because of the high numbers of *C. burnetii* bacteria shed after an abortion, the high abortion rates and the fact that abortion waves and human cases were found in the same area, dairy goat farms are believed to be the main source of the Q fever outbreaks in 2007 and 2008 (Steenbergen et al., 2007; Delsing and Kullberg, 2008; Schimmer et al., 2009).

Ongoing research

The large human Q fever outbreaks in 2007 and 2008 stimulated the start of a large multidisciplinary research portfolio, aimed at generating better knowledge about the background and transmission of *C. burnetii* to be able to take adequate control measures.

Analyses of the first thirteen outbreaks of abortion on dairy goat farms showed that the average number of goats per farm was 900, of which 20% aborted. The average number of sheep on the only two infected sheep farms was 400 and the abortion rate was 5%.

In 2008, all 15,772 blood samples from small ruminants to be tested for *Brucella melitensis*, were also tested for Q fever using an ELISA (Ruminants Serum Q Fever

LSI Kit, LSI, Lissieu, France). From those samples, 12,363 were of ovine and 3,409 of caprine origin. Based on these blood samples, seroprevalence for sheep in the Netherlands was 2.4% (95% confidence interval (CI): 2.1–2.7) and for goats 7.8% (95% CI: 6.9–8.7).

Dairy sheep and dairy goat farmers were also given the opportunity to test bulk milk samples using a PCR (TaqvetTM *Coxiella burnetii*, TaqMan Quantitative PCR, LSI, Lissieu, France). In total, 306 bulk milk samples were tested and 79 (26%) were positive.

In the autumn of 2008, a voluntary vaccination campaign against Q fever in dairy goats started in the infected area in Noord-Brabant, where most of the human cases occurred. Some dairy goat farmers reported side effects after vaccination, like fever, reduced appetite and reduced milk yield, especially on infected farms. Before start of the 2009 vaccination campaign, these side effects were investigated.

The shedding of *C. burnetii* after vaccination is being measured using a PCR on vaginal swabs. The effect of vaccination can also be monitored by measuring shedding of *C. burnetii* in bulk milk samples, using a PCR and the serological response after vaccination is also monitored.

The Central Veterinary Institute (CVI, Wageningen-UR, Lelystad) in the Netherlands, recently started to culture *C. burnetii* and started investigations on the possible infection routes in goats. Molecular characterization of *C. burnetii* (MLVA-typing) originating from placentas of aborted goats is also carried out by the CVI.

At the end of 2009, a serological survey among farm animal veterinary practitioners will be carried out and the results will be compared with previous results (Richardus et al., 1984).

Ongoing studies address the factors involved in the 2008 epidemic at national, regional and local level, the efficacy of the 2008 voluntary vaccination campaign in small ruminants and the nationwide occurrence of *C. burnetii* antibodies in the community and in small ruminants. From the human epidemiological perspective, a case control study is currently underway in the main affected region in the province of Noord-Brabant. Routinely collected sera of pregnant women from the affected regions over the period June 2007–July 2008 are retrospectively screened for Q fever to study the effect of infection on pregnancy outcome. An integrated human-veterinary study has started, in which small ruminant farmers and their animals will be screened for presence of *C. burnetii* antibodies. In addition, environmental samples will be obtained from a subset of these farms and the transmission of *C. burnetii* will be further investigated.

Measures implemented

Before June 2008, abortion outbreaks were reported on a voluntary basis to the Animal Health Service (GD) and confirmed by immunohistochemistry (Wouda and Dercksen, 1997). Since June 2008, Q fever in small ruminants is notifiable in the Netherlands. Notification criterion for farms with over 100 breeding animals is an abortion wave, defined as an abortion percentage over 5% of all pregnant small ruminants. For smaller holdings, three or more abortions in a 30-day period is used for notification of authorities.

Although a definitive source of human infections has not been identified, dairy goat farms are believed to be the main source of the human Q fever outbreak in 2007 and 2008 (Steenbergen et al., 2007; Delsing and Kullberg, 2008; Schimmer et al., 2009). Because of that, all owners of non-pregnant sheep and goats in the area, where most of the human cases had occurred in 2008, were given the opportunity to vaccinate their animals on a voluntary basis. In the autumn 2008, approximately 35,000 goats were vaccinated with Coxevac® (CEVA Santé Animale), a Phase I vaccine containing inactivated *C. burnetii*. The aim of the vaccination was to reduce shedding of *C. burnetii* and thus, environmental contamination, trying to reduce human exposure. In the spring 2009, the Dutch government implemented a compulsory vaccination campaign in the infected area, the province of Noord-Brabant and parts of the provinces of Gelderland, Utrecht and Limburg. This vaccination campaign is compulsory for dairy sheep and dairy goat farms with over 50 animals and for farms with intensive animal–human contact.

Since February 2009, a stringent hygiene protocol became mandatory for all professional dairy goat and dairy sheep farms in the Netherlands, independent of their Q fever status. The protocol includes some mandatory and some voluntary measures, aiming to preventing environmental contamination. Farmers are obliged to fight against vermin, are not allowed to take out manure from their stables for at least one month after the lambing season, are obliged to cover manure during storage and transport and will have to plough it under immediately or after composting it for at least three months. Aborted foetuses and placentas have to be rendered and records of all measures taken have to be kept for at least one year. Farmers are advised to take some voluntary measures to improve general hygiene. They are stimulated to bring in fresh straw every day during the lambing period and to submit aborted foetuses for pathological examination. Farmers are also encouraged not to admit pregnant women, children and elderly people into their stables.

Conclusions

After two serious outbreaks of human Q fever in 2007 and 2008, the Netherlands is facing a third outbreak in 2009 (Schimmer et al., 2009). This new surge in Q

fever cases increases public pressure on the dairy goat industry. The mandatory vaccination campaign that started in April 2009 is aiming to reducing the occurrence of abortion waves and the shedding of *C. burnetii* and thus, environmental contamination, trying to reduce human exposure in 2010. The results of the large portfolio of multidisciplinary research will eventually lead to the implementation of improved control measures.

Chapter 3

Demography of Q fever seroprevalence in sheep and goats in the Netherlands in 2008

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Abstract

At the end of 2007, the first year of what later turned out to be one of the largest Q fever outbreaks in the world with ultimately almost 3,500 human patients notified in three years time, dairy goats were suspected to be the possible cause. However, current information on the Q fever prevalence in small ruminants in The Netherlands was lacking.

A serological survey, using an indirect ELISA, was carried out in 15,186 sheep and goats in The Netherlands in 2008. In total, 2.4% (95% CI: 2.2–2.7) of the sheep and 7.8% (95% CI: 6.9–8.8) of the goats was seropositive for antibodies against *Coxiella burnetii*. In 14.5% (95% CI: 12.5–16.5) of the sheep flocks and 17.9% (95% CI: 14.2–21.5) of the goat herds at least one seropositive animal was found. In sheep flocks with at least one seropositive sheep, the within herd seroprevalence was 14.8% (95% CI: 12.6–17.0). In goat herds with at least one seropositive goat, the within herd seroprevalence was 29.0% (95% CI: 24.6–33.3).

The seropositive sheep were equally distributed across the country. The seroprevalence in goats in the south-eastern part of The Netherlands, the area where most of the human Q fever cases were notified, was significantly higher than the seroprevalence in goats in the rest of The Netherlands. Dairy sheep and dairy goats had a significantly higher chance of being seropositive than non-dairy sheep and goats. During pregnancy and in the periparturient period, small ruminants tested significantly more often seropositive than in the early- or non-pregnant period.

The seroprevalence as well as the true prevalence among small ruminants in The Netherlands were lower than prevalences reported elsewhere. The seroprevalence among sheep was also lower than reported in an earlier Dutch study in 1987. The Q fever seroprevalence was highest in pregnant and periparturient dairy goats in the south-eastern part of The Netherlands, which coincides with the region with the highest human incidence of Q fever.

Introduction

Q fever is an almost ubiquitous zoonosis caused by the obligate intracellular bacterium *Coxiella burnetii*. This bacterium is able to infect several animal species as well as people (Babudieri and Moscovici, 1952; Arricau-Bouvery and Rodolakis, 2005). Domestic ruminants are the primary animal reservoir of *C. burnetii*, but infections are also found in rodents, birds and arthropods (Babudieri and Moscovici, 1952), and in addition to ruminants, cats are also able to shed the bacterium (Marrie et al., 1988). The main symptom in infected sheep and goats is abortion, mainly during late pregnancy, and under these circumstances the placenta may contain up to 10^9 hamster infective doses of *C. burnetii* per gram of tissue (Babudieri, 1959). The main route of transmission of the bacterium from animals to people is by aerosols (Marrie, 1990a; Maurin and Raoult, 1999; Schimmer et al., 2009).

Q fever was first described as a febrile illness in abattoir workers in Brisbane, Australia, in 1933 (Derrick, 1937). In 1955, Kaplan and Bertagne described its existence in 51 countries on five continents, but not in The Netherlands. In a Dutch survey in 1954, no evidence was found for the existence of Q fever in humans and cattle (Wolff and Kouwenaar, 1954). Q fever was first diagnosed in The Netherlands in three human patients in 1956 (Dekking and Zanen, 1958), and became a notifiable disease in humans in 1978. Between 1978 and 2006, 1–32 human patients were diagnosed annually, with an average of 17 (Schimmer et al., 2009). In 2007, a Q fever outbreak in humans started with 176 notified human patients that year (Van Steenbergen et al., 2007). Within three years, ultimately almost 3,500 human Q fever patients were notified (van der Hoek et al., 2010a). Dairy goats were the suspected source of the human Q fever outbreak (Van Steenbergen et al., 2007).

Historically, the seroprevalence of Q fever in sheep and goats in The Netherlands was considered to be low (Houwers and Richardus, 1987). Since 2005, abortion waves due to *C. burnetii* have been diagnosed in The Netherlands on dairy goat and dairy sheep farms, by immunohistochemistry on sections of placenta (Wouda and Dercksen, 2007). However, current information on the Q fever prevalence in small ruminants was lacking. The aim of this study was to determine the Q fever seroprevalence in sheep and goats in The Netherlands, and to determine possible risk factors for infection. The likeliness that small ruminants were the source of the human outbreak is discussed.

Materials and methods

Study population in The Netherlands

At the time of this survey in 2008, the sheep and goat industry in The Netherlands was relatively small with less than one million breeding ewes and about 260,000 breeding goats. There were slightly more than 50,000 registered farms where sheep and goats were kept.

In 2008, there were about 10,000 dairy sheep in The Netherlands, kept on about 40 farms that all had at least 50 dairy sheep per farm. Most of the sheep in The Netherlands were kept for meat production, both on professional sheep farms (> 50 sheep) and on hobby farms. For the purpose of this study we called meat producing sheep, non-dairy sheep. The highest density of sheep was found in the coastal provinces.

The dairy goat industry started in the 1980s after the introduction of a system of milk quota in the dairy cattle industry. The goat industry grew from 53,000 animals in 1984 to about 260,000 animals in 2008. The highest density of goats was found in the south-eastern part of The Netherlands. There were about 360 dairy goat farms, all with more than 200 animals per farm. On the conventional dairy goat farms, the animals were housed inside throughout the year. About 17,000 goats were kept on organic farms and had access to pasture (Van den Brom and Vellema, 2009).

Serological survey

The Netherlands is officially free from *Brucella melitensis*. To keep the officially recognized free status, annually a representative sample of about 16,000 female small ruminants are serologically tested for brucellosis with negative results. About 3,500 samples are obtained by randomly selecting 480 herds and obligatory sampling of up to 13 animals older than 1 year per herd. The sample size of 13 is based on a within herd prevalence of at least 20%, which should be sufficient to detect an infection with 95% confidence. About 12,500 samples are convenience samples obtained from farms participating in the accreditation scheme for maedi-visna virus (MVV) or caprine arthritis encephalitis virus (CAEV), and up to 13 samples per submission have to be obligatory tested for brucellosis. The samples were submitted throughout 2008 (about 1,200 per month) and none of the serum samples were taken from the animals that had been vaccinated against Q fever. Farms were sampled only once a year. The samples were serologically tested for the presence of antibodies against *C. burnetii* with an indirect ELISA (Ruminants Serum Q Fever LSI Kit, LSI, Lissieu, France). This ELISA test uses antigen obtained from an European ovine strain. The test was used according to the manufacturer's instructions. The sensitivity of the test was reported to be 93.9% (95% CI: 89.4–97.2) and the specificity 98.4% (95% CI: 96.6–99.5). A farm was considered *C. burnetii* positive when at least one animal presented a positive serology. Throughout the paper (apparent) test prevalences are reported unless specifically mentioned otherwise. The Netherlands is divided into twelve provinces and ninety-two two-digital postal code areas. A postal code area was called positive when at least one farm tested positive for *C. burnetii*. The human Q fever outbreak that started in 2007 was mainly situated in the south-eastern part of The Netherlands. For the final analysis of this study, this south-eastern part of The Netherlands, consisting of the provinces of Limburg, Noord-Brabant and Gelderland, was compared with the rest of The Netherlands.

Statistical analysis

Statistical analyses were carried out using STATA 12.1 (STATA, 2011). Univariate logistic regression analysis (logistic) was used to determine the association between the categorical risk factors and the serostatus for *C. burnetii* on animal and herd level.

The factors that were considered were region, health status of the flock or herd, stage of gestation of the animal, and whether a dairy or non-dairy animal. The prevalence per region was calculated per two-digital postal code area, per province and for the south-eastern part of The Netherlands, in comparison with the rest of The Netherlands. The MVV and CAEV health status of sheep flocks and goat herds, respectively, were investigated as a potential risk factor. Stage of gestation was determined by combining the date of birth (age) and the month of sampling of the sheep and goats tested in this study. Given a clear seasonal breeding pattern of Dutch sheep and goats, the stage of gestation of the sampled animal was determined.

Logistic regression analysis (logistic) was performed to determine the multivariate association between risk factors and serostatus for *C. burnetii*. For the final multivariable analysis, all potential risk factors were forced into the model.

In the model, standard errors were adjusted for clustering of observations within the farms by using the robust estimator of variance (vce (cluster "herd")). All two-way interactions were tested. The goodness of fit of the model was determined by Pearson's goodness of fit test (estat gof), the proportion of correctly classified observations and McFadden's pseudo R^2.

Finally, because a negative binomial distribution may fit better to the low prevalence found in the study, the data were aggregated on herd-level and a negative binomial regression (nbreg) was run. All potential risk factors were forced into the model. The goodness of fit of the model was determined by the likelihood ratio test of alpha (to determine the fit relative to a Poisson distribution) and McFadden's pseudo R^2.

Results

In 2008, a total of 15,186 blood samples from female small ruminants were tested for antibodies against *C. burnetii*, being 12,052 sheep samples from 1,208 farms, and 3,134 goat samples from 442 farms. The sheep samples originated from 140 dairy sheep and from 11,912 non-dairy sheep. The goat samples originated from 1,290 dairy goats and 1,844 non-dairy goats. Based on information from the national small ruminant identification and registration database, Dutch sheep and goats were demonstrated to be seasonal breeders (Figure 3.1a and b), and therefore animals sampled between January and May were regarded pregnant or in the periparturient period and animals sampled between June and December were regarded to be in the non-pregnant or early pregnant period.

Sheep

A total of 2.4% (95% confidence interval (CI): 2.2–2.7) of the sheep tested positive for antibodies against *C. burnetii*. The seroprevalence in dairy sheep was 5.7% (95% CI: 1.9–9.5) and 2.4% (95% CI: 2.1–2.7) in non-dairy sheep (Table 3.1).

In 14.5% (95% CI: 12.5–16.5) of the flocks at least one sheep was seropositive. The herd seroprevalence of dairy sheep farms was significantly higher (38.5% (95% CI: 17.7–64.5)) than the herd seroprevalence of non-dairy sheep farms (14.2% (95% CI: 12.4–16.3)).

In a positive flock, the average within herd seroprevalence was 14.8% (95% CI: 12.6–17.0) (Table 3.1). Positive sheep were found in 66 out of 86 two-digital postal code areas. In six areas, no sheep were tested. The positive postal code areas were found throughout the country. No difference was found in seroprevalence per province.

The results of the multivariable analysis (Table 3.2) showed that a dairy sheep had a 2.1 (95% CI: 1.4–3.0) times higher risk to be seropositive than a non-dairy sheep. Sheep sampled during pregnancy and in the periparturient period had a 3.6 (95% CI: 2.8–4.7) times higher risk to be seropositive than sheep sampled during early- or non-pregnancy. Other factors, such as region and MVV health status, did not significantly differ in the final multivariable model, nor did any of the two-way interactions. Both the predictive value of the model and Pearson's goodness of fit test showed a poor fit of the model to the data. The results for the risk factors of the negative binomial regression model on herd-level were very similar (results not shown).

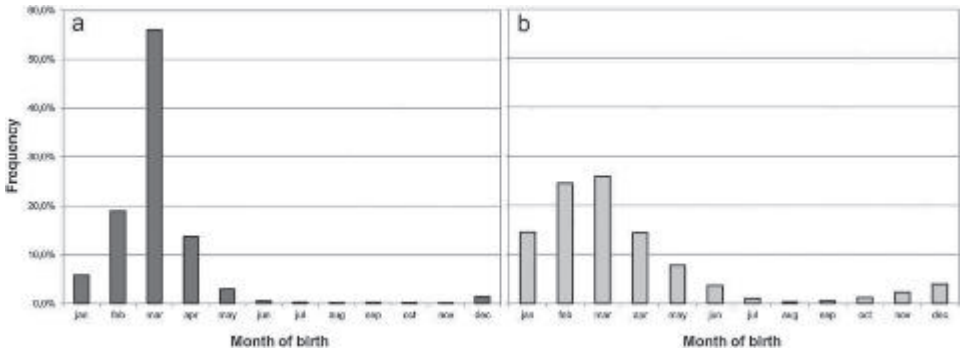

Figure 3.1. (a) The frequency distribution of the month of birth of sheep tested for Q fever in 2008. Information is based on the national small ruminant identification and registration database and indicates that sheep are seasonal breeders. (b) The frequency distribution of the month of birth of goats tested for Q fever in 2008. Information is based on the national small ruminant identification and registration database and indicates that goats are seasonal breeders.

Table 3.1. Results of descriptive analysis of the seroprevalence of Q fever in sheep and goats in the Netherlands in 2008.

	Sheep			Goats		
	n	Seroprevalence (%)	95% CI	n	Seroprevalence (%)	95% CI
Overall (animal seroprevalence)	12,052	2.4	2.2 - 2.7	3,134	7.8	6.9 - 8.8
Dairy	140	5.7	1.9 - 9.5	1,290	14.7	12.8 - 16.6
Non-dairy	11,912	2.4	2.1 - 2.7	1,844	3.0	2.2 - 3.8
Farms (herd seroprevalence)	1,208	14.5	12.5 - 16.5	442	17.9	14.2 - 21.5
Dairy	13	38.5	17.7 - 64.5	103	44.7	35.4 - 54.3
Non-dairy	1195	14.2	12.4 - 16.3	339	9.7	7.0 - 13.4
Within herd prevalence on a positive farm	1,976	14.8	12.6 - 17.0	846	29.0	24.6 - 33.3
Within herd prevalence on a positive dairy farm	36	22.2	8.6 - 35.8	588	32.1	28.4 - 35.9
Within herd prevalence on a positive non-dairy farm	1,940	14.7	13.1 - 16.3	258	21.7	16.7 - 26.7

Goats

A total of 7.8% (95% CI: 6.9–8.8) of the goats tested positive for antibodies against *C. burnetii*. Dairy goats showed a significantly higher (14.7% (95% CI: 12.8–16.6)) seroprevalence than non-dairy goats (3.0% (95% CI: 2.2–3.8)).

In 17.9% (95% CI: 14.2–21.5) of the herds, at least one goat was seropositive. The herd seroprevalence of dairy goat farms was significantly higher (44.7% (95% CI: 35.4–54.3)) than of non-dairy herds (9.7% (95% CI: 7.0–13.4)). In a positive goat herd, the within herd seroprevalence for Q fever was 29.0% (95% CI: 24.6–33.3). The within herd seroprevalence in positive dairy goat herds was significantly higher (32.1% (95% CI: 28.4–35.9)) than in non-dairy herds (21.7 (95% CI: 16.7–26.7)) (Table 3.1).

Positive goats were found in forty out of 83 two-digital postal code areas. In nine two-digital postal code areas no goats were tested. The seroprevalence in goats in the province of Noord-Brabant was higher than in the other provinces and significantly higher than in most of the other provinces in The Netherlands (Figure 3.2).

The results of the multivariable analysis (Table 3.2) showed that goats kept in the south-eastern region (Figure 3.2), consisting of the provinces of Limburg, Noord-Brabant, and Gelderland, had a 2.2 (95% CI: 1.3–3.9) times higher risk to be seropositive than goats kept in other regions of The Netherlands. A dairy goat had a 4.0 (95% CI: 2.2–7.2) times higher risk to be seropositive than a non-dairy goat. Goats sampled during pregnancy and in the periparturient period had a 2.2 (95% CI: 1.2–3.7) times higher risk to be seropositive than goats sampled during early- or non-pregnancy. Being CAEV accredited was not significantly associated with the serostatus for Q fever. Also, none of the two-way interactions was significantly

Table 3.2. Final results of multivariable logistic regression analysis of risk factors for the serostatus of Q fever in sheep and goats in the Netherlands in 2008.

	Sheep	Pseudo r^2=0.05 Pearson's g.o.f.[c] P= 0.01			Goats	Pseudo r^2=0.10 Pearson's g.o.f.[c] P< 0.01		
Variable	Seroprevalence (%)	OR	95% CI	P	Seroprevalence (%)	OR	95% CI	P
Region								
Rest Netherlands	2.5	1.00			5.3	1.00		
South-eastern part	2.4	1.18	0.86 - 1.62	0.29	11.4	2.23	1.26 - 3.94	<0.01
Health status								
MVV[a]/CAEV[b]-free	2.1	1.00			8.4	1.00		
not MVV[a]/CAEV[b]-free	3.3	1.33	0.89 - 2.00	0.17	6.3	0.84	0.43 - 1.65	0.62
Type of farm								
non-dairy	2.4	1.00			3.0	1.00		
dairy	5.7	2.08	1.44 - 3.01	<0.01	14.7	4.01	2.23 - 7.21	<0.01
Gestation period								
early- or non-pregnant	1.2	1.00			6.2	1.00		
pregnant/periparturient	3.3	3.64	2.84 - 4.65	<0.01	11.9	2.15	1.24 - 3.72	<0.01

[a] MVV= maedi visna virus (sheep only)
[b] CAEV= caprine arthritis encephalitis virus (goats only)
[c] g.o.f.= goodness of fit

differed in the multivariable analysis. Both the predictive value of the model and Pearson's goodness of fit test showed a poor fit of the model to the data. The results for the risk factors of the negative binomial regression model on herd-level were very similar (results not shown).

Discussion

The Q fever prevalences for sheep and goats found in this study were considered representative for the registered small ruminant population in The Netherlands. Both for goats and sheep, selection bias was thought to be minimal because participation in the monitoring scheme for *B. melitensis* is obligatory. Only a very small proportion of the small ruminants in The Netherlands was not officially registered and thus not part of this study, and these animals were likely to be kept mainly on locations with only a few animals. Trading of not officially registered small ruminants is almost impossible and therefore these animals were considered to have had a very limited role in the epidemiology of infections.

In this survey, the chosen sample size was based on the compulsory *B. melitensis* monitoring programme. On farms with less than thirteen small ruminants, all animals were sampled and the results provided a good impression of the herd and within herd prevalence. On larger farms, thirteen animals were tested and, based

Figure 3.2. The Q fever seroprevalence in goats (mean, 95% CI) per province of The Netherlands, in 2008. In the south-eastern region, consisting of the provinces of Limburg (LB), Noord-Brabant (NB) and Gelderland (GL), most of the ultimately almost 3,500 human Q fever patients were notified. LB, Limburg; NB, Noord-Brabant; GL, Gelderland; ZL, Zeeland; ZH, Zuid-Holland; UT, Utrecht; NH, Noord-Holland; FL, Flevoland; OV, Overijssel; FR, Friesland; DR, Drenthe; GR, Groningen.

on a within herd prevalence of 20%, this sample size per farm should be sufficient to detect an infection on herd and flock level with a 95% confidence. Taking into account that during a Q fever outbreak in goats abortion rates up to 90% are described (Arricau-Bouvery and Rodolakis, 2005; Van den Brom and Vellema, 2009), high seroprevalences were expected on infected farms. Therefore, the influence of sample size on herd seroprevalence in goats was thought to be minimal in this survey. Given that the prevalence in infected sheep flocks may be lower, it

is possible that in sheep the sample size could have resulted in underestimation of the herd prevalence on the larger farms (>13 sheep).

The fit of the multivariable logistic regression models on animal level was poor, probably due to the low prevalence (many zeros). However, the estimates for the risk factors seemed robust and were not affected when a better fitting distribution (negative binomial) was applied on herd level data.

In 1954, no evidence was found for the existence of Q fever in 745 ruminants tested (Dekking and Zanen, 1958). A survey in 1987 also used an indirect ELISA, and showed antibodies against *C. burnetii* in 3.5% of 3,603 sheep from 191 flocks. A total of 52 flocks (27.2%) had one or more seropositive sheep. This limited survey also included 498 goats of 0.5–1 year old, and 96 adult goats, and showed that less than 1% of goats had antibodies against *C. burnetii* (Houwers and Richardus, 1987).

In The Netherlands, the seroprevalence among sheep was similar throughout the country, and relatively low with 2.4% (95% CI: 2.2–2.7). In similar surveys in other countries, the sheep and flock seroprevalences were higher (Martinov et al., 1989b; McQuiston and Childs, 2002; Dolcé et al., 2003; Masala et al., 2004; Psaroulaki et al., 2006; García-Pérez et al., 2009).

The seroprevalence in goats of 7.8% (95% CI: 6.9–8.8) was lower than prevalences found in similar surveys in other countries (Martinov et al., 1989b; Hatchette et al., 2002; McQuiston and Childs, 2002; Psaroulaki et al., 2006). Also, for both sheep and goats the true prevalence, meaning test prevalence corrected for sensitivity and specificity of the ELISA test used, was lower (results not shown) than prevalences reported in other countries.

Goats in the south-eastern part of The Netherlands (Figure 3.2) had a two times higher risk to be seropositive. In this area, most of the human cases, and most of the abortion outbreaks in dairy goat herds have been reported. This area also had the highest density of goats per square kilometre. The seroprevalence in goats of the province of Noord-Brabant was higher than in most of the other provinces in The Netherlands. A relatively high seroprevalence was also found in the provinces of Drenthe and Limburg (Figure 3.2), but the numbers of tested animals in these provinces were relatively small, which resulted in wide confidence intervals.

However, the results of surveys from other countries were difficult to compare with our results, because of different study populations, and the use of different tests with different test characteristics. Whether the study population was dairy or not and pregnant or not may have a strong influence on the prevalence estimates. Complement fixation (CF) and ELISA were mostly used tests for detecting antibodies

against *C. burnetii* (McQuiston and Childs, 2002; Dolcé et al., 2003; Masala et al., 2004; García-Pérez et al., 2009; Kennerman et al., 2010). ELISA was more sensitive than CF and described as suitable for seroepidemiological studies (Kovácová and Kazár, 2000) and was therefore used in our study.

The significantly higher prevalence in dairy goats compared to non-dairy goats was probably due to the way goats were kept. On most dairy goat farms, the animals were kept inside barns throughout the year, and the average herd consisted of about 900 animals. This may have facilitated the spread of *C. burnetii* within the herds. Non-dairy goats were kept outside part of the year and often in small groups, with less intense contact than dairy goats. This hypothesis was supported by the within herd prevalence on positive dairy goat farms (32.1% (95% CI: 28.4–35.9)) compared with the within herd prevalence on positive non-dairy goat farms (21.7% (95% CI: 16.7–26.7)). Furthermore, the herd seroprevalence of dairy goat farms (44.7% (95% CI: 35.4–54.3)) was significantly higher than the herd seroprevalence of non-dairy goat farms (9.7% (95% CI: 7.0–13.4)). A serological survey in 2009, on only dairy goat farms in The Netherlands, showed that 43.1% of 123 farms and 21.4% of 2,828 dairy goats were seropositive (Schimmer et al., 2011).

For the significantly higher herd prevalence in dairy sheep compared to non-dairy sheep, the same explanation may apply, although dairy sheep are kept in lower numbers than dairy goats, and most of the dairy sheep are kept outside, at least for a few months a year (Van den Brom and Vellema, 2009).

All sheep and goat sera were collected during the year 2008. The highest seroprevalences of antibodies against *C. burnetii* were found in animals tested during pregnancy and in the periparturient period. In pregnant animals, massive *C. burnetii* multiplication can take place during the last weeks of the pregnancy (Sánchez et al., 2006). This possibly explained the higher seroprevalence in Dutch sheep and goats during pregnancy and in the periparturient period versus the early-/non-pregnant period in 2008.

Several human outbreaks of Q fever have been described and related to small ruminants. In Bulgaria, an outbreak was related to grazing and travelling sheep and goats (Panaiotov et al., 2009). In Germany, a Q fever outbreak in a rural community was related to sheep (Lyytikäinen et al., 1998). The large human Q fever outbreak in The Netherlands started in 2007 in the south-eastern part of the country, and was suspected to be related to dairy goat farms with *C. burnetii* induced abortions (Van Steenbergen et al., 2007; Schimmer et al., 2009). In 2008, also in the south-eastern region compared to the other part of The Netherlands, a significantly higher proportion of dairy goat farms tested RT-PCR positive in bulk tank milk samples (Van den Brom et al., 2012a). It was in the same area that we found a significantly higher seroprevalence in goats when compared to other parts

of The Netherlands (Figure 3.2 and Table 3.2). One can argue that this higher seroprevalence reflected a recent infection which caused environmental contamination, and human exposure, however causality could not be proven from the results in this study. Sheep in The Netherlands seemed to play a minor role in the human Q fever outbreak, given that no regional differences were found and the seroprevalence was very low.

Conclusions

The seroprevalences of antibodies against *C. burnetii* among small ruminants in The Netherlands in 2008 were relatively low compared with similar seroprevalence surveys in other countries. However, the results from international surveys were difficult to compare with our results, because of different study populations, and the use of different tests with different test characteristics.

The Q fever seroprevalence in goats was significantly higher in the south-eastern part of The Netherlands than in the other parts of The Netherlands. Dairy sheep and goats had an increased risk to be seropositive for Q fever compared to non-dairy sheep and goats. Sheep and goats tested during pregnancy and in the periparturient period had a significantly higher risk to be seropositive than animals tested in early-pregnancy or non-pregnant period. The higher prevalence in dairy goats in the south-eastern part of the country possibly reflected a recent infection, which caused environmental contamination, and consequently human exposure in the same region.

Conflict of interest

None of the authors of the above manuscript have declared any conflict of interest which may arise from being named as an author of the manuscript.

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Chapter 4

Coxiella burnetii in bulk tank milk samples from dairy goat and dairy sheep farms in the Netherlands in 2008

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Abstract

In 2007, a human Q fever epidemic started, mainly in the south eastern part of The Netherlands with a suspected indirect relation to dairy goats, and, to a lesser degree, to dairy sheep. This article describes the Q fever prevalences in Dutch dairy goat and dairy sheep bulk tank milk (BTM) samples, using a real-time (RT) PCR and ELISA. Results of BTM PCR and ELISA were compared with the serological status of individual animals, and correlations with a history of Q fever abortion were determined. When compared with ELISA results, the optimal cut-off value for the RT-PCR was 100 bacteria/ml. In 2008, there were 392 farms with more than 200 dairy goats, of which 292 submitted a BTM sample. Of these samples, 96 (32.9 per cent) were PCR positive and 87 (29.8 per cent) were ELISA positive. All farms with a history of Q fever abortion (n = 17) were ELISA positive, 16 out of 17 were also PCR positive. BTM PCR or ELISA positive farms had significantly higher within-herd seroprevalences than BTM negative farms. In the south eastern provinces, the area where the human Q fever outbreak started in 2007, a significantly larger proportion of the BTM samples was PCR and ELISA positive compared to the rest of The Netherlands. None of the BTM samples from dairy sheep farms (n = 16) were PCR positive but three of these farms were ELISA positive. The higher percentage of BTM positive farms in the area where the human Q fever outbreak started, supports the suspected relation between human cases and infected dairy goat farms.

Introduction

Q fever is a zoonosis caused by *Coxiella burnetii*, which is an aerobic, obligate intracellular, Gram-negative, highly resistant bacterium that may infect mammals, birds, arthropods and man (Babudieri and Moscovici 1952; Arricau-Bouvery et al., 2005; Berri et al., 2007). In domestic ruminants, the primary animal reservoir of *C. burnetii*, the main clinical sign of Q fever is abortion. *C. burnetii* is mainly shed after parturition or abortion in birth products, but shedding also occurs in urine, faeces and milk (Arricau-Bouvery et al., 2003; Guatteo et al., 2007; García-Pérez et al., 2009).

In 2007, a human Q fever epidemic started in the south eastern part of The Netherlands within three years resulting in almost 3,500 officially notified human patients (van der Hoek et al., 2010a), and an indirect relation to dairy goats was suspected (Van Steenbergen et al., 2007). Because of the precautionary principle, the Dutch government decided to implement measures on infected dairy sheep and goat farms, making it necessary to distinguish between infected and non-infected farms. In order to demonstrate an infection with *C. burnetii* in animals, individual tests like ELISA and real-time PCR (RT-PCR) in various matrices and immunohistochemistry (IHC) performed on placentas are available (Kovácová and Kazár 2000; Wouda and Dercksen 2007; García-Pérez et al., 2009; Muskens et al., 2011). Taking into account the size of the Dutch dairy goat farms with an average number of around 900 adult animals per farm (Van den Brom and Vellema 2009), a monitoring programme based on repeated individual testing is expensive and difficult to perform. However, for dairy cattle farms, bulk tank milk (BTM) sampling for different diseases, as neosporosis, salmonellosis and bovine viral diarrhoea (BVD)., has been shown to be a good method to measure the disease status of lactating animals (Veling et al., 2002; Zimmer et al., 2002; Bartels et al., 2005) and this has also been demonstrated for *C. burnetii* (Kim et al., 2005; Muskens et al., 2011). For cattle, it has been shown that shedding of *C. burnetii* occurs in milk, faeces and vaginal fluid. From these shedding routes, shedding by milk is the most continuous one (Guatteo et al., 2007; 2011).

The aim of this study was (1) to determine the agreement between the results of a commercially available ELISA and RT-PCR in the same BTM samples and individual serum samples from dairy goat and dairy sheep farms with and without a history of IHC-confirmed Q fever abortions and (2) to describe the Q fever prevalence on farm level by testing BTM samples using this ELISA and RT-PCR, related to the results of individual blood samples and of IHC-confirmed Q fever abortions.

Materials and methods

Study population in The Netherlands

In 2008, there were 40 professional dairy sheep farms and 392 dairy goat farms with more than 200 dairy goats per farm, containing approximately 260,000 goats in total (Van den Brom and Vellema 2009). No vaccination for *C. burnetii* was performed on these farms before sampling.

Sampling

BTM samples

In 2008, all 392 dairy goat and 40 dairy sheep farmers in The Netherlands were asked to submit a BTM sample to be tested for *C. burnetii*, using an ELISA and a RT-PCR.

Serum samples

Serum samples were submitted from randomly selected farms as part of the annual *Brucella melitensis* monitoring programme. Per farm, 13 samples from animals older than one year were tested. This number of animals is sufficient taking into account that during a Q fever abortion outbreak in goats, abortion rates up to 90 per cent are described (Palmer et al., 1983; Hatchette et al., 2003; Arricau-Bouvery and Rodolakis 2005; Van den Brom and Vellema 2009) and high seroprevalences are therefore expected on infected farms. The within-herd seroprevalences were determined on 77 of the farms that submitted a BTM sample. Correlations between the ELISA and PCR BTM results and the within-herd seroprevalences were investigated.

Farms with a history of abortion caused by C. burnetii

In The Netherlands, abortion herd prevalences exceeding 5 per cent were notifiable in 2008. *C. burnetii* was first diagnosed as abortifacient agent on a dairy goat farm in 2005. The diagnosis was made by IHC detection of *C. burnetii* in sections of fetal membranes of representative cases. Since that time, suspected cases of Q fever abortions were tested by IHC. IHC was performed using the EnVision+ system (DAKO). For the first incubation step, sheep-anti-*C. burnetii* IgG1, labelled with horseradish peroxidase (HRP) was used, which was kindly provided by the Moredun Research Institute, Scotland, UK. The following step was incubation with rabbit anti-HRP and consecutively with the DAKO Envision+ system antirabbit. The immunoperoxidase staining was done with diaminobenzidine using the DAKO Liquid DAB+ Substrate-Chromogen System and sections were counterstained with haematoxylin (Wouda and Dercksen 2007). On dairy goat farms where Q fever abortion was confirmed between 2005 and 2008, the relationship with the results of the BTM samples was investigated.

ELISA

In this study, BTM and serum samples were tested for the presence of antibodies to *C. burnetii* with an indirect ELISA (Ruminants Serum Q Fever LSI Kit, LSI). The ELISA test is based on antigen obtained from an European ovine strain. The test was used according to the manufacturer's instructions. Briefly, serum was diluted 1:400, and milk was diluted 1:20 in dilution buffer, and both were transferred to 96 wells ELISA plates (total volume 100 µl), coated with antigen. The serum samples were incubated for one hour at 37°C and the milk samples overnight at 4°C. The plates were washed four times and incubated with 100 µl antiruminant IgG peroxidase conjugate for one hour at 37°C. After washing four times, the wells were incubated with 100 µl tetramethylbenzidine substrate for 10 minutes at 22°C in darkness. Colour development was stopped by the addition of 100 µl stop solution (0.5 M H_2SO_4). Optical density values were measured at 450 nm (OD450). Sample/-positive percentages (S/P per cent) were calculated using the following formula $(OD_{sample}-OD_{negative\ control})/(OD_{positive\ control}-OD_{negative\ control})$ x 100 per cent. The resulting S/P per cent for serum samples were divided in two different classes: negative (S/P per cent <40) or positive (S/P per cent≥40). For BTM samples, the different classes were as follows: negative (N; S/P per cent <30), low positive (LP; 30≤S/P per cent <100), positive (P; 100≤S/P per cent <200) and high positive (HP; S/P per cent≥200).

PCR

The BTM samples were tested using a commercial RT-PCR assay (LSI Taqvet *C. burnetii*, Laboratoire Service International) which targets the repetitive transposon-like region of the bacterium. The test was used according to the manufacturer's instructions. DNase RNase free water was used as negative control sample. The external positive control sample was delivered with the kit and contained 105 *C. burnetii*/ml (Strain CB01, INRA). DNA was extracted using the QIAmp DNA mini kit (Qiagen S.A) according to the manufacturer's instructions. The extraction was performed directly from 200 µl of raw milk. The PCR assays were performed using ABI Prism sequence Detection System 7500 (Applied Biosystems). For positive samples with a typical amplification curve, the results were given in Ct (cycle threshold) values. The samples presenting a typical amplification curve with a Ct value below 40 were considered to be positive. Each sample was also tested with a specific primer set for the ruminant household gene glyceraldehyde 3-phosphate dehydrogenase. Titres of *C. burnetii*/ml were quantified. For each sample, quantification was based on a reference line generated in each test from decimal dilutions of the positive control. The results are presented in four classes: negative (N; no bacteria), weak positive (WP; 1≤PCR <100 bacteria/ml), high positive (HP; 100≤PCR <10,000 bacteria/ml), very high positive (VH; PCR≥10,000 bacteria/ml).

Statistical data analysis

Farm prevalences for the presence of antibodies and the repetitive transposon-like regions of *C. burnetii* were calculated. Corresponding 95 per cent CI were calculated with WinEpiscope 2.0 (Thrusfield et al., 2001). Potential risk factors were analysed by logistic regression (logistic, STATA/SE 11.2). Bonferroni method was used for multiple comparisons between ELISA or PCR classes and number of positive goats per farm (Oneway, Bonferroni, STATA/SE 11.2).

Results

Descriptive data for the BTM ELISA and RT-PCR

A total of 308 BTM samples from dairy sheep and dairy goat farms were tested by RT-PCR and ELISA. From the 292 goat BTM samples, 87 (29.8 per cent [95 per cent CI: 27.2 to 32.5]) were ELISA positive and 96 (32.9 per cent [95% CI: 30.2 to 35.6]) were PCR positive (Table 4.1). From the 16 sheep BTM samples, three (18.8 per cent [95 per cent CI: 4.0 to 33.6]) were ELISA positive and none were PCR positive. These BTM results were also used to determine the characteristics of the tests.

Results of BTM ELISA versus BTM PCR

Log-transformed quantitative PCR data were compared with ELISA S/P ratios and a correlation coefficient (r) of 0.90 was calculated (Figure 4.1). Different cut-off levels of the BTM PCR were taken as reference values. After this, for each PCR cut-off level Receiver Operator Curve (ROC) curves were plotted for the different BTM ELISA S/P ratios. For the chosen PCR cut-offs of 1, 10, 20, 50, 80, 100, 120, 200, 500, 1,000, 2,000, 5,000 and 10,000, the highest area under the ROC curve of the ELISA was at a PCR cut-off of 100 bacteria/ml (Figure 4.2). For this reference value, the area under the ROC curve of the ELISA S/P ratio was 0.968 and the maximum proportion of agreement was reached at ELISA cut-off of 93 per cent S/P ratio reaching a sensitivity of 88.2 per cent and a specificity of 94.6 per cent. At cut-off levels of 30, 100 and 200 S/P ratios, as indicated by the manufacturer, the sensitivity and specificity was 95.6, 85.3 and 8.8 per cent, respectively, and 89.6, 95.0 and 99.6 per cent, respectively.

Table 4.1. PCR and ELISA results of dairy goat BTM samples.

		PCR				
		Negative	Weak	Positive	Very High	Total (95% CI)
ELISA	n	196	28	61	7	292
Negative	205	64.7%	4.5%	1.0%		70.2% (67.6-72.8)
Low Positive	19	0.7%	3.1%	2.7%		6.5% (5.1- 7.9)
Positive	61	1.4%	2.1%	15.4%	2.1%	20.9% (18.5-23.3)
High Positive	7	0.3%		1.7%	0.3%	2.4% (1.5- 3.3)
Total	292	67.1%	9.6%	20.9%	2.4%	100.0%
(95% CI)		(64.4-69.8)	(7.9-11.3)	(18.6-23.3)	(1.5-3.3)	

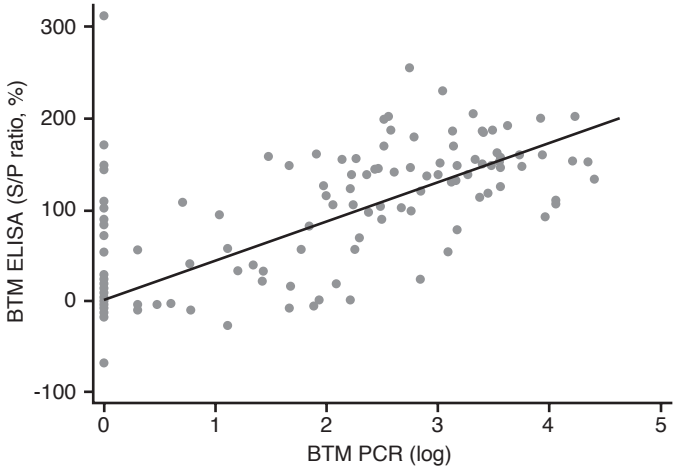

Figure 4.1. Comparison of semiquantitative PCR results (as log value) and ELISA results (S/P-ratio) of 292 goat and 16 sheep bulk tank milk samples. Note that the results of many samples are zero in both tests.

Individual serum samples

The overall percentage seropositive goats was 17.7 per cent. From the 77 herds, 40 (51.9 per cent [95 per cent CI: 41.9 to 61.9]) herds contained one or more positive animals out of 13 sampled animals. From these herds with positive samples, the mean prevalence was 4.4 and the median was four positive animals out of 13.

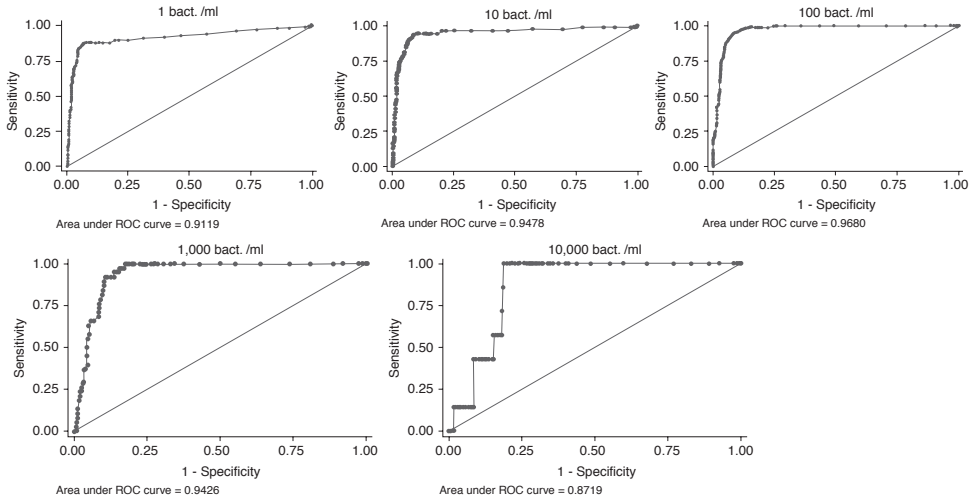

Figure 4.2. ROC plots of the BTM antibody ELISA with use of the BTM RT-PCR as reference value. Different plots indicate different cut-off levels of the BTM PCR of respectively 1, 10, 100, 1,000 and 10,000 bacteria per ml.

Table 4.2. Serological results of individual goats, average number of positive animals per farm and herd prevalences in relation to BTM PCR results of these farms.

BTM PCR	Number of farms	Average number of positive animals per farm	Herd prevalence	Number of serological positive goats per farm out of 13					
				0	1	2	3	4	≥5
Negative	45	0.80	6.2%	32	6	3	1	0	3
Weak	6	1.17	9.0%	3	1	1	0	1	0
Positive	24	5.13	39.4%	2	1	2	3	2	14
Very High	2	5.50	42.3%	0	0	0	0	1	1
Total	77	2.30	17.7%	37	8	6	4	4	18

BTM, bulk tank milk

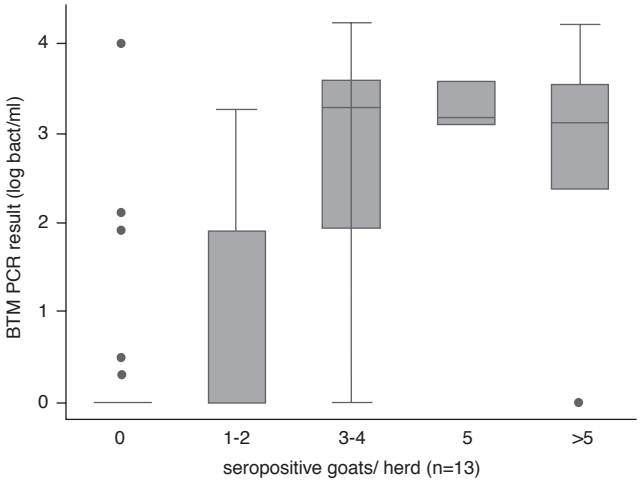

Figure 4.3. Comparison of the BTM PCR results (log value) and the number of seropositive animals, from 13 sampled animals, serologically tested by ELISA, per herd. Note that the results of many farms are 0 for both PCR and within-herd seroprevalence.

Correlation BTM ELISA and individual serum samples

Different cut-offs of within-herd seroprevalences were taken as reference for estimating the sensitivity and specificity of the BTM ELISA with different S/P cut-off levels. For the chosen cut-off levels of prevalence (8, 15, 23, 46 and 62 per cent), the area under the ROC curve was highest (0.8774) for a within-herd seroprevalence of 15%. In that situation, the proportion of agreement was highest (88.3 per cent) at BTM ELISA cut-off of 46 per cent S/P ratio. At this cut-off, the sensitivity of the BTM ELISA was 84.3 per cent and the specificity was 91.1 per cent. The correlation coefficient between within-herd seroprevalences and BTM ELISA S/P ratio was $r = 0.72$.

Table 4.3. Serological results of individual goats, average number of positive animals per farm and herd prevalences in relation to BTM ELISA results of these farms.

BTM ELISA	Number of farms	Average number of positive animals per farm	Herd prevalence	Number of serological positive goats per farm out of 13					
				0	1	2	3	4	≥5
Negative	46	0.70	5.4%	35	6	1	1	0	3
Low Positive	8	2.25	17.3%	2	0	3	1	1	1
Positive	22	5.59	43.0%	0	2	2	2	2	14
High Positive	1	4.00	30.8%	0	0	0	0	1	0
Total	77	2.30	17.7%	37	8	6	4	4	18

BTM, bulk tank milk

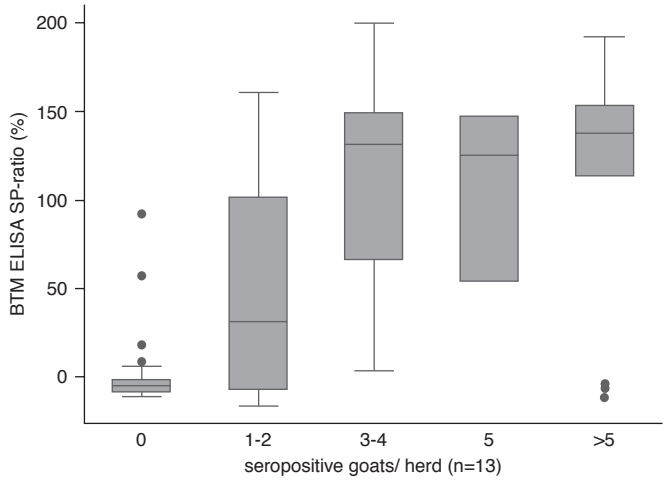

Figure 4.4. Comparison of the BTM ELISA S/P ratio and the number of seropositive animals, from 13 animals sampled, serologically tested by ELISA, per herd. Note that the results of many farms are zero for both within-herd seroprevalence and ELISA S/P ratio.

Correlation BTM PCR/ELISA and history of Q fever abortion

Results of the BTM PCR and ELISA in herds with IHC confirmed Q fever abortion were compared with results of herds without notified Q fever abortions. From 17 goat herds with a history of abortion, 16 (94.1 per cent [95 per cent CI: 83.2 to 100.0]) were BTM PCR positive and 17 (100.0 per cent) were BTM ELISA positive. In herds without notified Q fever abortion (n = 275), 80 (29.1 per cent [95 per cent CI: 27.8 to 30.4]) were BTM PCR positive ($P < 0.001$), and 70 (25.5 per cent [95 per cent CI: 24.3 to 26.7]) were BTM ELISA positive ($P < 0.001$). Although the time period between abortion and BTM sampling differed from zero to three years, both ELISA and PCR results did not change by year of abortion.

Q fever prevalence in BTM (PCR, ELISA) and individual samples (ELISA)
A total of 308 BTM samples from dairy sheep and dairy goat farms were tested by RT-PCR and ELISA, and 1,053 serum samples from 81 dairy sheep and dairy goat farms were tested for antibodies against *C. burnetii*. From these 1,053 samples, 181 (17.2 per cent (95 per cent CI: 14.9 to 19.5) were serological positive.

Between 2005 and 2008, IHC-confirmed Q fever abortion was diagnosed on 21 dairy goat and two dairy sheep farms before BTM sampling in 2008. One dairy goat farm had an outbreak of Q fever abortion after submitting the BTM sample and classified as having no history of Q fever abortion at the time of BTM sampling.

Dairy sheep
A total of 16 out of 40 dairy sheep farms submitted a BTM sample. None was PCR positive and three (18.8 per cent [95 per cent CI: 4.0 to 33.6]) were ELISA positive for *C. burnetii*.

From four dairy sheep farms which were all BTM ELISA and PCR negative, in total 52 serum samples were available. Four serum samples (7.7 per cent [95 per cent CI: 0.0 to 14.9]) originating from one farm, were seropositive. On this farm, no abortion caused by *C. burnetii* was notified. Two dairy sheep farms had a history of abortion waves caused by *C. burnetii*. One of these two farms submitted a BTM sample. This BTM sample was ELISA positive and PCR negative.

Dairy goats
A total of 292 out of 392 dairy goat farms submitted BTM samples, of which 158 (54.1 per cent) originated from farms that were situated in the south eastern provinces of The Netherlands. 96 BTM samples (32.9 per cent [95 per cent CI: 30.2 to 35.6]) were PCR positive (Table 4.1). 75 (78.1 per cent (95 per cent CI: 69.9 to 86.4) from the 96 BTM PCR positive goat farms were situated in the south eastern provinces of The Netherlands. In this region, 50.2 per cent (95 per cent CI: 46.5 to 53.9) of the investigated BTM samples was PCR positive, compared with 15.7 (95 to CI: 12.6 to 18.8; $P < 0.001$) in the other provinces of The Netherlands.

87 (29.8 per cent [95 per cent CI: 27.2 to 32.5]) BTM samples were ELISA positive (Table 4.1). Of this, 68 were situated in the south eastern provinces. In this region, 43 per cent (95 per cent CI: 39.3 to 46.7) of the BTM samples was ELISA positive, compared with 15.7 per cent (95 per cent CI: 12.6 to 18.8; $P < 0.001$) in the other provinces of The Netherlands.

From 77 dairy goat farms, 1,001 individual serum samples were available and the average seroprevalence on these farms was 17.7 per cent (95 per cent CI: 15.3 to 20.0). The within-herd seroprevalences were 42.3 per cent, 39.4 per cent, 9.0 per cent and 6.2 per cent on BTM PCR very high positive, high positive, weak positive and

negative farms, respectively (Table 4.2). The within-herd seroprevalences differed significantly between BTM PCR (very) high positive farms and BTM PCR weak positive farms (Figure 4.3). No significant difference in within-herd seroprevalence was found between BTM PCR weak positive farms and negative farms.

The within-herd seroprevalences were 30.8 per cent, 43.0 per cent, 17.3 per cent and 5.4 per cent on BTM ELISA high positive, positive, low positive and negative farms, respectively (Table 4.3). The within-herd seroprevalences differed significantly between BTM ELISA high positive, positive, and low positive farms on the one hand, compared with BTM ELISA negative farms on the other hand (Figure 4.4).

From the 292 BTM samples, 17 were obtained from farms with a confirmed history of Q fever abortion. From these 17 farms, 16 (94.0 per cent) were BTM PCR positive and all of them were BTM ELISA positive.

Discussion

The first aim of this study was to determine the agreement between the results of a commercially available ELISA and RT-PCR in the same BTM samples and individual serum samples from dairy goat and dairy sheep farms with and without a history of IHC-confirmed Q fever abortions, Therefore, the BTM PCR and ELISA test results were compared with each other and with the within-herd seroprevalences as determined by individual serology. Since Muskens et al. (2011) showed a correlation between PCR prevalence and ELISA prevalence in herds, it was calculated at what PCR level the correlation between BTM PCR and ELISA results was the maximum. It appeared that for the chosen cut-off levels of the PCR, the cut-off level of 100 bacteria/ml results in the highest area under the ROC curve. This cut-off level is the same as the cut-off level that is recommended by the manufacturer as cut-off between weak- and high positive results. Using this cut-off, the proportion of agreement is at most at ELISA S/P ratio of 93 per cent, which is near the cut-off level of 100 which is recommended by the manufacturer, giving a sensitivity of 88.2 per cent and a specificity of 94.6 per cent. Therefore, the cut-off levels as given by the manufacturer are used for the descriptive part of the study. Taking into account that there is no real gold standard for quantifying *C. burnetii* on BTM level and PCR and ELISA are based on different principles, the agreement between PCR and ELISA results in BTM is sufficient. In the absence of a gold standard or reference value, both sensitivity and specificity are relative. In addition to comparison with PCR results, BTM ELISA results were also compared with individual seroprevalences. The correlation between BTM ELISA results and within-herd seroprevalences was highest at a seroprevalence cut-off of 15 per cent and a BTM ELISA cut-off of 46 per cent S/P-ratio. For these criteria, the specificity and sensitivity were 91.0 per cent and 84.3 per cent, respectively, which is sufficient for large-scale monitoring. Under these conditions, the BTM ELISA has a lower

sensitivity than reported in a recent study for cattle in The Netherlands but a much higher specificity (Muskens et al., 2011). However, the latter used a cut-off for ELISA of 30 per cent and prevalence cut-off of 10 per cent.

In this study, PCR and ELISA results are coherent when the tests are not used for finding the last positive animal but for detecting within-herd prevalences of 15 per cent or more, which is only slightly different from findings in cattle herds in a recent study in The Netherlands with the same tests (Muskens et al., 2011).

The second aim of this study was to describe the Q fever prevalence on farm level by testing BTM samples using the above-mentioned ELISA and RT-PCR, related to the results of individual blood samples and of IHC-confirmed Q fever abortions.

None of the dairy sheep BTM samples was PCR positive. This was also found in Switzerland (Fretz et al., 2007) but differs from a study in the Basque Country where 22 per cent of the sheep flocks tested positive by PCR (García-Pérez et al., 2009). However, results from different countries are difficult to compare, both as a result of different test protocols and different epidemiological circumstances (Guatteo et al., 2011). It might be that in the present study, the bacterium was totally absent on the dairy sheep farms, at the time of sampling, but it could also be that the bacterium resided in other matrices than milk (Rodolakis et al., 2007; Astobiza et al., 2011). Since infected sheep mainly shed *C. burnetii* in milk during a short period after parturition, sampling shortly after lambing might have led to higher prevalences (Rodolakis et al., 2007; Roest et al., 2011a).

BTM samples were submitted by 292 (76.2 per cent of all) Dutch dairy goat farms in 2008, and 96 (32.8 per cent) BTM samples were PCR positive. This percentage is higher than found in Switzerland, where none of the 39 BTM samples from goat farms was PCR positive (Fretz et al., 2007). In Iran, only 1 of 56 BTM samples from 20 goat breeding farms was PCR positive (Rahimi et al., 2010). In the south eastern provinces of The Netherlands, significantly more dairy goat BTM samples (50.2 per cent) were PCR positive compared with the remaining provinces (15.7 per cent). The within-herd seroprevalence of farms with very high positive or positive BTM PCR results were 39.4 per cent and 42.3 per cent, respectively. For farms with PCR BTM negative or weak positive results, the within-herd seroprevalence was 6.2 per cent and 9.0 per cent, respectively, which was significantly lower. No significant differences in within-herd seroprevalences were found between PCR weak positive farms and PCR negative farms, indicating that the cut-off value of the RT-PCR of 100 bacteria/ml, as given by the manufacturer, is a reliable indication of the infection status of the herd or flock. Analysing the feasibility of the PCR for dairy goat BTM samples, in this study, the area under the curve was highest at a cut-off value of 100 bacteria/ml, which would therefore be the preferred cut-off.

BTM samples were also tested for antibodies. Until now no studies have been published describing the diagnostic performance of a Q fever BTM ELISA for large numbers of dairy goat farms. From 292 of all BTM samples, 87 (29.8 per cent) were ELISA positive. In the south eastern provinces of The Netherlands, significantly more farms were ELISA positive compared with the other provinces which is in line with the BTM PCR results. A clear correlation between within-herd seroprevalences and ELISA BTM results was found; within-herd seroprevalences were significantly higher on ELISA BTM positive farms (43.0 per cent) than on ELISA BTM negative farms (5.4 per cent). Unexpectedly, on three BTM ELISA and BTM PCR negative farms 6, 6 and 9 seropositive animals were found, respectively. This might be caused by unintended biased sampling. A negative BTM PCR combined with high within-herd seroprevalences may also have been caused by former *C. burnetii* infections without current shedding. It is useful to continue monitoring on these farms during a longer period.

In this study, by BTM testing, all 17 farms with an IHC-confirmed *C. burnetii* abortion outbreak were detected by ELISA and one was missed by PCR which could be explained by the interval between abortion and testing. When, on the contrary, only the IHC-confirmed farms were regarded as true positives, both PCR and ELISA were largely lacking specificity.

PCR testing of BTM samples has some limitations: a single BTM PCR test result only gives information about shedding in milk at one particular moment. A positive BTM PCR can be caused by only a few shedding animals, and shedding via other routes (Rodolakis et al., 2007) is not determined in this way. However, the results of this study demonstrate a clear correlation between BTM PCR and ELISA and individual serology. BTM testing is a proper tool for Q fever monitoring purposes in dairy goats.

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Chapter 5

Bulk tank milk surveillance as a measure to detect *Coxiella burnetii* shedding dairy goat farms in the Netherlands between 2009 and 2014

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submitted for publication

Abstract

In the period 2005-2009, *C. burnetii* was a cause of abortion waves at twenty eight dairy goat farms and two dairy sheep farms in the Netherlands. Two years after the first abortion waves, a large human Q fever outbreak started mainly in the same region, and aborting small ruminants were regarded as most probable source. To distinguish between infected and non-infected herds, a surveillance program started in October 2009, based on PCR testing of bulk tank milk (BTM) samples, which had never been described before. The aim of this study was to analyse the effectiveness of this surveillance program, and to evaluate both the effect of culling of pregnant dairy goats on positive farms, and of vaccination on BTM results.

BTM samples were tested for *C. burnetii* DNA using a real-time PCR, and results were analysed in relation to vaccination, culling, and notified *C. burnetii* abortion records. In spring and autumn, BTM samples were also tested for antibodies using an ELISA, which results were evaluated in relation to the compulsory vaccination campaign.

Between October 2009 and April 2014, 1,660 (5.6%) out of 29,875 BTM samples from 401 dairy goat farms tested positive for *C. burnetii* DNA. The percentage of positive samples dropped from 20.5% in 2009 to 0.3% in 2014. In a multivariable model, significantly higher odds of being PCR positive in the BTM surveillance program were found in the months February until November compared to January, and in farms of which all pregnant dairy goats were culled. Finally, the risk for *C. burnetii* BTM PCR positivity significantly decreased after multiple vaccinations. BTM ELISA results were significantly higher after vaccination than before. ELISA results were higher after multiple vaccinations compared to a single vaccination, and ELISA results on officially declared infected farms were significantly higher compared to non-infected farms.

In conclusion, BTM surveillance is an effective and useful tool to detect *C. burnetii* shedding dairy goat herds.

Introduction

Q fever is a zoonosis caused by *Coxiella burnetii*, an aerobic, obligate intracellular, Gram-negative, highly resistant bacterium that may infect mammals, birds, and arthropods (Babudieri and Moscovici, 1952; Arricau-Bouvery et al., 2005; Berri et al., 2007). In infected domestic ruminants, the primary animal reservoir of *C. burnetii*, the main symptom is abortion, and after abortion or parturition, shedding mainly takes place in birth products, but also occurs in urine, faeces and milk (Arricau-Bouvery et al., 2003; Guatteo et al., 2007; Garcia-Pérez et al., 2009; Roest et al., 2012).

In the period 2005-2009, *C. burnetii* has been demonstrated as cause of abortion waves at twenty eight dairy goat farms and two dairy sheep farms in the Netherlands (Vellema and Van den Brom, 2014). In 2007, a large human Q fever outbreak started mainly in the same region where abortion waves occurred, and aborting small ruminants were increasingly regarded as the most probable source (van der Hoek et al., 2010; Van Steenbergen et al., 2007). Until April 2014, more than 4,200 human patients were notified (www.RIVM.nl).

In 2008, the second year of the outbreak, several measures on dairy sheep and dairy goat farms were implemented, aiming at preventing environmental contamination and human infections (Van den Brom and Vellema, 2009). When, however, the number of human Q fever patients further increased, additional measures were implemented, and after a voluntary start at the end of 2008, compulsory vaccination of all dairy sheep and dairy goats was carried out in the outbreak area in 2009, and from 2010 onwards in the whole country, with a phase 1 vaccine (Coxevac®, CEVA Santé Animale), which was not licensed at that time and only limitedly available. To distinguish between infected and non-infected herds, a surveillance program started in October 2009, based on PCR testing of bulk tank milk (BTM) samples. At the end of 2009, the government decided to cull all pregnant sheep and goats on officially declared *C. burnetii* BTM PCR positive farms. For non-pregnant sheep and goats on *C. burnetii* BTM PCR positive farms, a breeding ban for life was implemented.

The aim of this study was to analyse all results from BTM testing of dairy goat farms in the Dutch surveillance program and, retrospectively 1) to determine the usability of BTM surveillance to detect *C. burnetii* shedding dairy goat herds, 2) to evaluate the effect of culling of pregnant dairy goats on *C. burnetii* BTM PCR positivity on initially positive farms, and 3) to evaluate the effect of vaccination.

Materials and methods

Study population and bulk tank milk samples

In autumn 2008, all 329 Dutch dairy goat farmers with more than 200 dairy goats per farm were requested to submit a BTM sample to be tested for *C. burnetii*, using a real-time PCR (rt-PCR, LSI Taqvet *C. burnetii*®, Laboratoire Service International, Lissieu, France), which targets the repetitive transposon-like region of the bacterium. The test was performed according to the manufacturer's instructions and results are presented in three classes: negative (N; <100 bacteria/mL), high positive (HP; 100≤PCR <10,000 bacteria/mL), very high positive (VH; PCR≥10,000 bacteria/mL), as proposed by Van den Brom et al. (2012a). In October 2009, a surveillance program based on BTM testing became mandatory for all dairy sheep and dairy goat farms with more than fifty animals, and BTM samples were tested twice a month during the lambing season, between December 1st and June 30th. Outside the lambing season, BTM samples were tested monthly on non-infected farms and twice a month on infected farms. In spring and autumn, BTM samples were also tested using an indirect ELISA (Ruminants Serum Q Fever LSI Kit, LSI, Lissieu, France) according to the manufacturers' instructions, and ELISA results were evaluated in relation to the compulsory vaccination, which has to be executed annually before the first of August (breeding season). ELISA results were expressed by sample/-positive percentages (S/P%), calculated using the following formula: $(OD_{sample} - OD_{negative\ control}) / (OD_{positive\ control} - OD_{negative\ control}) \times 100\%$. S/P percentages for BTM samples were divided in four classes: negative (N; S/P% <30), low positive (LP; 30≤S/P% <100), positive (P; 100≤S/P% <200) and high positive (HP; S/P%≥200). In this study, we only analysed data from dairy goat farms, because the number of dairy sheep farms in the Netherlands was very limited.

Officially declared C. burnetii infected herds

According to legislation, *C. burnetii* BTM PCR positive results at GD Animal Health had to be confirmed by the national reference laboratory (Central Veterinary Institute, CVI, Lelystad), and after confirmation, an official BTM sample was collected by the Dutch Food and Consumer Product Safety Authority to be tested by CVI. When the official BTM sample was found positive, a farm was officially declared *C. burnetii* positive.

Farms with a history of C. burnetii abortion

Coxiella burnetii was first diagnosed as the cause of abortion waves on two dairy goat farms in 2005, and confirmed by immunohistochemistry in sections of foetal membranes. Immunohistochemistry was performed using the EnVision+ system (DAKO Corporation, California, USA) (Wouda and Dercksen, 2007; Van den Brom et al., 2012a). Between 2005 and 2008, *C. burnetii* was detected in placental membranes of aborted foetuses submitted from fifteen dairy goat farms. In the Netherlands, small ruminant abortion rates exceeding five per cent became

notifiable in 2008. From that moment onwards, in 2008 and 2009, on eleven dairy goat farms which notified abortion problems, *C. burnetii* was confirmed as the cause (Van den Brom et al, 2012b).

Vaccination records
In the Netherlands, a vaccination campaign against *C. burnetii*, with a phase 1 vaccine (Coxevac® (CEVA, Santé Animale)), started in 2008. At that time, because of a lack of vaccine, voluntary vaccination was only carried out on 26 dairy goat farms located within a radius of 45 km of the centre of the outbreak area. In 2009, vaccination of all dairy goats in the high incidence area was mandatory, and in total, goats on 142 farms were compulsory or voluntary vaccinated that year. Since 2010, vaccination of all dairy goats was mandatory in the whole country. The vaccinations have to be executed annually before the first of August, or after animals reach the age of three months. Date of vaccination of each individual goat is compulsorily registered in the national identification and registration database for small ruminants, and were available for this study. For calculation purposes, the date of vaccination of the lactating goats was used.

Statistical analysis
For analytical purposes, all data were combined and checked. Farms that appeared to keep sheep instead of goats during a part of the analysed period were removed from the dataset (n = 8). On religious grounds, two dairy goat farmers requested and were exempted from compulsory vaccination, and data from these farms were excluded as well.

Two different datasets were composed. The first dataset contained combined results of bi-weekly or four-weekly BTM PCR results combined with results from farms with abortion waves, officially infected farms and vaccination data. The complete dataset consisted of 29,875 PCR results obtained between October 2009 and May 2014 from 401 goat farms. For descriptive purposes, all observations were included. For analytical purposes, only observations of PCR results between July 2010 and the end of 2013 were included. Observations prior to July 2010 were not included in the analyses because before this date interventions took place on farms that tested PCR positive, including the culling of pregnant goats.

The second dataset contained ELISA results combined with results from farms with abortion waves, from infected herds and vaccination data. After removing double observations and ELISA results that were not obtained as part of the BTM surveillance program, 3,006 observations remained from in total 401 different dairy goat farms. In addition, for the analyses of ELISA results, the period from the start of the BTM surveillance program until the end of 2013 remained for analytical purposes, and eventually 2,703 observations were included.

Regarding both datasets, descriptive results are presented comparing before and after vaccination, and before and after culling, using STATA 13®. Percentage of goat farms with a PCR positive result are presented for all dairy goat farms together and for three subgroups of dairy farms:

1. Farms that were officially declared to be infected between October 2009 and July 2010 versus the other dairy goat farms;
2. Farms with a history of *C. burnetii* abortions versus the other dairy goat farms;
3. Farms that tested three times negative versus farms that tested positive (>100) or farms that tested high positive (>1,000) in at least one of the first three months of the surveillance program in 2009.

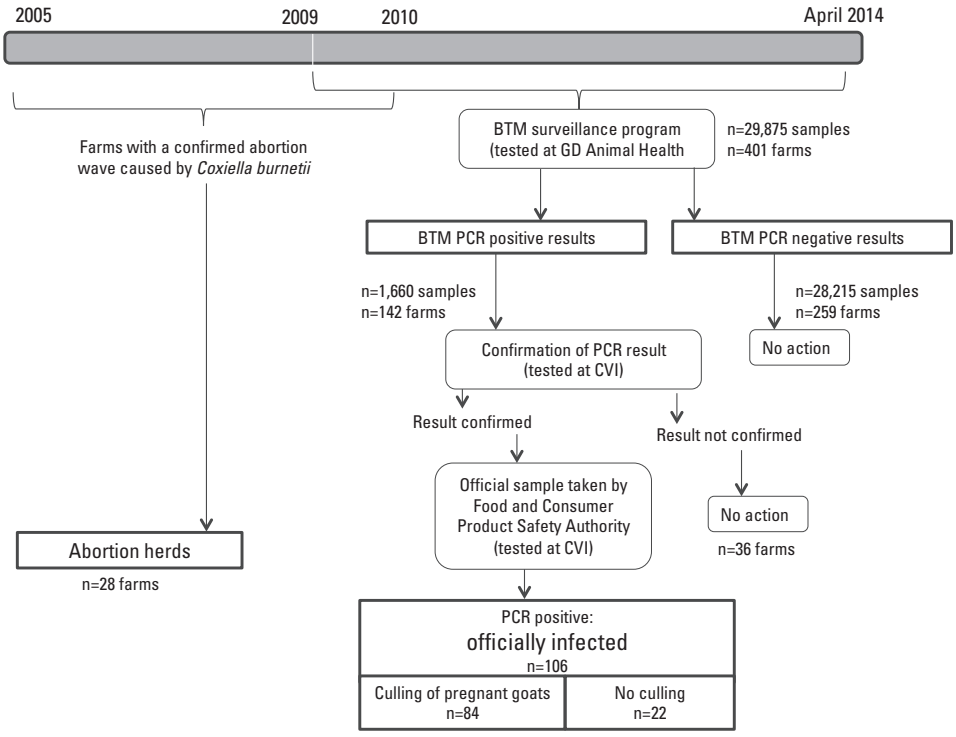

Figure 5.1. Bulk tank milk surveillance results in diagram form. Abortion waves caused by *C. burnetii* were confirmed on 28 dairy goat farms between 2005 and 2010. Between October 2009 and April 2014, 29,875 BTM samples from 401 dairy goat farms were tested by PCR at GD Animal Health. Samples that tested positive (1,660 from 142 farms) were submitted to the Central Veterinary Institute (CVI), Lelystad, and after confirmation, the Dutch Food and Consumer Product Safety Authority (NVWA) collected an official BTM sample which was tested by PCR at CVI. If this official BTM sample tested positive, a farm was declared officially infected (n=106). Between October 2009 and July 2010, pregnant dairy goats on 84 officially declared infected farms were culled. After July 2010, culling was not applicable for officially declared infected farms (n=22).

ELISA results were normally distributed and therefore means and confidence intervals were used for descriptive purposes.

To evaluate the effect of culling and vaccination on PCR or ELISA results, multivariable multilevel linear and logistic analyses were performed (mixed and melogit in STATA®). PCR result (binary variable) and ELISA SP value (continuous variable) were included as dependent variable in the models. Variables that were included as independent variable were prior versus post vaccination (both models), numbers of vaccinations (both models), farms that were officially declared to be infected versus all other farms (both models) and month of sampling (PCR). By using multilevel models, the model was corrected for the fact that BTM results within the same herd were more comparable than BTM results between different farms. The best model was evaluated by comparing the log likelihood between the

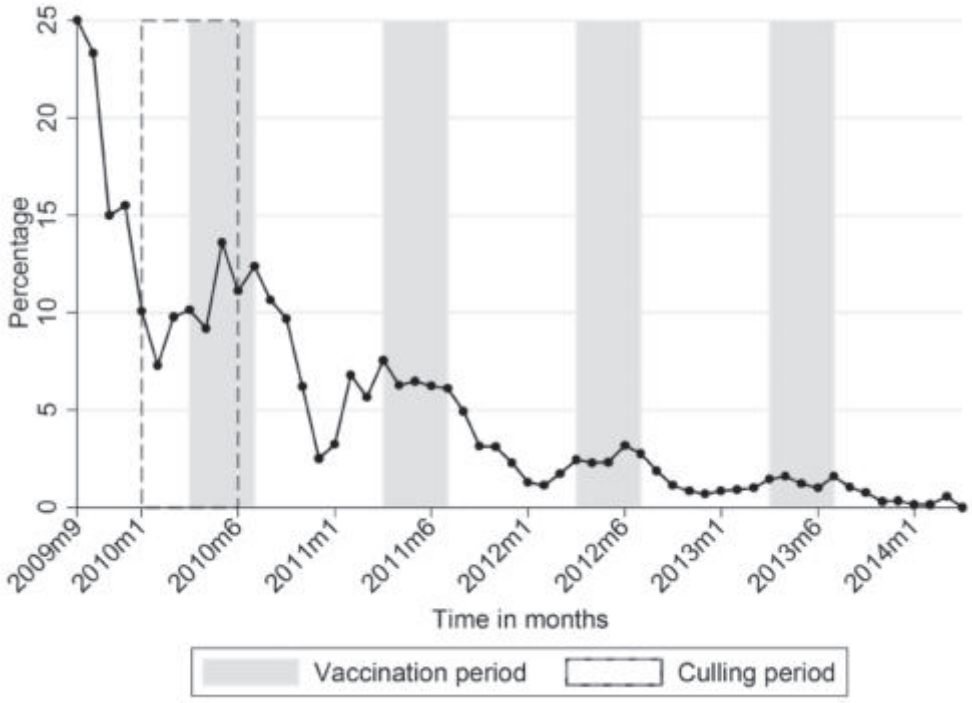

Figure 5.2. Percentage of Dutch dairy goat farms with a *C. burnetii* BTM PCR positive result (>100 bacteria/mL) between October 2009 and April 2014. After a voluntary start in October 2008, and a partly mandatory vaccination in 2009, since 2010, mandatory vaccination is executed on all dairy goat farms annually before the first of August. Vaccination is carried out between April and August (Vaccination period). Between December 2009 and July 2010, pregnant dairy goats were culled (Culling period) on all officially declared *C. burnetii* PCR positive farms.

full and the nested model, using the likelihood ratio test. Residuals of the models were monitored to evaluate whether models that were used met the assumptions of linear and logistic regressions, and the model fit was evaluated using the R^2.

Results

Between October 2009 and April 2014, 29,875 BTM samples from 401 dairy goat farms were tested for *C. burnetii* by PCR, and 1,660 (5.6%) of these samples from 156 different farms tested positive (Figure 5.1). The majority of positive samples was found in 2009 when 20.5% was positive, and this percentage declined to 0.3% in 2014 (Figure 5.2). Descriptive results of *C. burnetii* infections in the Netherlands between 2005 and April 2014 are presented in Table 5.1.

Table 5.1. Descriptive results of *C. burnetii* infections in the Netherlands between 2005 and April 2014.

	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014
Number of dairy goat farms (>50 animals)	~350	~350	~350	392	392	375	348	343	336	332
Number of farms with *C. burnetii* abortion	2	6	7	7	6	0	0	0	0	0
BTM (%)	NA	NA	NA	292 (74.5)	392 (100)	375 (100)	348 (100)	343 (100)	336 (100)	332 (100)
Newly BTM PCR positive farms (%)	NA	NA	NA	66 (32.9)	51 (13.0)	30 (8.0)	7 (2.0)	1 (0.3)	0 (0.0)	1 (0.3)
Repeatedly BTM PCR positive farms (%)	NA	NA	NA	NA	52 (78.8)	81 (69.2)	59 (40.1)	36 (23.4)	21 (13.5)	5 (3.2)
Newly officially infected farms (%)	NA	NA	NA	NA	72 (18.4)	35 (9.3)	4 (1.1)	2 (0.6)	0 (0.0)	0 (0.0)
Repeatedly officially infected farms (%)	NA	NA	NA	NA	NA	0 (0.0)	7 (7.0)	4 (3.8)	4 (3.8)	0 (0.0)
Vaccination (%)*	NA	NA	NA	26 (6.6)	142 (36.2)	375 (100)	348 (100)	343 (100)	336 (100)	332 (100)
Notified human Q fever patients	17$	17$	168	1,000	2,354	504	81	66	19	19&

BTM, bulk tank milk; NA, not applicable; * farms with religious reasons not to vaccinate (n=2) were excluded from the dataset; $ annually an average of 17 human Q fever cases was notified between 1978 and 2006; &until August 27th 2014.

Descriptive results of *C. burnetii* infections between 2005 and April 2014. Between 2005 and 2008, there were approximately 350 dairy goat farms in the Netherlands. From 2009 on, exact numbers are known. Between 2005 and 2010, abortion storms caused by *C. burnetii* were detected 28 times on dairy goat farms. In 2008, BTM samples were tested by RT-PCR for the first time on a voluntary base. From 2009 on, all dairy goats farms were mandatory tested using a *C. burnetii* BTM PCR. A total of 106 dairy goat farms was officially declared infected. Culling of pregnant animals on *C. burnetii* BTM PCR positive farms was performed between December 2009 and July 2010. Vaccination against *C. burnetii* started in 2008, was intensified in 2009, and became mandatory nationwide in 2010. The number of notified human Q fever patients between 2007 and August 27th 2014 was 4,211.

Not all farms participated in all rounds of the mandatory BTM surveillance program because of various reasons, e.g. some farmers started farming and others stopped farming during the surveillance period, and on some farms all adult goats did have a dry period. From 355 dairy goat farms at least 25 samples per farm were tested, and out of these, 222 farms (63%) tested always negative, 64 farms (18%) tested positive one to five times, 21 farms (6%) tested positive six to ten times, 25 farms (7%) tested positive eleven to twenty times, and 23 farms (6%) tested positive more than twenty times. Based on BTM PCR results, 106 dairy goat farms (67, 33, 4, and 2 in 2009, 2010, 2011, and 2012, respectively) were officially declared infected (Figure 5.1).

BTM PCR results are presented for the entire group of goat farms and for stratified farms based on 1) BTM results during the first three rounds of the surveillance program, 2) whether or not pregnant goats were culled, and 3) abortion history.

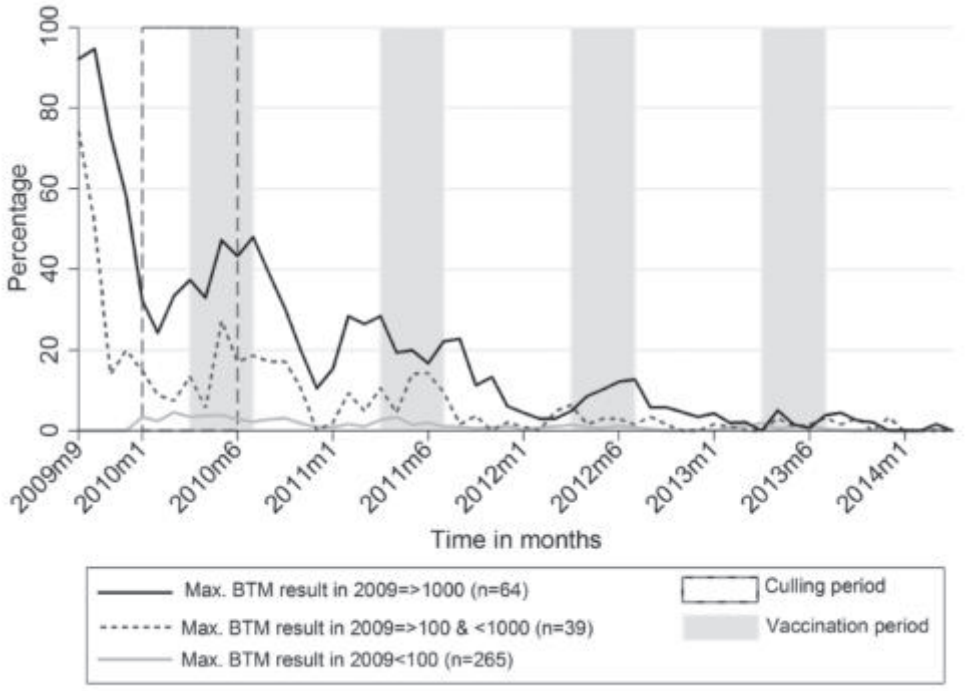

Figure 5.3a. Percentage of Dutch dairy goats farms with a *C. burnetii* BTM PCR positive result (>100 bacteria/mL), divided into three groups based on their highest *C. burnetii* BTM PCR result in 2009. After a voluntary start in October 2008, and a partly mandatory vaccination in 2009, since 2010, mandatory vaccination is executed on all dairy goat farms annually before the first of August. Vaccination is carried out between April and August (Vaccination period). Between December 2009 and July 2010, pregnant dairy goats were culled (Culling period) on all officially declared *C. burnetii* PCR positive farms.

The different groups of goat farms are schematically presented in Figure 5.1. Figure 5.2 shows the development of the percentage of dairy goat farms with a *C. burnetii* BTM PCR positive result during the surveillance period. In the first three rounds of the BTM surveillance program in 2009, 28% of all dairy goat farms tested BTM PCR positive in at least one of the rounds. In both groups with positive results, the percentage of PCR positive farms decreased in time (Figure 5.3a).

On 84 dairy goat farms that were officially declared infected between October 2009 and July 2010, all pregnant animals were culled. Alterations in BTM PCR results in time are presented in Figure 5.3b.

Out of the 28 dairy goat farms with an abortion wave between 2005 and 2010, 24 participated in the mandatory BTM surveillance (Table 5.2). On one farm, two *C. burnetii* abortion waves were confirmed, one in 2006 and another in 2009, and the

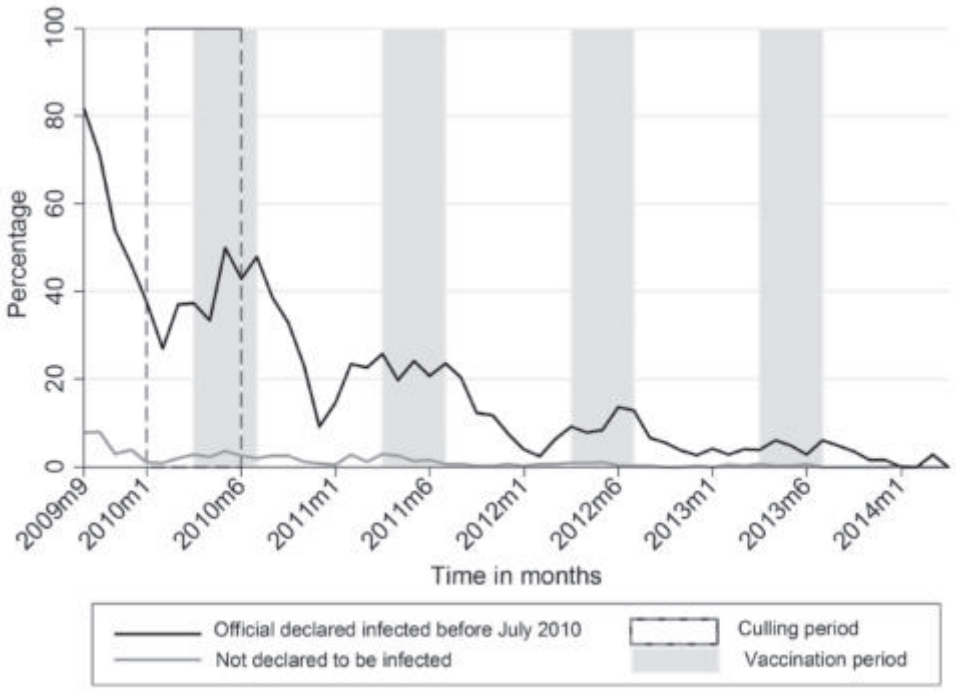

Figure 5.3b. Percentage of Dutch dairy goats farms with a *C. burnetii* BTM PCR positive result (>100 bacteria/mL), divided into two groups, an officially declared *C. burnetii* infected and a non-infected group. After a voluntary start in October 2008, and a partly mandatory vaccination in 2009, since 2010, mandatory vaccination is executed on all dairy goat farms annually before the first of August. Vaccination is carried out between April and August (Vaccination period). Between December 2009 and July 2010, pregnant dairy goats were culled (Culling period) on all officially declared *C. burnetii* PCR positive farms.

three remaining farms stopped farming before the start of the compulsory BTM surveillance program. Based on their BTM PCR results, thirteen out of the 24 participating farms were officially declared infected in 2009 or 2010. Alterations in BTM results from these farms in time are presented in Figure 5.3c. Dairy goat farms with an abortion wave confirmed to be caused by *C. burnetii* were significantly (Chisq: $p < 0.001$) more likely to be officially declared *C. burnetii* infected based on BTM. In addition, farms with an abortion wave caused by *C. burnetii* in more recent years (2008/2009) were significantly more often declared *C. burnetii* infected compared to farms with an abortion wave between 2005 and 2007 (Chisq: $p = 0.02$).

In a multivariable model in which all observations of the BTM surveillance program between July 2010 and April 2014 were included, significantly higher odds of testing BTM PCR positive were found in the months February until November, varying between 1.7 and 8.5, compared to January. The odds increased during the

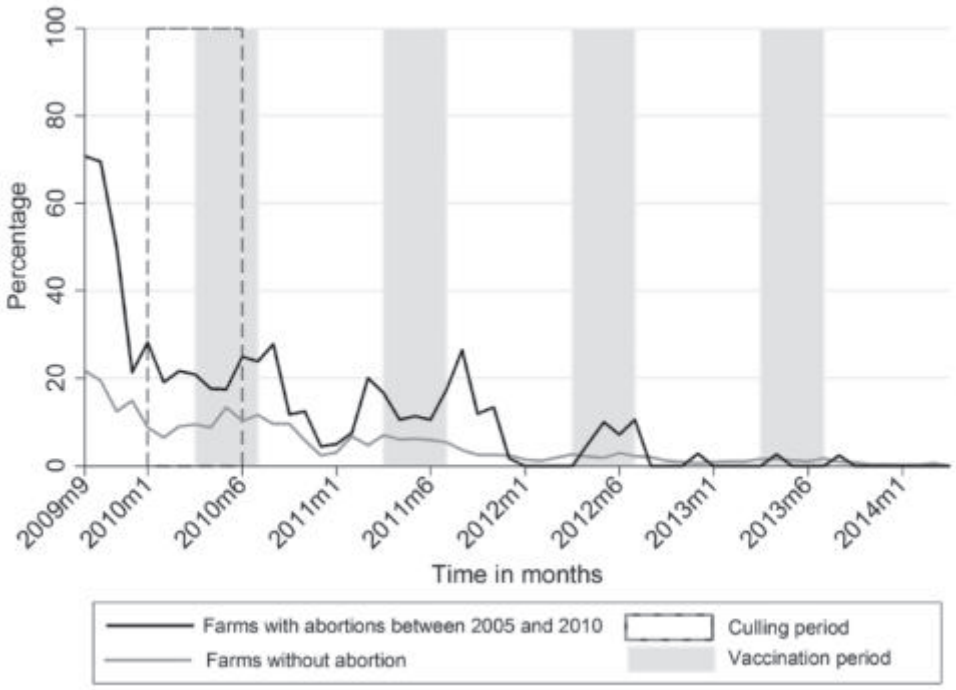

Figure 5.3c. Percentage of Dutch dairy goats farms with a *C. burnetii* BTM PCR positive result (>100 bacteria/mL), divided into two groups, farms with abortion caused by *C. burnetii* and farms without notified abortion caused by *C. burnetii*. After a voluntary start in October 2008, and a partly mandatory vaccination in 2009, since 2010, mandatory vaccination is executed on all dairy goat farms annually before the first of August. Vaccination is carried out between April and August (Vaccination period). Between December 2009 and July 2010, pregnant dairy goats were culled (Culling period) on all officially declared *C. burnetii* PCR positive farms.

Table 5.2. Descriptive information on dairy goat farms with an abortion storm caused by *Coxiella burnetii*.

Farm	Date of abortion	BTM ELISA	BTM PCR results % positive (n)						Nvac	Infected
		2008	2008	2009	2010	2011	2012	2013	<2010	
1	03-2005	255	100 (1)	25 (4)	5 (20)	0 (20)	0 (20)	0 (18)	2	0
2	09-2005	150	100 (1)	25 (4)	31 (13)	11 (19)	0 (19)	0 (20)	1	0
3	03-2006	NA	NA	50 (4)	24 (17)	0 (17)	7 (14)	0 (12)	2	0
4	04-2006	184	100 (1)	100 (4)	63 (16)	NA	NA	NA	1	1
5	06-2006	160	0 (1)	25 (4)	10 (20)	0 (20)	0 (19)	0 (19)	2	0
6	08-2006	138	100 (1)	0 (3)	14 (21)	0 (19)	0 (19)	0 (21)	1	0
7	02-2007	72	0 (1)	0 (3)	0 (18)	0 (15)	0 (19)	0 (20)	0	0
8	02-2007	197	100 (1)	0 (3)	5 (21)	0 (19)	0 (16)	0 (15)	1	0
9	03-2007	NA	NA	33 (3)	5 (21)	0 (17)	0 (11)	0 (15)	1	0
10	04-2007	161	100 (1)	75 (4)	16 (19)	36 (22)	0 (18)	0 (17)	2	0
11	04-2007	154	100 (1)	60 (5)	12 (25)	0 (17)	10 (20)	0 (19)	1	1
12	05-2007	89	100 (1)	67 (3)	0 (11)	8 (13)	0 (15)	0 (15)	1	1
13	06-2007	138	100 (1)	100 (3)	10 (21)	0 (19)	0 (19)	0 (21)	0	0
14	02-2008	77	100 (1)	0 (3)	0 (22)	0 (18)	0 (20)	0 (20)	2	0
15	04-2008	133	100 (1)	75 (4)	12 (25)	0 (18)	0 (18)	0 (18)		1
16	05-2008	138	100 (1)	50 (4)	12 (25)	0 (20)	0 (21)	0 (21)	2	1
17	07-2008	154	100 (1)	75 (4)	13 (24)	4 (27)	0 (23)	0 (20)	2	1
18	08-2008	0	0 (1)	33 (3)	100 (3)	NA	NA	NA	0	1
19	09-2008	108	100 (1)	100 (4)	96 (25)	77 (26)	38 (26)	4 (25)	2	1
20	02-2009	0	0 (1)	50 (4)	0 (3)	0 (7)	0 (17)	0 (21)	0	1
21	03-2009	39	0 (1)	20 (5)	0 (1)	NA	NA	NA		
22	05-2006 04-2009	148	0 (1)	100 (3)	0 (26)	0 (19)	0 (19)	0 (21)	1	1
23	05-2009	NA	NA	100 (2)	8 (24)	55 (22)	0 (23)	5 (19)	1	1
24	09-2009	0	100 (1)	100 (4)	92 (25)	62 (26)	4 (26)	0 (21)	1	1

Infected, officially declared *C. burnetii* positive based on BTM PCR; Nvac, number of vaccination.

lambing season between January and April and increased even further with the highest odds in July, August and September. Most vaccinations were performed in July. The odds of testing PCR positive after the culling period, between 1 July 2010 and April 2014, was significantly higher in farms of which all pregnant dairy goats were culled compared to farms which were not officially declared infected during the culling period. Finally, the risk for *C. burnetii* BTM PCR positivity significantly decreased after multiple vaccinations (Table 5.3).

A total of 3,006 BTM ELISA results were available from 401 dairy goat farms. Average ELISA results before and after vaccination, both for officially declared infected and non-infected farms, are presented in Figure 5.4. Average ELISA BTM results on officially infected dairy goat farms were consistently high positive, even before first vaccination. In non-infected dairy goat herds, average BTM ELISA results increased more gradually until the second vaccination, and remained high

Table 5.3. Final results of a multivariable model for risk factors associated with *Coxiella burnetii* BTM PCR positive results.

		OR	95% Confidence interval		P
Month	January	1.0			
	February	1.7	1.1	2.8	0.02
	March	1.8	1.1	2.8	0.02
	April	2.7	1.7	4.3	<0.001
	May	2.9	1.9	4.6	<0.001
	June	3.2	2.0	5.1	<0.001
	July	4.8	3.2	7.3	<0.001
	August	8.5	5.6	12.8	<0.001
	September	6.3	4.1	9.8	<0.001
	October	4.1	2.7	6.4	<0.001
	November	2.5	1.6	4.0	<0.001
	December	0.9	0.5	1.4	0.57
Number of vaccinations	1	1.0			
	2	0.3	0.3	0.5	<0.001
	3	0.1	0.1	0.2	<0.001
	4	0.03	0.02	0.05	<0.001
	5	0.01	<0.01	0.02	<0.001
	6	<0.01	<0.01	<0.01	<0.001
Infected	No	1.0			
	Yes	211.7	77.8	576.2	<0.001

Constant, probability of a dairy goat farm to have a *C. burnetii* BTM PCR positive result; Infected, officially declared *C. burnetii* positive based on BTM PCR; OR, odds ratio.

positive thereafter. Average BTM ELISA results before the first vaccination were significantly higher in officially infected dairy goat herds compared to non-infected herds (estimate 140; 95% CI: 120-161). Because the progression in SP ELISA values evolved differently in officially infected and non-infected herds, a stratified analysis on type of herd was performed. In the multivariable model for officially infected dairy goat herds, BTM ELISA results were significantly higher after than before vaccination (+30; 95% CI: 19-41) and increased even further after the first time that the herd was vaccinated. After the first vaccination, the average ELISA results remained stable at a high level and did not increase significantly after multiple vaccinations. In the multivariable model for non-infected dairy goat herds, BTM ELISA results were significantly higher after compared to before vaccination as well (+24; 95% CI: 18-29). In addition, after every next vaccination, ELISA results increased further to a high and stable level after five vaccinations (Table 5.4).

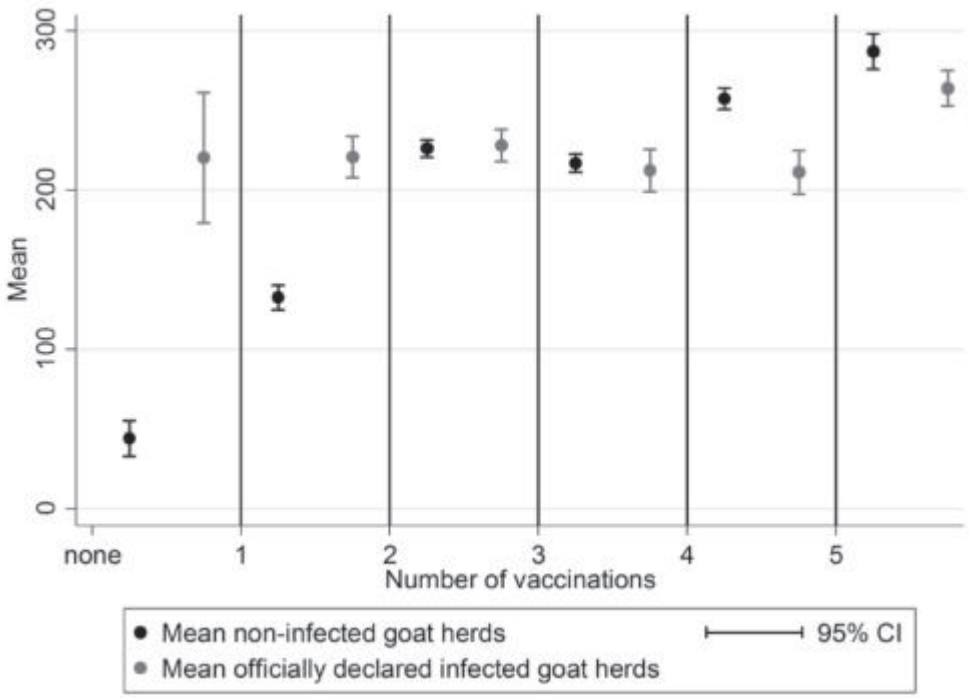

Figure 5.4. Average ELISA OD results before and after vaccination, both for officially declared infected and non-infected dairy goat herds.

Table 5.4. Final results of a multivariable model for risk factors associated with *Coxiella burnetii* BTM ELISA results.

		Officially infected herds		Non-infected herds	
		Coefficient (95% confidence interval)	P-value (z-test)	Coefficient (95% confidence interval)	P-value (z-test)
Before vaccination		Reference		Reference	
After vaccination		29.7 (18.8-40.6)	<0.001	23.7 (18.4-29.1)	<0.001
Number of vaccinations	0	Reference		Reference	
	1	47.5 (24.7-70.4)	<0.001	104.7 (95.9-113.4)	<0.001
	2	57.7 (35.1-80.3)	<0.001	196.1 (187.3-205.0)	<0.001
	3	40.7 (17.8-63.3)	<0.001	190.5 (181.7-199.4)	<0.001
	4	36.4 (12.8-60.1)	0.003	223.5 (213.6-233.5)	<0.001
	5	66.0 (38.9-93.1)	<0.001	235.7 (220.6-250.7)	<0.001
Constant		154.3 (133.1-175.5)	<0.001	15.6 (7.5-23.7)	<0.001

Constant, average BTM ELISA result on a not officially *C. burnetii* declared infected dairy goat farm before the first vaccination; Infected, officially declared *C. burnetii* positive based on BTM PCR.

Discussion

The human Q fever outbreaks in 2007 and 2008 stimulated the start of a large multidisciplinary research portfolio, aiming at generating knowledge to take adequate control measures (Van den Brom and Vellema, 2009). Voluntarily submitted BTM samples from dairy goat farms were tested by PCR and ELISA in 2008 and 2009, as part of this research portfolio, although at that time it was unknown how to interpret the results. After comparing BTM PCR and ELISA results with the serological status of thirteen animals per farm, and determining correlations with a farm history of abortion caused by *C. burnetii*, the highest area under the Receiver Operator Curve of the ELISA was at a PCR cut-off of 100 bacteria/mL (Van den Brom et al., 2012), and this value was used during the BTM surveillance program which started in October 2009. At that moment, there was no experience with BTM surveillance programs for *C. burnetii*, and the only publications were a study estimating the *C. burnetii* prevalence in dairy goats in Switzerland (Fretz et al., 2007) and one estimating the *C. burnetii* prevalence in dairy sheep in Spain (Garcia-Perez et al., 2009). Initially, the aim of the BTM surveillance program in the Netherlands was to declare dairy goat farms *C. burnetii* free after at least one

year of BTM PCR negative results. However, in December 2009, it was decided to interpret non-negative results as positive results and these results were used to declare herds *C. burnetii* infected. Following this decision, additional control measures, such as culling of all pregnant dairy goats, and a lifetime breeding ban for the remaining goats in officially declared infected herds were implemented. Keeping this history at the back of one's mind, it seemed worthwhile to retrospectively analyse the BTM surveillance program to determine its usability to detect *C. burnetii* shedding in dairy goat herds, and additionally, to evaluate implemented measures, as culling of pregnant goats and vaccination.

In this retrospective cohort study, 28% of all dairy goat farms tested *C. burnetii* BTM PCR positive in at least one of the first three rounds of the surveillance program in 2009. Until April 2014, the percentage of BTM PCR positive farms decreased to 0.3%. Compared to January, all months of the year except December, had a higher risk for BTM PCR positivity. The same applies for dairy goat farms which were officially declared infected between September 2009 and June 2010. After repeated vaccinations with Coxevac®, the odds to become *C. burnetii* BTM PCR positive were reduced.

Compared to 2009, a slightly higher proportion of dairy goat farms was BTM PCR positive in 2008 (32.9% (95% CI: 30.2-35.6)), although that prevalence was measured in voluntarily submitted BTM samples from 74.4% of the Dutch dairy goat farms (Van den Brom et al., 2012a). Since surveillance programs for *C. burnetii* based on BTM samples from dairy goat farms is a relatively new phenomenon, only a small number of studies from other countries described results of *C. burnetii* BTM testing. In these countries (Boarbi et al., 2014; Kampen et al., 2012; Rahimi et al., 2011; Fretz et al., 2007), lower prevalences were found compared to the Netherlands at the start of the surveillance program. However, results of studies from other countries are difficult to compare with our results, because of different study designs, and the use of tests with different characteristics. To the best of our knowledge, the Netherlands was the first country where a *C. burnetii* BTM surveillance program was implemented, aiming at distinguishing between infected and non-infected dairy goat farms at a nationwide level.

The odds of becoming BTM PCR positive differ in different months of the year. Significantly higher odds were found in the months February until and including November compared to January, with a steady rise from February to August, after which the OR declines to 2.5 in November, and to 0.9 in December. A possible explanation for the observed difference in risk in time could be the fact that Dutch dairy goats are seasonal breeders (Van den Brom et al., 2013a). This seasonality of sexual activity, mainly influenced by day length, breed and feeding, but also by stress and unknown factors, leads to a start of the breeding season in July, and after a gestation period of five months, kidding starts on most farms not earlier

than January and may last until June. In infected goats, a massive multiplication of *C. burnetii* can take place at the end of pregnancy (Sanchez et al., 2006), and shedding of the bacterium starts at parturition (Roest et al., 2012).

After a voluntary start in 2008, compulsory vaccination started in the high incidence area in 2009, and was mandatory since 2010 for all dairy goats in the Netherlands. This vaccination had to be done annually before the first of August. Vaccination did not only influence body temperature but also gave locally adverse reactions at the vaccination site, a variable reduction in milk yield (Vellema et al., 2010a), and could act as stress factor, possibly affecting luteal function (Hesselink, 1993) and non-specific immune response (Sejian and Srivastava, 2010). In 2010, it has been shown that up to nine days after vaccination with Coxevac®, vaccine-derived *C. burnetii* DNA could be detected in individual milk samples from dairy goats, and in the discussion of their paper the authors emphasize that a larger study was required to evaluate the effect of vaccination on BTM PCR results, suggesting that BTM could also become *C. burnetii* PCR positive by vaccination itself (Hermans et al., 2011). However, these results could not be repeated on four farms where milk samples from twenty dairy goats per farm were individually repeatedly tested after vaccination, using the same PCR test with the same cut-off as used for the mandatory *C. burnetii* BTM surveillance program. Additionally, in BTM samples from these four farms also no increased PCR results could be detected in the weeks following vaccination (Vellema et al., 2010b). The higher odds to become *C. burnetii* BTM PCR positive during the breeding season could indicate the possibility of shedding of *C. burnetii* during estrus, as also has been described for *Chlamydia abortus*, another intracellular abortifacient agent in small ruminants (Livingstone et al., 2009; Papp et al., 1994).

When we included the factor before or after vaccination in our multivariable model, part of the variance that was assigned to the high risk months was assigned to the period after vaccination. However, inclusion of this parameter did not improve model fit, and based on the available data it was not possible to distinguish between the amount of variation that was explained by estrus and by vaccination on the *C. burnetii* BTM PCR result. Based on the above mentioned arguments it was decided to include a parameter describing months instead of estrus and post-vaccination in our model.

Our results also show that the probability to become *C. burnetii* BTM PCR positive significantly decreases with an increasing number of vaccinations (Table 5.2). Vaccination seems to be very effective, especially when administered before the first pregnancy (Hogerwerf et al., 2011), and because all dairy goats have been mandatory vaccinated since 2010, this decrease was expected. Vaccinated, chronically infected goats can intermittentally shed *C. burnetii* and the trigger for that is not always clear (Van den Brom et al., 2013b). Shedding under these

circumstances probably mainly takes place in milk (Van den Brom et al., 2013b), but could also take place in birth products. Because of the life time breeding ban for the remaining goats in infected herds, massive shedding during parturition or abortion, and subsequent environmental contamination was not likely to occur anymore. Presence of chronically infected goats on officially declared *C. burnetii* BTM PCR positive farms can also explain why these farms are significantly more likely to have a positive BTM PCR result in the observed period. Although in a herd with a mandatory breeding ban for life individually shedding dairy goats probably do not pose a risk for public health, they can be responsible for maintanance of the infection within the herd. Therefore, detecting and removal of individual shedders as decribed before (Van den Brom et al., 2013b) should be considered on BTM PCR positive farms.

Farms with an abortion wave in 2008 and 2009 were more likely to become officially declared infected compared to farms with an abortion wave before 2008. (Table 5.2). In this study, it was not possible to access the cause of this. Both natural immunization, like has been described for *Chlamydia abortus* (Longbottom et al., 2013) and repeated vaccination could have played a role. Vaccination seems very effective in preventing abortion and reducing shedding of *C. burnetii*. In the Netherlands, Q fever in small ruminants is still a notifiable disease, and since the start of the mandatory vaccination program in 2010, no abortion caused by *C. burnetii* has been detected in small ruminants (van Engelen et al., 2014).

In 2009, the percentage of BTM positive results on farms without notified *C. burnetii* abortion waves was relatively high. An explanation for this could be that not all infections with *C. burnetii* will lead to abortion waves. Massive shedding of *C. burnetii* at parturition is the main source of environmental contamination. High incidences of human Q fever patients have been described around farms with notified abortion compared to BTM PCR positive farms without notified abortion (Van der Hoek et al., 2011a; 2012b).

Before the start of the vaccination campaign in 2008, BTM ELISA results showed that 87 (29.8%) out of 292 voluntarily submitted BTM samples from dairy goat farms were serologically positive (Van den Brom et al., 2012a). Our study shows that within a year after vaccination BTM ELISA results were significantly higher compared to the period before. In addtion, BTM ELISA results were significantly higher in offically infected dairy goat herds compared to non-infected herds. In a Belgian study, no significant difference was found in ELISA BTM results before and after vaccination on positive farms (Boarbi et al., 2014). In our study, offically infected dairy goat herds were consistently high positive, although an increase in average BTM ELISA values was found after the first vaccination. Thereafter, ELISA results in officially infected herds did not increase significantly anymore with more vaccinations performed, which is in consistence with the results of Boarbi et al.

(2014). In non-infected herds, BTM ELISA results increased when the herd was vaccinated more times. After non-infected herds had been vaccinated two times, BTM ELISA results were no longer significantly different from BTM ELISA results in officially declared infected herds (results not shown).

In this study, we did not analyse animal movements between farms as a possible source of infection, because officially declared infected farms were not allowed to sell goats with a breeding ban for other puposes than slaughter, and yearlings could only be sold when mandatory vaccinated before their first pregnancy on the farm where they were born.

In conclusion, BTM testing is an effective and useful tool to detect *C. burnetii* shedding in dairy goat herds. Repeated vaccination was an effective measure to reduce the number of *C. burnetii* BTM PCR positive farms. On farms where pregnant goats were culled, an increased risk existed of becoming BTM PCR positive afterwards. This increased risk was probably caused by chronically infected and intermittently shedding goats that were non-pregnant at the time of culling, and remained on those farms. The fact that several measures to prevent environmental contamination were implemented at the same time, disables the possibility to measure the effect of each particular measure separately. It is likely that the package of implemented measures has most probably caused the end of the human Q fever outbreak in the Netherlands (Dijkstra et al., 2012).

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Chapter 6

Detection of *Coxiella burnetii* in the bulk tank milk from a farm with vaccinated goats, by using a specific PCR technique

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Abstract

Q fever is a zoonotic disease, caused by the obligate intracellular bacterium *Coxiella burnetii*. Between 2007 and 2010, Q fever has been a major public health concern in the Netherlands, with almost 3,500 human cases reported and dairy goats considered to be the most probable source. At the end of 2009, the Dutch government decided to cull all pregnant dairy sheep and dairy goats based on bulk tank milk *C. burnetii* positive farms, aiming to preventing shedding and to reducing environmental contamination. On bulk tank milk *C. burnetii* PCR positive farms, a life-time breeding ban was implemented for all remaining non-pregnant small ruminants. This study describes test results on a bulk tank milk *C. burnetii* PCR positive dairy goat farm on which all goats had been vaccinated against Q fever with an inactivated phase one vaccine since 2008. All pregnant dairy goats of this farm were culled in 2010, after which bulk tank milk was negative in the *C. burnetii* PCR. One year later, however, this farm became bulk tank milk *C. burnetii* PCR positive again. From all lactating animals on the farm (n = 350), individual milk samples were collected and tested using a commercial real-time PCR assay. Individual milk samples from five dairy goats appeared to be *C. burnetii* PCR positive. These positive goats had been born on the farm between 2002 and 2006. At postmortem examination, out of 33 mostly tissue samples per animal, only milk and mammary tissue samples were *C. burnetii* PCR positive. Moreover, immunohistochemical examination did not reveal the source of *C. burnetii*. After culling of these *C. burnetii* PCR milk positive animals, the bulk tank milk remained negative in *C. burnetii* PCR until the end of the observation period. The results indicate that vaccination of Q fever infected dairy goat farms does not completely prevent intermittent shedding of *C. burnetii* in probably previously infected goats. Further research is needed to investigate how and where *C. burnetii* multiplies in such intermittently shedding animals.

Introduction

Between 2007 and 2010, Q fever, a zoonotic disease caused by *Coxiella burnetii*, had been of major public health concern in the Netherlands, with almost 3,500 human cases reported (Van der Hoek et al., 2010a). Dairy goats were considered as the most likely source (Steenbergen et al., 2007). In small ruminants, *C. burnetii* is mainly shed during and after parturition or abortion in birth products, but shedding also occurs in urine, faeces, vaginal mucus and milk (Arricau-Bouvery et al., 2003; Guatteo et al., 2007; García-Pérez et al., 2009; Stuen and Longbottom, 2011).

In June 2008, Q fever was made a notifiable disease for small ruminants kept for milk production, and, in addition to hygiene measures, a vaccination programme, which had initially started on a voluntary basis, was made compulsory. In October 2009, a Q fever monitoring programme was implemented on all dairy sheep and dairy goat farms, based on a real time PCR for detection of *C. burnetii*, to be performed on bulk tank milk samples (van den Brom et al., 2012a). A few months later, because of the precautionary principle, the Dutch government ruled the culling of all pregnant dairy sheep and dairy goats on bulk tank milk *C. burnetii* PCR positive farms and a breeding ban for remaining animals on infected farms was implemented.

Unexpectedly, in one farm where all dairy goats had been vaccinated, since 2008, against *C. burnetii* with an inactivated phase one vaccine (Coxevac® ; CEVA Santé Animale, Libourne, France), one year after culling of all pregnant dairy goats, positive results were obtained when testing bulk tank milk samples in the *C. burnetii* PCR. The aims of the present study were (i) to identify the dairy goats responsible for the change in bulk tank milk *C. burnetii* PCR and the tissues, in which *C. burnetii* PCR positive samples could be detected and (ii) to provide a plausible explanation for the recurrent shedding in these animals.

Materials and methods

Dairy goat farm

The Q fever status of the dairy goat farm was first evaluated in August 2008. In that year, before the first Q fever vaccination, several samples collected on various occasions were found to be positive for *C. burnetii*: a bulk tank milk sample was *C. burnetii* PCR and ELISA positive, 64 of 100 blood serum samples were ELISA positive and 12 of 100 vaginal swabs were *C. burnetii* PCR positive. Vaccination of the whole herd against *C. burnetii* was carried out in October and November 2008 and repeated annually since that time. The farm was officially declared Q fever infected in December 2009, based on bulk tank milk *C. burnetii* PCR being positive in October 2009. As a consequence of that, all pregnant goats in the farm were culled up to March 2010, whilst a breeding ban was implemented for the remaining

non-pregnant goats. In the spring of 2011, the farm was again found to be positive in *C. burnetii* PCR in bulk tank milk. At that time, 350 female goats were present on the farm. These goats could be divided in three groups: (i) a group of 250 multiparous and repeatedly vaccinated dairy goats with a breeding ban, (ii) a group of 70 primiparous goats without a breeding ban bought in as kids in 2010 and (iii) approximately 30 primiparous goats born on the farm.

Sampling

Milk and blood samples
Bulk tank milk samples were tested by using a real time *C. burnetii* PCR (LSI Taqvet *C. burnetii*®, LSI, Lissieu, France) and an antibody *C. burnetii* ELISA (Ruminants Serum Q Fever LSI Kit, LSI, Lissieu, France) performed at the Animal Health Service in the Netherlands. A bulk tank milk sample was regarded to be *C. burnetii* PCR positive when ≥100 bacteria mL^{-1} were detected (van den Brom et al., 2012a). In the framework of a compulsory bulk tank milk-monitoring programme, which started in October 2009, monthly bulk tank milk samples were collected from every dairy sheep and dairy goat farm in the Netherlands. After confirmation of a *C. burnetii* PCR positive result in the same sample at the Central Veterinary Institute (Roest et al., 2011c) and a positive *C. burnetii* PCR result of an additional bulk tank milk sample, a farm was officially declared infected.

After the farm had been officially declared infected again, individual milk samples from both mammary glands of all lactating goats were collected aseptically (Fthenakis, 1994) and tested using the *C. burnetii* PCR, as described by van den Brom et al. (2012a). Blood samples from *C. burnetii* shedding goats were collected from the jugular vein and tested in the *C. burnetii* PCR (van den Brom et al., 2012a).

Pathological examinations
Dairy goats shedding *C. burnetii* in milk were euthanised, after blood samples and vaginal swabs had been collected. Samples of various tissues (Table 6.1) were collected and frozen at −70°C, until tested in *C. burnetii* PCR or fixed in 4% buffered formalin. Immunohistochemical examination, aiming to detect *C. burnetii* in sections of *C. burnetii* PCR positive tissues, was performed using the EnVision+ system (DAKO Corporation, Glostrup, Denmark), as described before (Wouda and Dercksen, 2007; van den Brom et al., 2012a). Paraffin embedded samples of *C. burnetii* PCR positive tissues from two animals were also submitted for a second *C. burnetii* immunohistochemical examination. Samples of *C. burnetii* PCR positive tissues from the three remaining goats were additionally paraffin embedded, cut at 4 µm and stained by conventional histological techniques.

Results

Monthly bulk tank milk samples collected between end 2008 and mid 2009 were found to *C. burnetii* PCR positive (Figure 6.1). Based on those results, the farm was officially declared infected with *C. burnetii*. Consequently, all pregnant goats were culled in January and March 2010, to prevent environmental contamination; however, bulk tank milk had already become *C. burnetii* PCR negative. After a year of negative *C. burnetii* PCR bulk tank milk results, the farm was officially declared *C. burnetii* negative. Nevertheless, unexpectedly, in February 2011, the farm became officially *C. burnetii* positive again (Figure 6.1).

In individual milk samples from 5 out of 350 dairy goats, *C. burnetii* was detected by PCR. These animals had been born on the farm between 2002 and 2006. One of these had been tested for *C. burnetii* previously and was found serologically positive in 2008, before the first vaccination.

At postmortem examination in April 2011, no gross abnormal findings were evident; moreover, evidence of pregnancy at the time of the examination or immediately prior to that was not found in any of the five animals. *C. burnetii* was detected only in mammary tissue samples by using PCR (Table 6.1). In immunohistochemical examination, *C. burnetii* antigen was not demonstrated in the mammary tissue

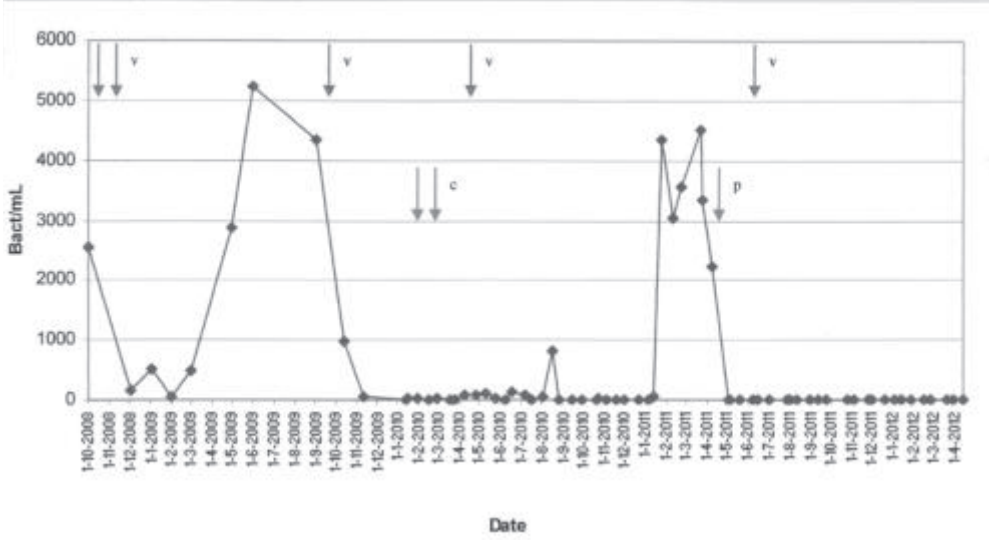

Figure. 6.1. *Coxiella burnetii* PCR results in bulk tank milk samples between 2008 and 2012 (V: vaccination with Coxevac® (Ceva Santé Animale, France), C: culling of pregnant dairy goats, P: removal of five *C. burnetii* shedding dairy goats from the farm for post mortem examination).

Table 6.1. Detailed results of *Coxiella burnetii* PCR performed in body fluids and tissue samples, collected from *C. burnetii* shedding goats.

	Goat numbers and respective dates of birth				
	Goat no. 1	Goat no. 2	Goat no. 3	Goat no. 4	Goat no. 5
	26-05-2005	18-05-2004	19-05-2002	31-05-2003	06-05-2005
Body fluids					
Blood	-	-	-	-	-
Vaginal swab	-	-	-	-	-
Uterine content	-	-	-	-	NA
1st milk sample (24-3-2011)	++++	++++	+++	+++	++
2nd milk sample (13-4-2011)	++++	++++	+++	+++	++
Urine	-	-	-	-	NA
Circulatory and haematopoietic systems					
Heart	-	-	-	-	NA
Bone marrow	-	-	-	-	NA
Spleen	-	-	-	-	NA
Thymus	-	-	-	-	NA
Reproductive system					
Ovaries	-	-	-	-	NA
Oviducts	-	-	-	-	NA
Uterus	-	-	-	-	-
Ileal lymph nodes	-	-	-	-	NA
Mammary glands	++++	++++	+++	++	++
Supramammary lymph nodes	-	-	-	-	-
Urinary tract					
Kidney	-	-	-	-	NA
Perirenal fat	-	-	-	-	NA
Bladder	-	-	-	-	NA
Alimentary tract					
Parotid glands	-	-	-	-	NA
Ileum	-	-	-	-	NA
Colon	-	-	-	-	NA
Ileo-caecal lymph nodes	-	-	-	-	NA
Upper respiratory tract					
Mucosa	-	-	-	-	NA
Tonsils	-	-	-	-	NA
Lymph nodes	-	-	-	-	NA
Lower respiratory tract					
Trachea	-	-	-	-	NA
Bronchi	-	-	-	-	NA
Lungs	-	-	-	-	NA
Lymph nodes	-	-	-	-	NA

-: negative result, +: 35≤cycle threshold (Ct) of PCR<40, ++: 30≤Ct<35, +++: 25≤Ct<30, ++++: Ct<25, NA: not available.
Note: in goats with results on the 1st milk sample +++ or ++++, extensive sampling was carried out at post mortem examination.

samples. Moreover, no histological findings supportive of active inflammatory process in the mammary tissue or in the supramammary lymph nodes were recorded.

After removal of the five *C. burnetii* shedding animals, bulk tank milk samples from the farm in the *C. burnetii* PCR became negative again. No shedding of *C. burnetii* has been detected in bulk tank milk samples since (Figure 6.1).

Discussion

C. burnetii presence was demonstrated on the farm described in this study for the first time in 2008, by using *C. burnetii* PCR and ELISA in bulk tank milk and serum samples and in vaginal swabs, although no increased incidence of abortions had been reported by the farmer, which might have been expected, as abortion is a salient sign of Q fever in small ruminants (Rodolakis et al., 2007; Wouda and Dercksen, 2007; Rousset et al., 2009a; van den Brom and Vellema, 2009). Nevertheless, the test results indicate that the farm, at that time, was already infected.

After 2007, a human Q fever outbreak in the Netherlands and the suggested association to dairy goats (Steenbergen et al., 2007) made the Dutch government to implement measures for Q fever infected farms in 2008 (van den Brom and Vellema, 2009; Van der Hoek et al., 2010a; Roest et al., 2011c). Because of the so called precautionary principle, pregnant dairy goats and dairy sheep in bulk tank milk *C. burnetii* PCR positive farms were culled before the kidding season end 2009 to beginning 2010.

In 2008, the bulk tank milk *C. burnetii* PCR was performed in sheep and goats in the Netherlands for the first time (van den Brom et al., 2012a). Since the start in 2008, the farm in this study submitted bulk tank milk samples regularly and, after vaccination in 2008, *C. burnetii* PCR results in bulk tank milk decreased as expected (Arricau-Bouvery et al., 2005; Astobiza et al., 2011). However, *C. burnetii* PCR results increased again during the kidding season of 2009. This reoccurrence could have been expected, as it has been described before for extensively infected herds, in which vaccination did not significantly and immediately reduce shedding (Astobiza et al., 2011). It has been described that *C. burnetii* can be shed in two successive parturitions by goats (Berri et al., 2002), with an intermittent shedding pattern (Rodolakis et al., 2007; Rousset et al., 2009a). In October 2009, the farm was officially declared infected. After Q fever vaccination, in September 2009, the *C. burnetii* PCR results in the bulk tank milk samples decreased and became negative in November 2009. Culling of pregnant goats in January and March 2010 had no effect on the bulk tank milk *C. burnetii* PCR results, because bulk tank milk had already been negative since November 2009. In August 2010, an initial positive bulk tank milk *C. burnetii* PCR result was not confirmed in a second testing. Possibly, the start of the reproductive season caused this positive bulk tank milk *C.*

burnetii PCR result, as, perhaps, goats with long-standing infection might have been shedding *C. burnetii* during oestrus. This phenomenon has been described in ewes after cases of chronic enzootic abortion, caused by *Chlamydia abortus*, also an obligate abortifacient agent (Gutierrez et al., 2011). After a year of negative, or non-confirmed, bulk tank milk *C. burnetii* PCR results, the farm was officially declared *C. burnetii* free. Nevertheless, within a month of the start of the kidding season in 2011, the bulk tank milk became *C. burnetii* PCR positive again and, after confirmation, the farm was officially declared infected again. Therefore, additional research on the farm started to find a plausible explanation for reactivation of the *C. burnetii* infection.

In milk samples from five goats, high numbers of *C. burnetii* were found. The fact that all shedding goats were born on the farm between 2002 and 2006, the indication that the farm had already been infected since, at least, 2008 and the seropositivity of one of these goats in 2008 suggest that these goats had been in contact with *C. burnetii* before their first vaccination with Coxevac® in 2008. Therefore, the animals had a long-standing infection. Unfortunately, the other four goats were not tested in 2008, which does not exclude a possible infection of these goats. In goats, mammary *C. burnetii* infection can be long-standing and with shedding of the organism in milk, perhaps even during successive lactation periods (Arricau-Bouvery et al., 2003). In none of the five shedding goats described above, evidence of recent pregnancy was detected during post-mortem examination. Shedding of *C. burnetii* in milk after kidding or abortion has been described (Arricau-Bouvery et al., 2003; Rodolakis et al., 2007). The reason why these five dairy goats started shedding *C. burnetii* is unknown, but kidding, despite a breeding ban, cannot be ruled out and might be a plausible cause of the recurrent shedding. Molecular studies have suggested that excretion of *C. burnetii* in the placenta of infected goats was limited until the next kidding season following an outbreak (Hatchette et al., 2003).

In this study, we did not find any major histopathological changes and were not able to detect *C. burnetii* antigen by immunohistochemical examination in *C. burnetii* PCR positive samples from the five shedding goats. After experimental infection of pregnant goats with *C. burnetii*, trophoblast cells of the choriallantoic membrane have been described as the first tissue affected by the pathogen. Previous studies have indicated that only mild histopathological changes could be observed in the liver, lung and spleen of goats after experimental infection (Sánchez et al., 2006). After abortion, the mammary glands showed major histopathological changes and *C. burnetii* was detected in all goats (Sánchez et al., 2006). In the present study, only mammary tissue was found to be *C. burnetii* PCR positive, possibly as a result of the long-standing mammary infection with the organism.

After removal of the shedding goats, bulk tank milk *C. burnetii* PCR results returned to negative and remained so for over a year. Nowadays, compulsory measures are

still applied in infected farms and *C. burnetii* shedding goats can be a risk for increased pathogen load in a farm. Results of this study indicate that detection and removal of shedding goats could be effective in returning bulk tank milk *C. burnetii* PCR to negative results for a prolonged period of time. This could be useful for infected herds aiming to become bulk tank milk *C. burnetii* PCR negative. The relatively high costs of a *C. burnetii* PCR and the high number (on average 900 goats) of animals in herds in the Netherlands (van den Brom and Vellema, 2009) may hinder efforts to detect and remove shedding animals on a large scale. To decrease costs, it may be suggested to explore possibilities of various sample pooling strategies, aiming at *C. burnetii* infected herds to becoming bulk tank milk *C. burnetii* PCR negative in a financially friendly way.

Concluding comments

On a bulk tank milk *C. burnetii* PCR positive dairy goat farm, shedding of the organism in milk was demonstrated in 5 goats, which, likely, were chronically infected. In all animals, at postmortem examination, only mammary tissue samples were found to be *C. burnetii* PCR positive. No major histopathological changes were found in the *C. burnetii* PCR positive tissue samples. Moreover, no *C. burnetii* antigen could be detected by immunohistochemical examination in *C. burnetii* PCR positive tissues. After removal of the *C. burnetii* shedding goats, bulk tank milk samples remained PCR negative for *C. burnetii* for over a year. This study indicates that detection and subsequent removal of goats shedding the organism is possible to eradicate *C. burnetii*. These results also indicate that Q fever vaccination of infected dairy goats does not completely prevent intermittent shedding of *C. burnetii.*

Conflict of interest statement

All authors declare to have no conflict of interest.

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Chapter 7

A probably minor role for land-applied goat manure in the transmission of *Coxiella burnetii* to humans in the 2007–2010 Dutch Q fever outbreak

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Submitted for publication

Abstract

In 2007, Q fever started to become a major public health problem in the Netherlands, with small ruminants as most probable source. In order to reduce environmental contamination, control measures for manure were implemented because of the assumption that manure was highly contaminated with *Coxiella burnetii*. The aims of this study were 1) to clarify the role of *C. burnetii* contaminated manure from dairy goat farms in the transmission of *C. burnetii* to humans, 2) to assess the impact of manure storage on temperature profiles in dunghills, and 3) to calculate the decimal reduction time of the Nine Mile RSA 493 reference strain of *C. burnetii* under experimental conditions in different matrices.

For these purposes, records on distribution of manure from case and control herds were mapped and a potential relation to incidences of human Q fever was investigated. Additionally, temperatures in dunghills from two dairy goat farms with a recent abortion wave caused by *C. burnetii* were measured and related to heat resistance of *C. burnetii,* as determined under experimental conditions in different matrices.

In 54 four-digit postal code areas in which manure from case herds was deposited, an average of 5.1 human cases per 100,000 residents was observed. In addition, in 103 postal code areas in which manure from control farms was deposited, an average of 3.6 human cases per 100,000 residents was observed. Results of negative binomial regression showed no significant association between the incidence of human Q fever cases and the source of manure (*P*-value 0.95). Temperature measurements in the core and shell of dunghills on two farms were above 40°C for at least ten consecutive days which would result in a strong reduction of *C. burnetii* over time.

Our findings indicate that there is no relationship between incidences of human Q fever and land applied manure from dairy goat farms with an abortion wave caused by *C. burnetii*. Temperature measurements in dunghills on two farms with *C. burnetii* shedding dairy goat herds further support the very limited role of goat manure as a transmission route during the Dutch human Q fever outbreak. It is very likely that the composting process within a dunghill will result in a clear reduction in the number of viable *C. burnetii*.

Introduction

Q fever is a zoonotic disease caused by the obligate intracellular bacterium *Coxiella burnetii*. Domestic ruminants are considered to be the most important source of infection. In cattle, the disease is mainly asymptomatic (Arricau-Bouvery and Rodolakis, 2005), but in sheep and goats abortion, stillbirth and retention of foetal membranes can occur (Maurin and Raoult, 1999; Wouda and Dercksen, 2007). The bacterium is shed in urine, milk, faeces, and is found in high numbers in birth products of infected animals, causing environmental contamination. The main route of transmission of the bacterium to humans is by aerosols (Marrie, 1990a; Maurin and Raoult, 1999; Schimmer et al., 2010).

Until 2007, about twenty human Q fever cases were notified in the Netherlands annually (Van Steenbergen et al., 2007). Since then, Q fever started to become a major public health problem with 168, 1,000, and 2,357 notified human cases in 2007, 2008 and 2009, respectively (Van der Hoek et al., 2012). These unprecedented annual outbreaks are largely explained by exposure of the general population to airborne *C. burnetii* contaminated dust particles originating from infected dairy goat herds with abortion storms (Schimmer et al., 2010; Van der Hoek et al., 2010b; Van der Hoek et al., 2011a; Van der Hoek et al., 2011a; Dijkstra et al., 2012; Van der Hoek et al., 2012b). To reduce shedding, and thus environmental contamination, control measures were implemented, such as compulsory vaccination of all dairy sheep and dairy goats, and measures to reduce potential transmission, for instance by prohibiting removal of manure from stables within thirty days after lambing, and compulsory covering of manure after removal from the stable to reduce potential transmission (Van den Brom and Vellema, 2009; Roest et al., 2011a).

These manure measures were implemented because of the assumption that manure played an important role in the transmission of *C. burnetii*. Not only urine and faeces (Arricau-Bouvery and Rodolakis, 2005), but especially birth products from infected small ruminants may contain large numbers of *C. burnetii*, leading to contamination of manure (Roest et al., 2012). In several outbreaks, manure was suspected as the most probable source of the outbreak (Georgiev et al., 2013). However, data confirming the contamination of manure by viable *C. burnetii* are lacking. In addition, no data are available that describe the anticipated reduction in the number of *C. burnetii* during storage, when composted. This is somewhat surprising as the manure control measures do have an impact on farm management and are implemented widely to avoid spread of *C. burnetii*.

The aims of this study were 1) to clarify the role of *C. burnetii* contaminated manure in the transmission of *C. burnetii* to humans, 2) to assess the impact of manure storage on temperature profiles in dunghills, and 3) to calculate the decimal reduction time of the Nine Mile RSA 493 reference strain of *C. burnetii* under experimental conditions in different matrices.

Materials and methods

Mapping manure distribution patterns

In the Netherlands, farmers have to register transport of manure from their farm to its destination. Based on these records, distributions of manure from dairy goat farms with notified abortion waves caused by *C. burnetii* in 2008 and/or 2009 were compared with distributions of manure from a group of control farms. These control farms were defined as dairy goat farms without notified abortions caused by *C. burnetii*, which never had a positive PCR result in the mandatory bulk tank milk (BTM) surveillance program between its start in 2009 up to and including 2014, and which were BTM ELISA negative in 2008, before goats on these farms were vaccinated against *C. burnetii* (Van den Brom et al., 2012a). Distribution of goat manure from both groups of farms in 2008 and 2009 was mapped. As a significantly higher incidence of Q fever patients has been demonstrated within a five km radius of an infected goat farm (Schimmer et al., 2010; Van der Hoek et al., 2010b; 2011a; 2012b), all destinations of goat manure within a ten km radius of a herd with a notified abortion wave were excluded. The purpose of this exclusion is to preclude shedding by goats on infected farms as a possible source of environmental contamination. Manure destination areas from either case or control herds were identified by their four-digit postal code.

For all included four-digit postal code areas, destination and amount of manure, and incidences of human Q fever patients in 2008 and 2009 were compared using descriptive statistics and negative binomial regression models (nbreg in STATA 13©). Human Q fever incidences were calculated for each four-digit postal code area by dividing the total number of Q fever patients in 2008 and 2009 by the number of residents present in the same area in 2009 based on CBS records (CBS, 2014). In the negative binomial regression, the number of human cases per four-digit postal code area was included as dependent variable, and amount of manure or residents per four-digit postal code in 2009 were included as exposure. Independent variables that were included were whether manure originated from a case or control herd, and amounts of manure that were dropt (categorical in four categories).

Participating farms

Two dairy goat farms (farms A and B), with a history of *C. burnetii* related abortion waves participated in this study. *C. burnetii* infection was confirmed by immunohistochemistry (Wouda and Dercksen, 2007; Van den Brom et al., 2012).

Farm A had a herd size of 2,505 goats and farm B of 1,568 goats. On both farms, all goats were kept in deep litter stables all year round. At the start of the study, both farms were *C. burnetii* BTM PCR positive (Van den Brom et al., 2012a) in the Dutch BTM surveillance program, which became mandatory for all dairy sheep and dairy goat farms from October 2009 onwards (Vellema and Van den Brom, 2014).

Temperature measurements and manure sampling

Temperature development in manure was measured for 97 consecutive days after removal from the stable on the two farms. Upon removal of manure from the deep litter stables, dunghills were made on both farms. On farm A, the dunghill was 10 metres (m) long, 4.5 m wide and 3.5 m high. On farm B, the dunghill was 30 m long, 12.5 m wide and 7 m high.

Temperature measurements were carried out using a temperature measurement lance, fabricated and calibrated for this experiment by Peekel Instruments BV, Rotterdam, the Netherlands (www.peekel.nl). The calibrated temperature measuring equipment was connected to a computer to enable continuous temperature measurement. Data were stored using Signa Soft 6000 software. Temperature measurements inside the dunghills on both farms were performed at two locations as shown in Figure 7.1a. The temperature of the core was measured at about 0.5 m from the concrete floor, while the shell temperature was taken at about 2.3 m from the concrete floor. Based on the results, an average daily temperature was determined for the core as well as for the shell of the dunghill.

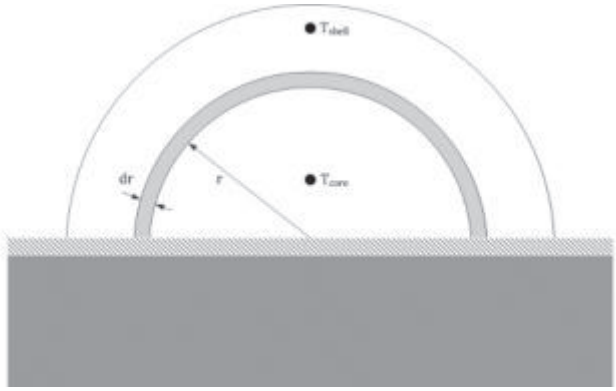

Figure 7.1a. Schematic drawing of dunghill cross section, placed on a concrete floor. Please note that in reality the shape of a dunghill is less smooth. Calculations were performed with a height of the dunghill of 2.5 m, a width of 5 m, and a length (into the paper) of 10 m. These dimensions approach those of the dunghill of farm A. The measurement locations for shell and core temperatures are indicated. r = radius [m], T = temperature [°C].

On the day of removal of manure from the stables, manure samples were obtained in the deep litter stable on three different depths from the surface: 0-2 centimetre (cm), 18-20 cm, and 38-40 cm, respectively. A durable plastic polymer guide tube was used as a cylindrical pathway to the sampling sites, to collect manure samples on different levels in the dunghills. On both farms manure samples were obtained from the surface layer (0-20 cm), middle layer (90-100 cm) and deep layer (190-200 cm), respectively.

Temperature profile estimates

In order to predict survival rates of *C. burnetii*, it is necessary to estimate the temperature profile between shell and core measurement locations. An energy balance was set up between both points, and for computational reasons, a simplification of the geometry of the dunghill was made as explained in Figure 7.1b.

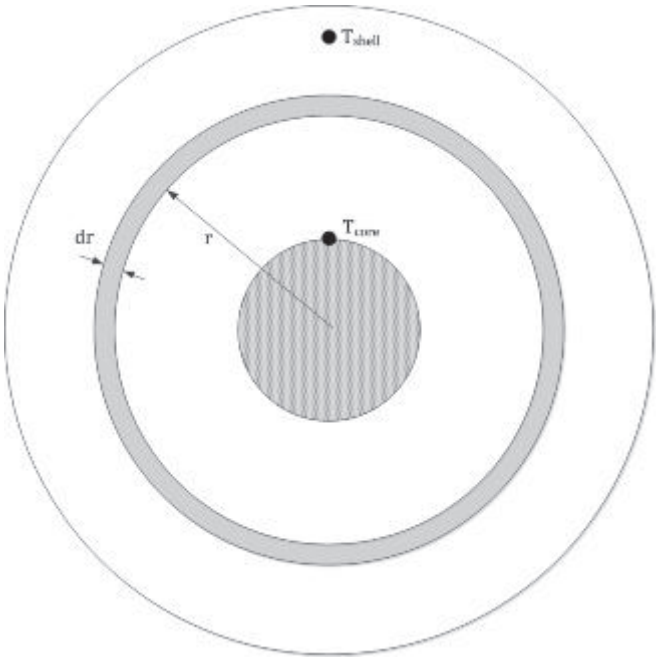

Figure 7.1b. Schematic drawing of dunghill as simplified for setting up an energy balance. The dunghill was modelled as a hollow cylinder of infinite length with an inner radius of 0.5 m and an outer radius of 2.5 m. Temperature prediction was only possible between both temperature measurement locations (T_{core} and T_{shell}). r = radius [m], T = temperature [°C].

Setting up an energy balance on a slice of thickness 'dr' at radius r in the geometry shown in Figure 7.1b gives:

accumulation = in − out + production

$$0 = \underbrace{\left(-2\pi rL\lambda\right)\frac{dT}{dr}\bigg|_{r}}_{in} - \underbrace{\left(-2\pi rL\lambda\right)\frac{dT}{dr}\bigg|_{r+dr}}_{out} + \underbrace{\left(2\pi rL\cdot dr\right)\dot{Q}'''_{prod.}}_{production} \qquad (1)$$

with:

$\dot{Q}'''_{prod}$ = rate of internal heat generation per unit volume $\left[\frac{W}{m^3}\right]$

λ = dung heat conductivity $\left[\frac{W}{m\cdot K}\right]$

r = radius $\left[m\right]$

L = dung hill length $\left[m\right]$

T = temperature $\left[°C\right]$

The first two terms of equation 1 represent conductive heat transfer in the slice according to Fourier's law of heat conduction (Mills, 1999). In the third term, heat production inside the slice is described. Rewriting equation 1 and solving the resulting differential equation gives the following result:

$$T(r) = T_{core} + \frac{\dot{Q}'''_{prod}}{4\lambda}\left(r_{core}^2 - r^2\right) + \frac{T_{shell} - T_{core} + \frac{\dot{Q}'''_{prod}}{4\lambda}\left(r_{shell}^2 - r_{core}^2\right)}{\ln\left(r_{shell}/r_{core}\right)}\ln\left(\frac{r}{r_{core}}\right) \qquad (2)$$

Equation 2 shows how the temperature inside the dunghill varies with its radius. This equation is only valid for $r_{core} \le r \le r_{shell}$. It was assumed that the rate of internal heat generation per unit volume ($\dot{Q}'''_{prod}$) does not depend on radius. In equation 2, temperatures of core and shell (T_{core} and T_{shell}), as well as the radius of core and shell (r_{core} and r_{shell}) are known. If $\dot{Q}'''_{prod}$ and the dung heat conductivity (λ) are also known, the temperature profile inside the dunghill can be calculated. Since these two parameters were not measured in the experiment, they need to be estimated. Looking at the terms in equation 2, which contain $\dot{Q}'''_{prod}$, it can be seen that for $r > r_{core}$ these terms would always be positive, meaning that they would increase the temperature at every value for r. Therefore, more conservative

temperature estimates would be obtained by setting $\dot{Q}'''_{prod}$ to zero in equation 2, neglecting internal heat generation altogether. Equation 2 then simplifies to equation 3 (Mills, 1999):

$$T(r) = T_{core} + \frac{T_{shell} - T_{core}}{\ln\left(r_{shell} / r_{core}\right)} \ln\left(\frac{r}{r_{core}}\right) \quad (3)$$

Using equation 3, a temperature profile could be calculated for each of the 97 days for which measurements were available for farm A. For this purpose, the dunghill was divided into 25 parts with a thickness of 10 cm and a length L, analogous in shape to the segmented part with thickness dr in Figure 7.1a. For all the segmented parts with a radius between r_{core} and r_{shell}, the temperature at each day in the middle of each segmented part was calculated using equation 3.

Extrapolation of decimal reduction time from literature

The decimal reduction time of *C. burnetii* in milk was measured by Enright et al. (1957) for temperatures between 143 (61.7°C) and 162 (72.2°C) degrees Fahrenheit. These data were fitted to the following equation (4):

$$^{10}\log(t) = -0.2258T + 17.3307$$

$t =$ time $\left[s\right]$

$T =$ temperature $\left[°C\right]$

Using extrapolation below 61.7°C (143°F), equation 4 was used in combination with the results from the temperature profile calculations in order to predict whether or not *C. burnetii* in a certain segmented part survived 97 days in the dunghill at Farm A.

Coxiella burnetii PCR in manure

Procedures for manure sample processing, DNA extraction, and qPCR detection of *C. burnetii* DNA have been described previously (de Bruin et al., 2012; 2013). Samples were scored as undetermined when no signals were observed for both *C. burnetii* and the internal control targets, indicating severe qPCR inhibition. In DNA extraction procedures, especially from complex environmental samples, many substances are co-extracted, which may interfere DNA amplification during qPCR. This can result in underestimations of the presence of DNA from a potential pathogen. To be able to estimate the number of *C. burnetii* organisms, differences between Cq values for internal control target *cry1*, obtained from samples and

positive controls (p.c), were corrected for qPCR inhibition effects by using the following formula: $\Delta Cq_{cry1} = Cq_{cry1\ sample} - Cq_{cry1\ p.c}$.

Values for $Cq_{cry1\ sample}$ and $Cq_{cry1\ p.c}$ resemble Cq values obtained from samples and positive controls, respectively. The value of ΔCq_{cry1} is a measure for qPCR inhibition in a particular sample. This value is subtracted from the Cq values for *C. burnetii* targets *IS1111* and *com1*, to correct for qPCR inhibition effects.

An important assumption using this procedure is that all targets are affected by qPCR inhibition in the same order of magnitude. We estimated the number of *C. burnetii* organisms present per gram manure, based on Cq values for target *com1,* and using a DNA standard for *C. burnetii* (Vircell (www.vircell.com), cat. Nr. MBC018).

Culture of Coxiella burnetii in naïve and spiked goat manure samples

To isolate *C. burnetii* from manure, 2 mL of manure was suspended in 10 mL of phosphate buffered saline (M: 0.01; pH: 7.2) and shaken for 10 minutes. The suspension was centrifuged for 10 minutes at 100 g. Supernatant was filtered stepwise over filters with pore sizes of 1.2 µm and 0.45 µm (Pall Cooperation, USA). Filtered material was centrifuged for 5 minutes, 15,000 g twice and the pellet was first suspended in 1 mL of culture medium without antibiotics (Eagle's minimal essential medium (EMEM) with 10% bovine serum albumin, 1% non-essential amino acids (NEAA), 1% glutamax) followed by resuspension in 100 µL of culture medium. This suspension was inoculated onto a culture of Buffalo Green Monkey (BGM) cells and incubated for 14 days at 37°C in a closed flask as reported earlier (Roest et al., 2012). Growth of *C. burnetii* was monitored by checking vacuolization of the BGM cells and confirmed by immunofluorescence staining, with the Nine Mile RSA 493 reference strain as positive control (Roest et al., 2012). To evaluate the ability to isolate and culture *C. burnetii* from manure (positive control experiment), a spiking experiment was set up: 1 to 1.5 gram *C. burnetii* PCR negative goat manure was suspended in 2 mL PBS. To eliminate contaminating flora, the suspension was heated for 30 min at 99°C. After cooling down, 8.68×10^9 *C. burnetii* Nine Mile strain bacteria were added. The number of bacteria was quantified according to Roest et al. (2012).

Calculated decimal reduction time

For the determination of the decimal reduction time (DRT) of *C. burnetii*, the Nine Mile RSA 493 reference strain was used in a concentration of 1×10^5 bacteria per mL. The DRT was determined in PBS, PBS with 1.8 w/v% urea, PBS with 1.8 w/v% ammonia and in goat manure extract (9.5 gram of goat manure in 28.5 mL PBS). To determine the concentration of *C. burnetii* in the suspension before and after time-temperature treatment, ten-fold dilutions of the samples were made and inoculated on BGM cells. Cells were incubated for 14 days as described above.

Growth of *C. burnetii* was monitored by PCR of the supernatant and finally by immunofluorescent staining (Roest et al., 2012). The different *C. burnetii* solutions were treated using the following time-temperature combinations: 5, 10 and 15 seconds with 70 and 72°C, and 3, 6 and 9 min with 60 and 65°C. Immediately after treatment, samples were cooled down to room temperature. Samples with PBS-urea, PBS-ammonia and goat manure extract were washed twice at 10 minutes of centrifuging at 14,000g and resuspension in 1 mL of PBS before inoculation onto BGM cells. All measurements were done in triplicate.

The DRT at a certain temperature can be calculated using the formula:

$$DRT = \frac{t_2 - t_1}{^{10}LOG(\frac{[start]}{[end]})}$$ (Bearns and Girard, 1958)

with $t_2 - t_1$ = the duration of treatment in which the change in concentration took place, and $^{10}LOG(\frac{[start]}{[end]})$ = the decimal reduction of the starting concentration to the concentration at the end. In this experiment, DRT was calculated as the average over three measurements over three time intervals per matrix at temperatures 60, 65, 70 and 72°C. The DRT in the matrix at other temperatures was extrapolated from DRT-temperature curve.

Results

Distribution of manure

In 2008 and 2009, records of all 3,357 notified human Q fever patients were available. Incidences of human Q fever patients are presented per four-digit postal code area in Figure 7.2a. In the same period, *C. burnetii* induced abortion waves were confirmed on twelve dairy goat farms (case herds). From these case herds, manure was removed 692 times in 2008 and 2009. This manure was distributed over 94 out of 3,972 four-digit postal code areas, and per area in which manure was distributed a median of 99,230 kg manure was distributed (25% percentile: 47,720-75% percentile: 202,540).

From 24 control herds, manure was removed 861 times in 2008 and 2009. This manure was distributed over 107 four-digit postal code areas. Per postal code area a median of 80,240 kg manure was distributed (25% percentile: 36,100-75% percentile: 199,260). After removal of the manure distributions in the 10 kilometre four-digit postal code areas around case herds, manure distribution of case herds remained in 54 postal code areas, and manure distribution of control herds remained in 103 postal code areas (Figure 7.2b and 7.2c).

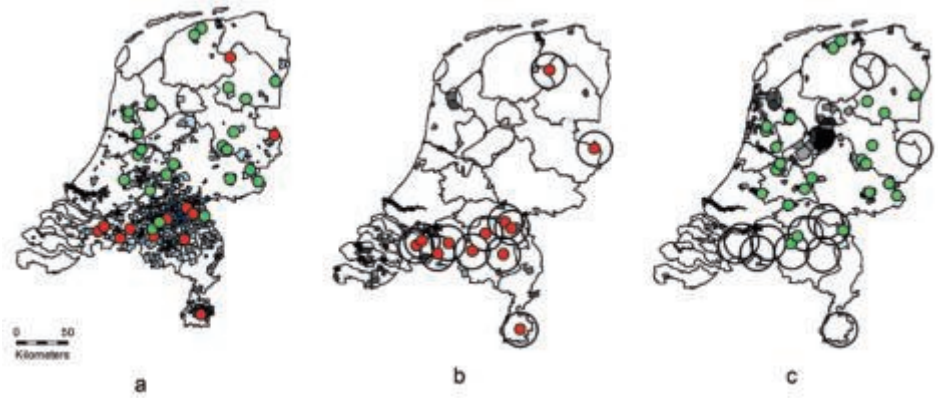

Figure 7.2. In Figure a, twelve dairy goat farms with abortion waves caused by *Coxiella burnetii* in 2008 and/or 2009 (case farms; red dots), and 24 dairy goat farms without notified abortion waves caused by *C. burnetii*, bulk tank milk (BTM) PCR negative results between 2009 and 2014, and BTM ELISA negative results in 2008 from which records of manure distribution were available (controls; green dots), as well as incidences (number of cases per 100,000 residents) of human Q fever patients (the darker area, the more human Q fever patients) are presented. In Figure b, distributions of manure from case farms to an area outside a radius of ten km around case farms (four-digit postcode areas; dark colored) are presented. In Figure c, distributions of manure from control farms outside a radius of ten km around case farms (four-digit postcode areas; dark colored) are presented.

In 54 four-digit postal code areas in which manure from case herds was distributed, there were on average 5.1 human cases per 100.000 residents (median 0; 25% percentile: 0-75% percentile: 0), in 2008 and 2009. In addition, in 103 postal code areas in which manure from control farms was dropped, there were on average 3.6 human cases per 100.000 residents (median 0; 25% percentile: 0-75% percentile: 0), in 2008 and 2009. In comparison, on average 99.8 human cases per 100,000 residents (median 9.3; 25% percentile: 0-75% percentile: 77.9) were found within a radius of ten kilometre around case farms, in 2008 and 2009.

Results of negative binomial regression showed no significant association between the incidence of human Q fever cases and the origin of manure (*P*-value 0.95). We also found no association with the amount of manure that was distributed and an interaction between case or control farms, and the amount of manure also tested non-significantly (*P*-value 0.81). In addition, to improve the certainty of our results, we varied the time period that was included (from January 1st 2008 until December 31th 2010, and from the moment that an abortion wave occurred until six months after this event) but all models showed non-significant results.

Temperature measurements and manure sampling

Temperature measurements inside dunghills were performed in the core and in the shell. In the shell of the dunghill on farm A, the highest temperature of 72°C was measured within four days after the start of the measurements. A shell temperature

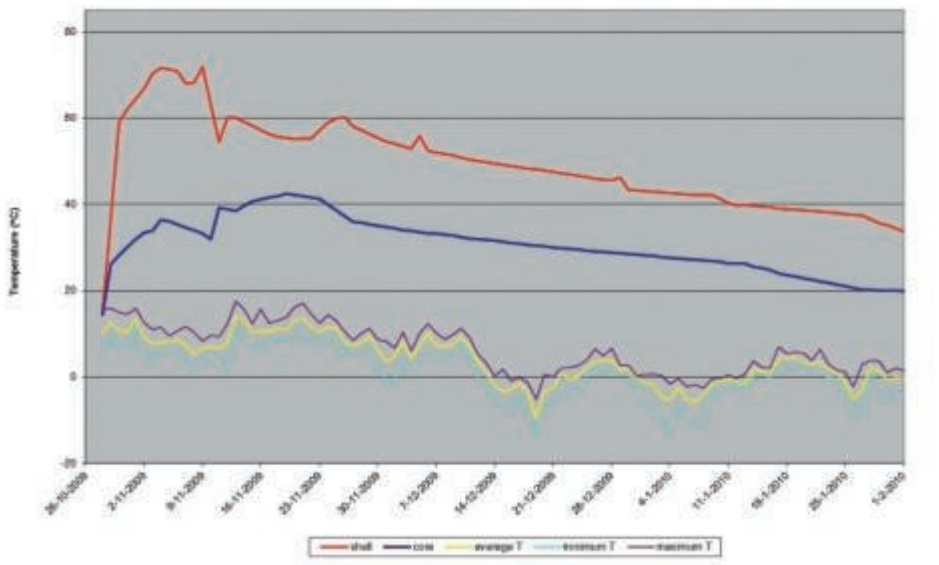

Figure 7.3a. Temperatures in the core (dark blue) and shell (red) of the dunghill on farm A. The average (yellow), the minimum (turquoise) and the maximum (purple) outside air temperature in Eindhoven, the Netherlands (www.knmi.nl) during the experiments are shown. All temperatures are in degree Celsius.

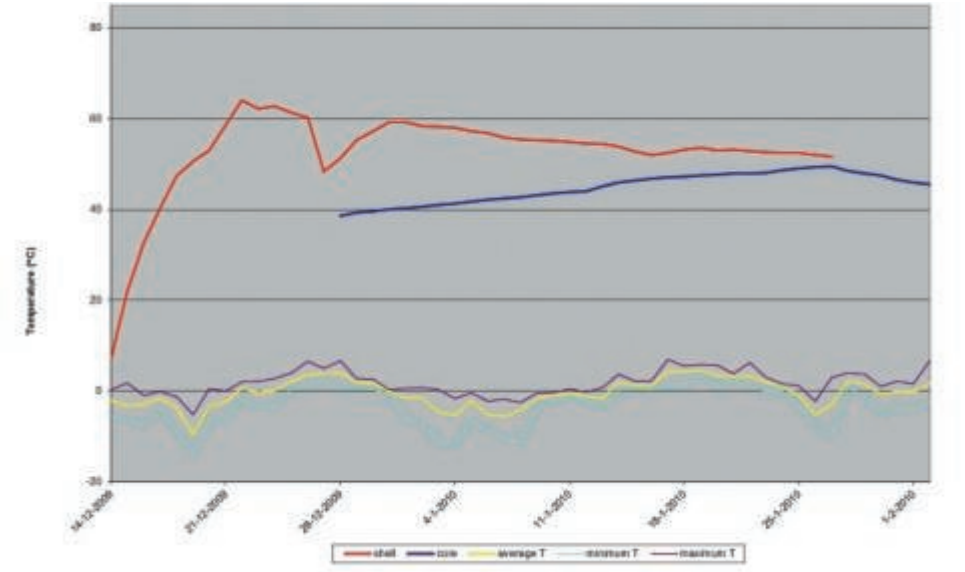

Figure 7.3b. Temperatures in the core (dark blue) and shell (red) of the dunghill on farm B. The average (yellow), the minimum (turquoise) and the maximum (purple) outside temperature in Eindhoven, the Netherlands (www.knmi.nl) during the experiments are shown. All temperatures are in degree Celsius.

above 60°C was measured for twelve consecutive days. The temperature in the core rose less quickly and reached a temperature above 40°C for ten consecutive days (Figure 7.3a).

In the shell of the dunghill on farm B, the highest temperature of 64°C was measured within five days after the start of the measurements. A shell temperature above 60°C was measured for five consecutive days. The temperature in the core of the dunghill on Farm B also rose less quickly than on farm A and reached a temperature above 50°C for ten consecutive days (Figure 7.3b).

Temperature profile estimates

Since temperature data for farm B were incomplete, caused by a technical problem, temperature profiles were only calculated for farm A. On this farm, the dunghill was 10 m long, 4.5 m wide and 3.5 m high at the start of the measurements. Temperature profiles were calculated using a height of 2.5 m since during the experiment the dunghill size settled to this height. The dung hill width used for the calculations was 5 m. Calculations were based on 97 consecutive days, starting on 28th October 2009.

Examples of temperature data obtained from the measurements and calculated as a result of the heat transfer models of equations 2 and 3 are shown in Figure 7.4. Depending on the values of $\dot{Q}'''_{prod}$ and λ, the temperature values from equation 2 may vary, but the general trend remains unaltered.

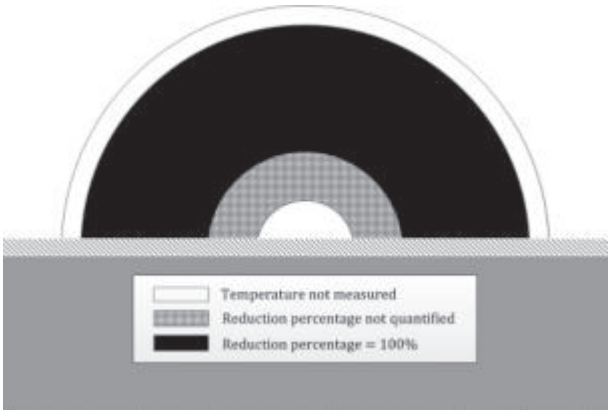

Figure 7.4. Estimated temperature profiles inside the dunghill at Farm A on 4th November 2009. Cases for Q_{prod} = 0 (only conduction, equation 3) and for Q_{prod} = 50 W/m^3 and λ = 2 W/m·K (conduction and heat production, equation 2) are shown. The λ value of wet soil is taken (Mills, 1999), the value for Q_{prod} was estimated based on heat transfer calculations using the outdoor air temperature on 4th November 2009.

Table 7.1. Estimated temperature profiles in 18 segmented parts of dunghill A.

	Temperature [°C][#]				Longest consecutive period above a certain temperature [°C][&]			Reduction [%][$]	% of the volume of the dunghill (cumulative)[@]
Half ring	<30	30-40	40-50	≥50	T (days)	Max	Average		
1*									0.16
2*									0.48 (0.64)
3*									0.8 (1.44)
4*									1.12 (2.56)
5*									1.44 (4)
6	36	46	15	0	>40(15)	43	42	^	1.76 (5.76)
7	26	47	24	0	>40(17)	44	43	^	2.08 (7.84)
8	21	44	32	0	>40(32)	46	44	^	2.4 (10.24)
9	17	42	38	0	>40(38)	47	45	^	2.72 (12.96)
10	14	39	40	4	>40(44)	51	46	^	3.04 (16)
11	10	39	40	8	53(3)	53	53	100	3.36 (19.36)
12	6	38	33	20	≥55(3)	56	55	100	3.68 (23.04)
13	5	36	31	25	≥56(4)	58	57	100	4 (27.04)
14	3	33	33	28	≥55(8)	59	58	100	4.32 (31.36)
15	1	35	30	31	≥57(7)	61	59	100	4.64 (36)
16	1	34	30	32	≥55(10)	62	60	100	4.96 (40.96)
17	1	31	31	34	≥55(11)	64	60	100	5.28 (46.24)
18	1	26	35	35	≥56(11)	65	62	100	5.6 (51.84)
19	1	24	34	38	≥58(11)	67	63	100	5.92 (57.76)
20	1	23	33	40	≥56(12)	68	64	100	6.24 (64)
21	1	23	32	41	≥57(12)	69	65	100	6.56 (70.56)
22	1	22	31	43	≥58(12)	70	66	100	6.88 (77.44)
23	1	20	31	45	≥59(12)	71	67	100	7.2 (84.64)
24*									7.52 (92.16)
25*									7.84 (100)

[*] Temperature profiles in the segmented parts 1, 2, 3, 4, 5, 24 and 25 fell outside the scope of the two measurement locations in the dunghill (see Figure 5). These are therefore outside the range of validity of the temperature profile model.

[#] For each segmented part, the number of days that the estimated temperature in the dunghill fell within a certain temperature interval during the 97 days of the experiment is presented.

[&] The combination of the minimum daily temperature (T) with the longest consecutive time interval (days) that could achieve the maximum reduction percentage. In all cases, the highest temperature fell within this period. For the longest consecutive time period also the maximum and the average temperature are determined.

[$] Estimated reduction percentage of C. burnetii in the dunghill according to comparison with described decimal reduction time (DRT) in milk, as described by Enright et al. (1957) and extrapolated using equation 4.

[^] For the segmented parts 6-10, the reduction percentage of C. burnetii could not be quantified based on the calculated temperature profiles. Reduction percentages in these segmented parts are less than 100% when compared to DRT of C. burnetii in milk (Enright et al., 1957). Nevertheless, based on DRT in goat manure (see Table 3), survival of C. burnetii is just above 3 hours at a temperature of 40 degree Celsius. Therefore, total reduction of C. burnetii in the segmented parts 6-10 might also be possible.

[@] For each segmented part, its contribution (%) to the total volume of the dunghill is presented. Also, the cumulative percentage is presented.

It is clear from Figure 7.4 that the case which includes heat production inside the dung hill shows higher temperature values across the whole range, when compared to the case with only heat conduction. However, the choice of the parameters $\dot{Q}'''_{prod}$ and λ has a large impact on calculated temperature profiles, and thereby on the survival rates of *C. burnetii*. In order to mitigate the risk of overestimating the amount of bacteria that did not survive, equation 3 was used for all calculations.

For the segmented parts 6-23, results of the temperature profiles, determined reduction percentages of *C. burnetii* based on heat resistance of the bacterium in milk (Enright et al., 1957), and percentages of the volume of the dunghill of every segmented part, are presented in Table 7.1.

Coxiella burnetii DNA in manure samples

In total, 46 samples were obtained, 22 from farm A and 24 from farm B. Manure samples were categorized into manure location, deep litter stable or dunghill, respectively (Table 7.2). *C. burnetii* DNA was found in manure obtained at all depths of deep litter stables as well as from both dunghills during the whole sampling period. On Farm A, the number of *C. burnetii* per gram manure was between 10^3 and 10^5. On farm B, the number of *C. burnetii* per gram manure was between 10^4 and 10^7. The standard deviation ranged between 10^2 and 10^7. Due to the presence of multiple copies of the *IS1111* target within the *C. burnetii* genome (Seshadri et al., 2003; Klee et al., 2006), amplification of this target is expected to occur before amplification of the single-copy target *com1*. This was reflected in our data, where for samples showing positive results for both targets *com1* and *IS1111*, Cq values of *IS1111* were consistently lower than those of *com1*. Therefore, positive samples were categorized into two classes with increasing *C. burnetii* DNA content: (1) *IS1111* positive and (2) positive for both *IS1111* and *com1*. A number of manure samples showed severe qPCR inhibition in undiluted, and sometimes ten-fold diluted DNA samples. This resulted in the absence of a positive signal for internal control target *cry1*, or amplification curves that showed reduced amplification efficiencies. Samples with no signal for the internal control *cry1* and *C. burnetii* targets *IS1111* and *com1* are categorized as 'not determined'. For quantification purposes, differences between Cq values for internal control target *cry1,* obtained from samples and positive controls, were used to correct for qPCR inhibition effects where possible.

Culture of Coxiella burnetii in naïve and spiked goat manure samples

In none of the *C. burnetii* PCR positive goat manure samples from both farms, we were able to culture *C. burnetii*. In order to exclude technical problems, *C. burnetii* was cultured from *C. burnetii* spiked solutions of goat manure samples (positive results of the positive control) taken from the floor in the deep litter stable. Both in immediate culture as in samples after 48 hour incubation, *C. burnetii* could be cultured. Therefore, technical culture problems were excluded.

Table 7.2. *Coxiella burnetii* PCR results in manure from two dairy goat farms.

Farm	Manure location	*IS1111*	*IS1111 + com1*	Negative	Not determined
A	Dunghill	1	8		9
	Deep litter stable	2	4		
B	Dunghill	10	3	1	5
	Deep litter stable		2	1	

Number of C. burnetii positive samples categorized in manure location per farm. The category 'Not determined' reflects samples for which no signals were observed in the internal control, or C. burnetii targets.

Calculated decimal reduction time

Results of the calculated decimal reduction time (DRT) of the Nine Mile (NM) RSA 493 reference strain of *C. burnetii* under experimental circumstances are presented in Table 7.3. DRT in milk (Enright et al., 1957) was longer than we found in the measurements within goat manure, ammonia, urea and PBS. Extrapolation to a temperature of 40°C indicated that the DRT of the NM reference strain of *C. burnetii* in goat manure was just above 3 hours.

Table 7.3. Decimal reduction time (in seconds) of the Nine Mile reference strain of *Coxiella burnetii* at different temperatures in different matrices.

	DRT (in seconds)				
	NM in PBS	NM in 1.8% ammonia	NM in 1.8% urea	NM in manure from deep litter stable	NM in milk (Enright et al., 1957)
Temperature (t) (°C)	$10^{\wedge}$ (-0.1139t+8.7138)	$10^{\wedge}$ (-0.1355t+10.383)	$10^{\wedge}$ (-0.1222t+9.4457)	$10^{\wedge}$ (-0.0996t+8.0317)	$10^{\wedge}$ (-0.2253t+17,3307)
40	14381*	918333*	36116*	11161*	208305147*
50	1044*	4055*	2166*	1126*	1163322*
60	66,0	113,3	123,7	113,7	6497*
65	30,0	102,2	40,0	36,1	486§
70	3,3	3,8	4,6	11,5	36
72	4,3	5,2	6,3	7,3	13

*DRT, decimal reduction time; NM, Nine Mile reference strain of C. burnetii; PBS, Phosphate Buffer Saline; *Extrapolated DRT results; §Intrapolated DRT result.*

Discussion

During the human Q fever outbreak (2007-2010) in the Netherlands, which occurred primarily in the south-eastern part of the country, manure from dairy goat farms has been transported to several other parts of the country. We found no increased incidences of human Q fever related to distribution of manure originating from dairy goat farms with confirmed abortion waves caused by *C. burnetii*. Several studies have shown that living within a radius of five km from an infected farm

was an independent risk factor for acquiring human Q fever (Schimmer et al., 2010; Van der Hoek et al., 2010b; Van der Hoek et al., 2011a; Van der Hoek et al., 2011a; Dijkstra et al., 2012; Van der Hoek et al., 2012b). In these studies, distributions of manure from an infected farm with small ruminants were not described as risk factor for human Q fever, which is now supported by our study as well. In another Dutch study, distribution of goat manure was actually linked to human Q fever cases (Hermans et al., 2014). However, these results are difficult to compare with our results for several reasons. Hermans et al. (2014) did not include control herds, did not only include goat farms with abortion waves caused by *C. burnetii,* but also included herds that only tested PCR positive in the BTM surveillance program, and included distributions of manure to an area within a radius of five and ten km around infected farms while it is not possible to distinguish whether clusters of human Q fever patients are caused by transmission from land-applied goat manure or by airborne transmission from infected herds. Based on our results and bias in the study design of Hermans et al. (2014), we find it highly unlikely that land-applied goat manure played an important role as a source of human Q fever.

Although a large amount of *C. burnetii* DNA was present in manure samples from both participating farms with a recent history of *C. burnetii* related abortion, we were not able to culture *C. burnetii* from any of these manure samples. We were able to culture *C. burnetii* from spiked manure samples, demonstrating that technically it was possible to isolate *C. burnetii* from a complex matrix like manure. Although serial passages in experimental hosts is the most accurate procedure for determining the presence of small numbers of viable *C. burnetii* (Enright et al., 1957), our negative culture results suggest that no or only low numbers of viable *C. burnetii* were present in the manure samples.

The results of this study show that temperatures in the core and shell of the dunghills on farm A and B were above 40°C for at least ten consecutive days. Temperature measurements showed a higher temperature in the shell compared to the core. This difference probably is a result of the fact that successful composting is influenced by the availability of oxygen, and compulsory covering of a dunghill can therefore negatively influence the composting process. Temperature profiles calculated for farm A indicate a reduction in numbers of *C. burnetii* in the segmented parts 11-23 of 100%. In the segmented parts 6-10, temperatures were not high enough for a certain consecutive period of time to be certain that a total reduction of *C. burnetii* occurred. Temperature profiles of the segmented parts 1-5 and 24-25 fell outside the two measuring points (Figure 7.5), and we chose not to incorporate them and consequently neither could a reduction percentage be determined. Segmented parts 1-5, 6-10, 11-23, and 24-25 represent 4, 12, 68.6 and 15.4per cent of the total volume of the dunghill, respectively. The segmented parts for which temperature profiles could be determined (6-23) represent about 81 per cent of the volume of the dunghill. Because of a lack of measuring points in the

segmented parts 1-5 and 24-25, temperature profiles and therefore reduction percentages of *C. burnetii* could not be determined for about 19 percent of the volume of the dunghill. Based on these temperature profiles, and the DRT according to Enright et al. (1957), it can be concluded that in at least 85 per cent (68.6/80.6) of the volume of segmented parts 6-23 probably no *C. burnetii* could have survived the composting process.

Heat resistance of *C. burnetii* has been validated in infection studies in guinea pigs (Enright et al., 1957). In that study, two time-temperature combinations were finally found to be effective for pasteurization purposes and have subsequently been universally recognized: 30 minutes at 62.8°C (degrees Celsius, 145 degrees Fahrenheit) or 15 seconds at 71.7°C (161 degrees Fahrenheit) (Enright et al., 1957). These recommendations were simplified as: 30 minutes at 63°C or 15 seconds at 72°C, thus providing an extra safety margin. Assuming the 10log survival curve is a straight line, this would achieve eight decimal reductions (Cerf and Condron, 2006). For other matrices than milk, the decimal reduction time (DRT) of the *C. burnetii* Nine Mile (NM) RSA 493 reference strain has not been described before. In this study, DRT measured under experimental conditions appeared to be shorter in PBS, ammonia, urea, and goat manure, compared to the DRT of *C. burnetii* in milk (Enright et al., 1957). Extrapolation of these results to a temperature of 40°C, results in a DRT of the NM reference strain of *C. burnetii* in goat manure of just above 3 hours. In that case, survival of *C. burnetii* in the segmented parts 6-10 of the dunghill, based on the estimated temperature profiles, is very unlikely. A shorter DRT of the NM RSA 493 reference strain of *C. burnetii* in manure compared to milk can be caused by biological, physical, and chemical variables that may influence survival of bacterial pathogens in manure (Ziemer et al., 2010). Survival of several food borne pathogens such as *Escherichia coli* O157:H7 and *Salmonella enteritidis* has been investigated, and in properly composted manure microbial contamination seems to be minimized (Lung et al., 2001). Although, compared to pathogens like *Salmonella* spp., spore-forming bacteria seem to be able to survive pasteurization for a longer period (Bagge et al., 2010). Sharma et al. (2009) showed that despite reduction of antimicrobially resistant *E. coli,* antimicrobially resistant genes from these bacteria could be detected and therefore it was discussed whether using PCR should be preferred over cultivation-based methods for rapid identification of composting effectiveness.

As a precautionary principle, we applied a worst case scenario in all our calculations for the temperature profiles in the 25 segmented parts in which we mathematically segmented the dunghill on farm A. This means that we assumed that only heat conduction and no heat production in the dunghill took place. Furthermore, we did not perform extrapolation of temperature profiles outside the two measuring points, and we compared the temperature profiles to the higher DRT of *C. burnetii* in milk rather than comparing it with the lower DRT which we experimentally

measured in goat manure. Consequently, it is very likely that the percentage of surviving *C. burnetii* is lower in reality than the values presented in this study. In a follow-up study we would recommend to extend the number of temperature measuring points to at least five in order to be able to estimate temperature profiles more accurately, without extrapolation, for all 25 segmented parts in a dunghill. Under such conditions, it would also be possible to determine heat conduction as well as heat production in composting dunghills, making an even more accurate estimation possible. The five recommended measurement locations are: core, shell (dung hill top), shell (at concrete floor), halfway between shell and core (vertically), and halfway between shell and core (horizontally). This follow-up would not only be of interest for *C. burnetii*, but also for determining survival possibilities in a dunghill for other pathogens, especially those with zoonotic potential.

In conclusion, several studies have suggested that manure from ruminants played an important role in the transmission of *C. burnetii* to humans (Salmon et al., 1982; Rehacek and Tarasevich, 1988; Berri et al., 2003). Arricau-Bouvery and Rodolakis (2005) stated that manure from infected herds should be covered and composted or treated with lime or calcium cyanamide 0.4% before being spread on the field, and spreading should never be performed under windy circumstances. In our study, no relation could be found between distributions of goat manure and incidences of human Q fever. The same applies for epidemiological risk factor surveys, where manure was not found to be a risk factor for human Q fever. Although a large amount of *C. burnetii* DNA was present in manure samples from both farms, we were not able to culture *C. burnetii*. Even if viable *C. burnetii* had been present, composting would have resulted in a large reduction, taking into account core and shell time and temperature profiles, heat resistance of *C. burnetii* as described by Enright et al. (1957), and the decimal reduction time of the Nine Mile RSA 493 reference strain of *C. burnetii* in manure determined in this study. Thus, land-applied goat manure probably played a minor role in the transmission of *C. burnetii* to humans in the 2007–2010 Dutch Q fever outbreak, possibly partly due to a proper composting process.

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Chapter 8

Reduction of *Coxiella burnetii* prevalence by vaccination of goats and sheep, the Netherlands

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Abstract

Recently, the number of human Q fever cases in the Netherlands increased dramatically. In response to this increase, dairy goats and dairy sheep were vaccinated against *Coxiella burnetii*. All pregnant dairy goats and dairy sheep in herds positive for Q fever were culled. We identified the effect of vaccination on bacterial shedding by small ruminants. On the day of culling, samples of uterine fluid, vaginal mucus, and milk were obtained from 957 pregnant animals in thirteen herds. Prevalence and bacterial load were reduced in vaccinated animals compared with unvaccinated animals. These effects were most pronounced in animals during their first pregnancy. Results indicate that vaccination may reduce bacterial load in the environment and human exposure to *C. burnetii*.

Introduction

Q fever, which is caused by *Coxiella burnetii*, is a worldwide zoonotic infectious disease, and ruminants are the main reservoir for human infections (Norlander, 2000; Arricau-Bouvery and Rodolakis, 2005; Angelakis and Raoult, 2010). Ruminant infections may occasionally result in abortions, which are associated with shedding of large amounts of bacteria in placentas and birth fluids (Sánchez et al., 2006). Human infections have been reported mainly in persons handling infected animals and their products (Marrie et al., 1988; Tselentis et al., 1995; Armengaud et al., 1997; Lyytikäinen et al., 1998). However, this disease has not been perceived as a major public health risk for the general population. In 2007, a major epidemic occurred in the general population in the Netherlands (ProMED-mail, 2009), which resulted in > 2,300 reported cases in 2009. An explanation for the emergence of human Q fever was abortion clusters in goat herds beginning in 2005 within an intensified dairy goat production system (Van Steenbergen et al., 2007; Wouda and Dercksen, 2007; Klaassen et al., 2009; Schimmer et al., 2009; Van den Brom and Vellema, 2009; van der Hoek et al., 2010b). This hypothesis was substantiated by epidemiologic studies, which indicated a possible spatial link between dairy goat farms and human cases (Schimmer et al., 2010).

Reduction of the number of human cases was considered essential by public health authorities in the Netherlands. One of the intervention measures taken was vaccination of dairy goats against *C. burnetii* (Ministry of Agriculture, Nature and Food quality (MinLNV), 2010). This measure assumed that vaccination would reduce abortions and bacterial shedding to levels that would reduce the number of human cases in the following year. Vaccination began in 2008 and intensified in 2009. As the number of cases of *C. burnetii* infection in patients doubled in 2009, policymakers applied a precautionary principle and decided to cull all pregnant dairy goats or sheep on infected farms before the 2010 kidding season. This measure was implemented at the end of 2009 and thereby precluded any field analysis of vaccine efficacy in the spring of 2010. However, there was an opportunity to sample animals shortly after they were humanely killed. The purpose of this study was to quantify the effect of vaccination on bacterial load in excreta of pregnant animals.

Materials and Methods

Q fever in the Netherlands since 2005

Human Q fever cases in the Netherlands increased from 168 in 2007 to 1,000 in 2008 and 2,355 in 2009, mainly in Noord-Brabant Province (van der Hoek et al., 2010b). A campaign of voluntary vaccination of dairy goats began at the end of 2008 in the area of the 2007 human case cluster and was followed by mandatory vaccination of all dairy goat and dairy sheep on farms with > 50 animals in a larger area in 2009. This vaccination zone included Noord-Brabant Province and parts of

adjacent provinces because the supply of vaccine was not sufficient for all small ruminant farms in the Netherlands and because most human cases had occurred in that area (Figure 8.1) (Schimmer et al., 2009).

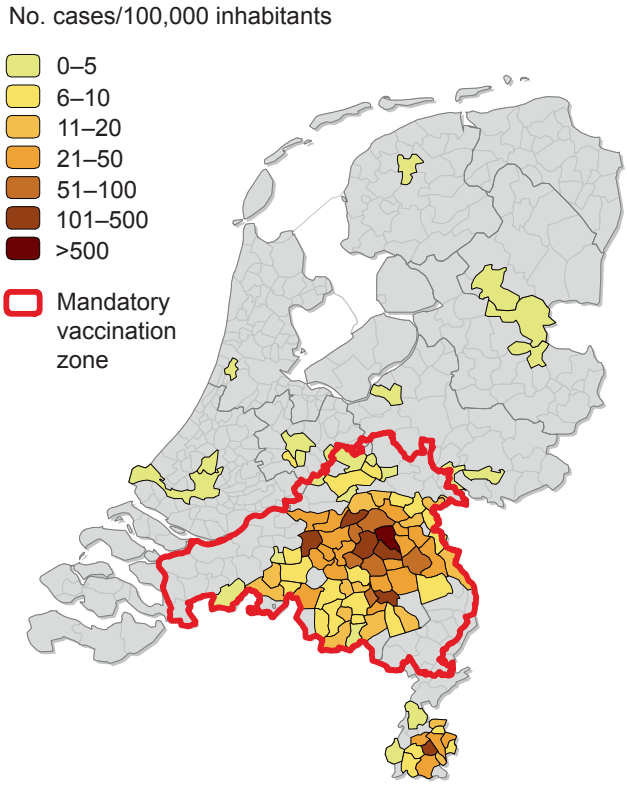

Figure 8.1. Density of 1,133 reported cases of acute Q fever in humans per municipality, the Netherlands, January 1-June 10, 2009. Area outlined in red is where vaccination of dairy goats and sheep was mandatory in 2009 (Noord Brabant Province and parts of adjacent provinces). Data were obtained from the National Institute for Public Health and the Environment, Statistics Netherlands, the Food and Consumer Product Safety Authority, and the Ministry of Agriculture, Nature and Food Quality.

Additional control measures implemented in the fall of 2009 were a bulk milk test every two weeks to detect *C. burnetii*–infected herds and to monitor *C. burnetii*–negative herds, movement and breeding bans for dairy goats or sheep, and culling of all pregnant dairy goats or sheep on infected farms. Health authorities considered a farm to be infected when two consecutive bulk milk samples were positive by PCR, as tested by two laboratories, including the national reference laboratory (Ministry of Agriculture, Nature and Food quality (MinLNV), 2010). Thus, culling

included pregnant goats in vaccinated herds and pregnant goats in unvaccinated herds located outside the vaccination zone. Culling was conducted from the end of December 2009 through May 2010.

Vaccine

The vaccine used was Coxevac® (Ceva Santé Animale, Libourne, France). This vaccine was not registered in the Netherlands at the time of the study, but authorities had issued a temporary exemption. The vaccine is a phase I vaccine containing inactivated *C. burnetii* strain Nine Mile (Committee for Medicinal Products for Veterinary Use, 2010). It was recommended that uninfected animals be vaccinated twice over a one-month interval before pregnancy. Although efficacy in dairy goats was not shown, the expected effects in vaccinated animals were reduced infection, abortion, and bacterial shedding if animals were infected after vaccination (Arricau-Bouvery et al., 2005; Guatteo et al., 2008; Rousset et al., 2009b).

Study design

For various reasons related to regulations of the national culling operation, unvaccinated dairy goats from five farms, vaccinated dairy goats from seven farms, and unvaccinated dairy sheep from one farm were included in this study. Farms were not randomly selected but were selected on the basis of convenience of culling date, vaccination status, and agreement of farmers to participate in the study. We sampled one hundred animals per farm, fifty pregnant and lactating animals (old animals), and fifty nulliparous animals (young animals). With this sample size, we expected to be able to detect a 20% difference in *C. burnetii* prevalence between vaccinated and unvaccinated animals and between old and young animals. We tested three types of samples: 1) uterine fluid, to detect animals with a high risk for shedding around parturition; 2) vaginal mucus, to be consistent with test results of other studies (Arricau-Bouvery et al., 2005; Guatteo et al., 2008; Rousset et al., 2009b); and 3) milk, because herds were monitored on the basis of results of bulk milk tests.

On the day before culling, animals were selected and marked on the farm by the study team; authorities identified pregnancies by using sonography. We selected pregnant animals that were closest to giving birth because it was expected that these animals had the highest number of *C. burnetii* in birth fluids, which would facilitate detection of infection (Sánchez et al., 2006). After animals were humanely killed on farms, marked animals were transported in a separate container to a rendering plant (Rendac BV, Son, the Netherlands), where they were unloaded onto a concrete floor and prepared for sampling.

Sampling

Uterine fluid was obtained by using a 9-mL monovette EDTA blood collection system (Sarstedt, Nümbrecht, Germany) and a Bovivet 2.10 mm × 60 mm needle

(Terumo Europe NV, Leuven, Belgium). Before obtaining uterine fluid, we made an incision in the linea alba cranial from the udder, moved part of the uterus to an extraabdominal position, and cleaned the uterus with alcohol-soaked cotton balls. We also cleaned the vulva with alcohol-soaked cotton balls and then obtained a swab sample from the vagina wall by using a dry and sterile cotton-tipped Cultiplast swab (LP Italiana SPA, Milan, Italy). These two samples were obtained from all selected animals. Additionally, from old animals we obtained a milk sample, which was collected into a 30-mL sterile tube. The teat was cleaned with alcohol-soaked cotton balls before sampling, and the first few streams of milk were discarded. All samples were frozen at −40°C within a few hours after sampling and were sent to the laboratory to be analyzed after the end of the culling period.

Diagnostic test

Quantitative real-time PCR was performed for all samples. Milk samples were analyzed at the Animal Health Service by using the Taqvet *Coxiella burnetii* TaqMan Quantitative PCR (Laboratoire Service International, Lissieu, France). Swabs and uterine samples were analyzed by the national reference laboratory by using an in-house real-time PCR specific for the *C. burnetii* insertion sequence 1111a gene (Klee et al., 2006). Results for the three sample types were given as positive, negative, or doubtful on the basis of cycle threshold (C_t) values, in which a value <36.01 was considered positive and a value >40 was considered negative. A negative result indicated that no specific signal was detected in a maximum of 40 cycles. Values between 36.01 and 40 were reported as doubtful on the basis of <100% reproducibility. For additional analysis, we considered all samples with C_t ≤40 as positive.

Statistical analyses

Vaccine efficacy was calculated for young and old animals separately for all 3 sample types according to the following equation: [% (positive test result, unvaccinated)- % (positive test result, vaccinated)] / [% (positive test result, unvaccinated)] (Dohoo et al., 2009)). This efficacy can be interpreted as the percentage of positive samples (C_t ≤40) prevented by vaccination in a vaccinated population.

Influence of vaccination and parity on test results of individual animals was examined by using logistic regression (Hosmer and Lemeshow, 2000) for the 3 sample types. We included vaccination status and parity group in the model as explanatory variables. Herd was included as a random factor to incorporate the fact that observations within a herd are dependent in the model. For uterine samples and vaginal swabs, we used the equation logit (fraction of positive test results) = parity (old) + vaccination status stratified by parity (young or old vaccinated) + random herd effect stratified by vaccination status (vaccinated or unvaccinated herds). For milk samples, we used the equation logit (fraction of

positive test results) = vaccination status (vaccinated) + random herd effect stratified by vaccination. Vaccine effect was quantified by calculating the odds ratio (OR).

For positive samples only, we tested whether vaccination had an effect on the relative amount of bacteria present in each sample type, as indicated by the C_t value. A C_t value closer to 0 indicates a higher bacterial concentration in the sample relative to a C_t value closer to 40. We performed survival analysis on samples with C_t values for which the C_t value at which a sample result becomes positive is considered the event. Hazard ratio (HR) indicates the rate at which samples from unvaccinated animals become positive compared with samples from vaccinated animals (Eisenberg et al., 2010). No correction for herd level was necessary and no correction for parity was possible because of the low number of bacterial shedders per group. For each of the three samples types, we used the equation C_t value (of positive samples only) = vaccination status (vaccinated).

Kaplan-Meier curves were plotted to show bacterial load in samples from old vaccinated, young vaccinated, old unvaccinated, and young unvaccinated animals. Statistical analyses were performed by using R software (R Development Core Team, 2009). For logistic regression, the function glmer() in lme4 in R software (Bates and Maechler, 2010) was used. For survival analysis, the functions Surv() and coxph() in Survival in R software (Thernau et al., 2009) were used. The model fit of all models was assessed by using the likelihood ratio test.

Results

Background information for individual farms

Information for each farm is shown in Table 8.1. Three farms (B, F, and K) did not have a history of animals with Q fever before the end of 2009 when their bulk milk PCR results changed from negative to positive during the monitoring period, which suggested a recent infection. Abortion caused by Q fever had been diagnosed in 2008 on sheep farm X. On all other farms, > 1 bulk milk ELISA or PCR results were positive for *C. burnetii* in 2008 or 2009. Animals in vaccinated herds were supposedly vaccinated twice in 2009, with the exception of farm M, where the first vaccination was given after abortions had occurred.

Effect of vaccination on bacterial shedding

Crude test results are summarized in Table 8.2. The percentage of *C. burnetii*–positive animals on each farm is shown in Figure 8.2. For vaccinated animals, 0.43% of uterine samples, 30% of vaginal swabs, and 4% of milk samples were positive (C_t < 36.01). For unvaccinated animals, 26% of uterine samples, 76% of vaginal swabs, and 33% of milk samples were positive. Prevalences within vaccinated herds and unvaccinated herds varied substantially (Table 8.2).

Table 8.1. Characteristics of goat and sheep farms sampled for *Coxiella burnetii*, the Netherlands, January-April 2010.*

Farm	No. animals culled	No. live animals	Vaccination period	Bulk milk sample PCR result and date of change, 2010[$]
Unvaccinated goats				
A	550	178	NA	+
B	102	530	NA	Mar
F	53	938	NA	Mar
K	121	649	NA	Feb
L	324	367	NA	+
Unvaccinated sheep				
X	128	378	NA	Jan
Vaccinated goats				
H	365	673	2009 Aug-Dec	Jan
M	719	3,557	2009 Dec-2010 Jan	+
P	625	1,750	2009 Sep-Dec	+
Q	685	281	2009 Aug-Oct	+
R	3,595	0	2009 Sept-Oct	+
S	180	358	2009 Oct	+
T	1,081	83	2009 Apr-Sep	+

*Data from Animal Health Service and the Food and Consumer Product Safety Authority. Animals were vaccinated with Coxevac (CEVA Santé Animale, Libourne, France). No. live animals is the number of non-pregnant animals remaining after culling. NA, not applicable; +, positive.
$Shown are farms that had a positive PCR result at the start of the culling period(+) and those for which a PCR result changed from negative to positive during the culling period (date).

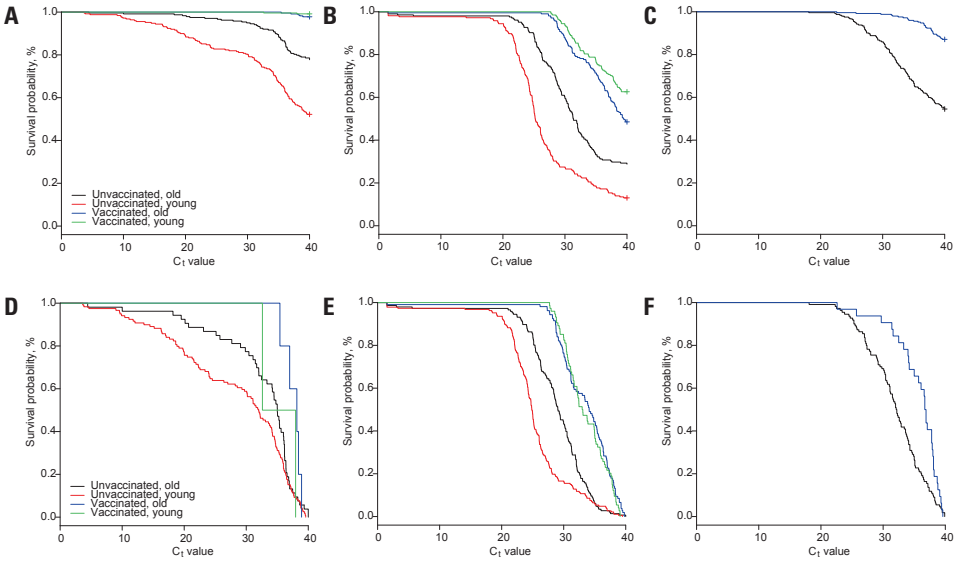

Figure 8.2. Kaplan-Meier curves for cycle threshold (C_t) values of all samples (A,C,E) and for samples with positive and doubtful results for *Coxiella burnetii* (C_t <40) (B,D,F), the Netherlands, January 1–June 10, 2009. A, B) Uterine fluid; C, D) vaginal mucus; E, F) milk. Old, pregnant and lactating; young, nulliparous.

Table 8.2. Quantitative PCR results and prevalence for samples positive for *Coxiella burnetii* for 957 animals in 13 small ruminant herds, the Netherlands, January-April 2010.

		Uterine fluid				Vaginal mucus				Milk			
Farm	Group	No.	Pos	D	% (95% CI)	No.	Pos	D	% (95% CI)	No.	P	D	% (95% CI)
Unvaccinated goats													
A	Young	46	0	0	0 (0-6)	0	0	0	NA	0	0	0	NA
	Old	47	0	2	0 (0-6)	0	0	0	NA	52	8	2	15 (6-25)
B	Young	74	35	16	47 (36-59)	76	75	0	99 (96-100)	0	0	0	NA
	Old	26	10	2	39 (20-57)	26	26	0	100	26	17	8	65 (47-84)
F	Young	49	35	4	71 (59-84)	53	53	0	98 (95-100)	0	0	0	NA
	Old	0	0	0	NA	0	0	0	NA	0	0	0	NA
K	Young	26	17	5	65 (47-84)	32	32	0	100	0	0	0	NA
	Old	28	12	3	43 (25-61)	39	39	0	100	34	33	0	97 (91-100)
L	Young	37	0	0	0 (0-8)	37	2	9	5 (0-13)	0	0	0	NA
	Old	58	0	0	0 (0-5)	58	1	3	2 (0-5)	51	2	3	4 (0-9)
Unvaccinated sheep													
X	Young	17	5	2	29 (8-51)	17	17	0	100	0	0	0	NA
	Old	79	11	13	14 (6-22)	82	76	1	93 (87-98)	79	19	18	24 (15-34)
Vaccinated goats													
H	Young	48	1	0	2 (0-6)	49	1	5	2 (0-6)	0	0	0	NA
	Old	50	0	0	0 (0-6)	50	6	11	12 (3-21)	37	0	0	0 (0-8)
M	Young	50	0	1	0 (0-6)	49	46	2	94 (87-100)	0	0	0	NA
	Old	47	1	3	2 (0-6)	48	47	0	98 (94-100)	47	5	12	11 (2-20)
P	Young	0	0	0	NA	0	0	0	NA	0	0	0	NA
	Old	0	0	0	NA	30	12	9	40 (23-58)	30	1	0	3 (0-10)
Q	Young	49	0	0	0 (0-6)	50	2	8	4 (0-9)	0	0	0	NA
	Old	49	0	1	0 (0-6)	50	2	12	4 (0-9)	50	0	2	0 (0-6)
R	Young	0	0	0	NA	0	0	0	NA	0	0	0	NA
	Old	10	0	0	0 (0-26)	0	0	0	NA	10	0	0	0 (0-26)
S	Young	46	0	0	0 (0-6)	50	4	6	8 (0-16)	0	0	0	NA
	Old	25	0	0	0 (0-11)	28	2	5	7 (0-17)	28	1	5	4 (0-10)
T	Young	49	0	0	0 (0-6)	0	0	0	NA	0	0	0	NA
	Old	47	0	0	0 (0-6)	0	0	0	NA	46	3	3	7 (0-14)

No., no. tested; pos, no. with positive result; D, no. with doubtful result; %, prevalence; CI, confidence interval; young, nulliparous; NA, not applicable; old, pregnant and lactating.

Vaccine efficacy for uterine sample results was 98% for young animals and 90% for old animals. Vaginal sample vaccine efficacy was much lower (57% and 28%) for young and old animals, respectively. Vaccine efficacy for milk sample test results was 72% (Table 8.3). All logistic regression model fits and survival model fits were better than those of null models according to likelihood ratio tests.

For vaccinated animals, uterine samples from young animals were 0.5% as likely to be positive for *C. burnetii* (OR 0.005, 95% CI 0.0002–0.1200), and uterine samples

from old animals were 3.2% as likely to be positive (OR 0.032, 95% CI 0.002–0.580) than samples from unvaccinated young animals. For unvaccinated animals, old animals were 44% as likely to be positive than young animals (OR 0.44, 95% CI 0.25–0.78) (Table 8.4). Results from the vaginal swabs were comparable; vaccinated young animals were 1.5% as likely to be positive for *C. burnetii* than unvaccinated young animals (OR 0.015, 95% CI 0.0006–0.3500). Milk from vaccinated old animals was 4% as likely to be positive for *C. burnetii* than milk from unvaccinated old animals (OR 0.04, 95% CI 0.003–0.380) (Table 8.5).

Effect of vaccination on C_t value

In uterine fluid, vaccinated animals had an HR that was half that of unvaccinated animals (HR 0.49, 95% CI 0.34–0.70), which indicated that unvaccinated *C. burnetii*–

Table 8.3. Efficacy of vaccination against *Coxiella burnetii* for 957 animals in 13 small ruminant herds, the Netherlands, January-April 2010.

	Uterine fluid				Vaginal mucus				Milk			
Group	No.	Pos	D	E, %	No.	Pos	D	E, %	No.	Pos	D	E, %
Unvaccinated												
Young	249	92	27	NA	215	178	9	NA	NA	NA	NA	NA
Old	238	33	20	NA	205	142	4	NA	242	79	31	NA
Subtotal	487	125	47	NA	420	320	13	NA	242	79	31	NA
Vaccinated												
Young	241	1	1	98	198	53	21	57	NA	NA	NA	NA
Old	228	1	4	90	206	69	37	28	248	10	22	72
Subtotal	470	2	5	NA	404	122	58	NA	248	10	22	NA
Total	957	127	52	NA	824	442	71	NA	490	89	53	NA

No., no. tested; pos, no. with positive result; D, no. doubtful; E, vaccine efficacy; young, nulliparous; NA, not applicable; old, pregnant and lactating.

Table 8.4. Multivariate logistic regression of prevalence of *Coxiella burnetii* in culled animals from 13 small ruminant herds, the Netherlands, January-April 2010.*

	Uterine fluid		Vaginal mucus	
Group	OR (95% CI)	p value	OR (95% CI)	p value
Unvaccinated				
Young	1	NA	1	NA
Old	0.44 (0.25-0.78)	<0.05	0.22 (0.08-0.64)	<0.05
Vaccinated				
Young	0.005 (0.0002-0.12)	<0.05	0.015 (0.0006-0.35)	<0.05
Old	0.03 (0.002-0.58)	<0.05	0.13 (0.006-3.01)	0.2

*A random herd effect was included. OR, odds ratio; CI, confidence interval; young, nulliparous; NA, not applicable; old, pregnant and lactating

positive animals had higher relative amounts of bacteria on the basis of C_t value. This effect was similar for vaginal mucus (HR 0.34, 95% CI 0.28–0.42) and milk (HR 0.54, 95% CI 0.39–0.75) (Table 8.6).

Table 8.5. Univariate logistic regression of prevalence of *Coxiella burnetii* in milk samples from culled animals in 13 small ruminant herds, the Netherlands, January-April 2010.*

Group	OR (95% CI)	p value
Old, unvaccinated	1	NA
Old, vaccinated	0.04 (0.003-0.38)	<0.05

*A random herd effect was included. CI, confidence interval; NA, not applicable; old, pregnant and lactating; OR, odds ratio.

C_t values for uterine fluid and vaginal mucus were lowest for *C. burnetii*–positive, unvaccinated young animals, which suggested that they had the highest relative amount of bacteria (Figure 8.2). C_t values were similar in bacteria-positive vaccinated animals, regardless of parity group, which indicated lower but similar shedding levels in all vaccinated animals. For milk samples, C_t values were lower for unvaccinated animals than for vaccinated animals.

Table 8.6. Univariate survival analysis of PCR C_t values for *Coxiella burnetii* in positive and doubtful samples from culled animals in 13 small ruminant herds, the Netherlands, January-April 2010.

	Uterine fluid		Vaginal mucus		Milk	
Group	HR (95% CI)	p value	HR (95% CI)	p value	HR (95% CI)	p value
Unvaccinated	1	NA	1	NA	1	NA
Vaccinated	0.49 (0.39-0.70)	<0.05	0.34 (0.28-0.42)	<0.05	0.54 (0.39-0.75)	<0.05

C_t, cycle threshold; HR, hazard ratio; CI, confidence interval; NA, not applicable

Discussion

This study showed that vaccination of dairy goats against Q fever with Coxevac® reduced the percentage of animals in which bacteria were detected and bacterial load in uterine fluid, vaginal swabs, and milk. Reduced prevalence was most prominent in uterine fluid and in young animals. Because shedding of bacteria may be quantitatively highest during parturition, abortion, and subsequent periods, these results suggest that vaccination may reduce environmental contamination, thereby contributing to reduction of risk for human exposure and associated human cases of Q fever.

Our findings are consistent with those of other studies. In a clinical trial of cattle, Guatteo et al. (2008) demonstrated that vaccine was effective in reducing the

probability of becoming a bacterial shedder when given to uninfected animals before pregnancy. Arricau-Bouvery et al. (2005) showed that vaccination of 17 goats in a clinical trial decreased excretion of *C. burnetii*. Rousset et al. (2009b) conducted a field study of a goat herd infected with *C. burnetii* and found that vaccination did not prevent shedding but did reduce bacterial load in vaginal swabs of primiparous animals.

Although these studies provided useful data on the effect of vaccination, these data were based on a limited number of observations. The advantages of our study were that it was based on a larger number of field samples (957 animals from thirteen herds) obtained from animals vaccinated under field conditions and that it tested uterine fluid, which is likely to be a good proxy for shedding at the time of kidding. A disadvantage of our study was its observational nature, in which vaccination was not conducted randomly at the herd, animal, parity, or infection levels, as would have been conducted in a clinical trial.

In unvaccinated herds *C. burnetii* was detected more often in uterine fluid of young animals than in old animals. However, no parity difference was observed for vaccinated herds. Rousset et al. (2009b) observed a reduced bacterial load in vaginal swabs in primiparous goats only. We also observed that the bacterial load was most reduced in young vaccinated animals. However, vaccinated young and old animals had similar bacterial loads in uterine fluid and vaginal mucus (Figure 8.2). Our results suggest that vaccination is more protective in nulliparous animals than in parous animals. Further investigations are required to determine whether the association between vaccination and bacterial shedding depends on vaccination before a first or subsequent pregnancy or on vaccination before or after natural exposure, and to elucidate underlying mechanisms.

As reported by Guatteo et al. (2008), the time of vaccination before or during breeding may affect its effectiveness. In our study, whether all animals had been vaccinated before breeding was not known. On one farm, all animals were vaccinated after breeding, and most vaccinated animals with a positive test result for *C. burnetii* came from this farm. When we excluded this farm from the analyses, we observed a stronger effect of vaccination, which indicated that the effect of vaccination could have been underestimated.

However, the efficacy of vaccination may also have been overestimated. With exception of the dairy sheep farm, all unvaccinated herds with a high prevalence of *C. burnetii*–positive uterine samples had no known history of Q fever until milk PCR results became positive during the culling period. This result suggested a recent introduction of the infectious agent. In other unvaccinated herds that had only a few positive uterine samples, *C. burnetii* was circulating before the culling period. All vaccinated herds appeared to have histories of *C. burnetii* infection.

This factor makes it difficult to conclude whether absence of positive uterine samples in vaccinated herds was caused by vaccination or was a combined effect of vaccination and an immune response after natural infection.

Another study limitation is that the stage of pregnancy can affect the amount of *C. burnetii*; bacterial load in secreta may increase sharply during the last stage of pregnancy (Sánchez et al., 2006). Although we attempted to select animals that were closest to giving birth, not all animals sampled were in the same stage of pregnancy, and the average duration of pregnancy may have differed from farm to farm. Because data about gestation stage were lacking, we did not include this factor in our analyses.

Goats and sheep in the Netherlands were vaccinated to reduce the number of human cases of Q fever. However, other countries use a different strategy. In Australia, persons at risk are vaccinated against Q fever (Gidding et al., 2009). In France, cattle are vaccinated to prevent economic losses caused by abortions (Courcoul et al., 2010). No substantial numbers of human cases of Q fever have been reported in these countries (EFSA, 2010). The effect of vaccination in the Netherlands on reduction of human exposure could not be quantified. However, the low number (≈350) of human cases in 2010 compared with those in 2009 (National Institute for Public Health and the Environment, 2010) suggests a beneficial effect of intervention measures. The relationship between bacterial shedding, environmental contamination, and human cases needs further investigation.

Our results showed that in uterine fluid, vaginal mucus, and milk, *C. burnetii* prevalence and load were reduced in vaccinated animals in the Netherlands. These effects were most pronounced in young primiparous animals. We can reasonably assume that vaccination under field conditions contributed to reduction of shedding of *C. burnetii* by dairy goats and dairy sheep, which in turn may contribute to reduction of the risk for human exposure.

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Chapter 9

Seroepidemiological survey for *Coxiella burnetii* antibodies and associated risk factors in Dutch livestock veterinarians

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Abstract

Since 2007, Q fever has become a major public health problem in the Netherlands and goats were the most likely source of the human outbreaks in 2007, 2008 and 2009. Little was known about the consequences of these outbreaks for those professional care providers directly involved. The aim of this survey was to estimate the seroprevalence of antibodies against *C. burnetii* among Dutch livestock veterinarians and to determine possible risk factors. Single blood samples from 189 veterinarians, including veterinary students in their final year, were collected at a veterinary conference and a questionnaire was filled in by each participant. The blood samples were screened for IgG antibodies against phase I and phase II antigen of *C. burnetii* using an indirect immunofluorescent assay, and for IgM antibodies using an ELISA. Antibodies against *C. burnetii* were detected in 123 (65.1%) out of 189 veterinarians. Independent risk factors associated with seropositivity were number of hours with animal contact per week, number of years graduated as veterinarian, rural or sub urban living area, being a practicing veterinarian, and occupational contact with swine. Livestock veterinarians should be aware of this risk to acquire an infection with *C. burnetii*. Physicians should consider potential infection with *C. burnetii* when treating occupational risk groups, bearing in mind that the burden of disease among veterinarians remains uncertain. Vaccination of occupational risk groups should be debated.

Introduction

Q fever is a zoonotic disease caused by the obligate intracellular bacterium, *Coxiella burnetii*, and ruminants are considered to be the primary source of infection for humans. In cattle, the disease is mainly asymptomatic (Arricau-Bouvery and Rodolakis, 2005), but in sheep and goats the main symptom is abortion, stillbirth and retention of foetal membranes (Zeman et al., 1989; Damoser et al., 1993; Maurin and Raoult, 1999; Hatchette et al., 2001; Wouda and Dercksen, 2007). The bacterium is shed in urine, milk, faeces and birth products of infected animals. The main route of transmission of the bacterium to humans is by aerosols (Marrie, 1990a; Maurin and Raoult, 1999; Schimmer et al., 2009).

Until 2007, about 20 Q fever cases were reported in the Netherlands annually (Van Steenbergen et al., 2007). In that year, Q fever became a major public health problem in the Netherlands with 168, 1,000 and 2,357 human cases notified in 2007, 2008 and 2009, respectively (van der Hoek et al., 2010a). These unprecedented annual outbreaks are largely explained by exposure of the general population living in the surroundings of infected dairy goat farms to airborne contaminated dust particles. Only 5% of the notified Q fever patients in the Netherlands report an occupation in agriculture, transporting or handling animal products, or animal care (Dijkstra et al., 2012). However, since its first description in abattoir workers in Australia in 1935 (Derrick, 1937), Q fever has been considered primarily an occupational zoonotic disease for abattoir workers, sheep shearers, livestock farmers, and especially veterinarians because of their direct contact with potentially infected animals (Marrie and Fraser, 1985; Richardus et al., 1987; Valencia et al., 2000; Abe et al., 2001; Monno et al., 2009; Whitney et al., 2009; Chang et al., 2010).

The aim of this survey was to estimate the seroprevalence of antibodies against *C. burnetii* among Dutch livestock veterinarians and to determine possible risk factors.

Materials and Methods

Human population and data collection

In November 2009, professional laboratory assistants collected a single blood sample from Dutch livestock veterinarians and final-year veterinary students attending a veterinary conference.

Each participant filled in a self-administered questionnaire to obtain epidemiological and clinical information. The questionnaire existed of three parts, and took approximately fifteen minutes to complete. The first part focused on demographic data and included age, gender, and residence in urban, sub urban or rural area. The second part consisted of occupation-related questions regarding work location, type of veterinary occupation, years in veterinary practice, contact with livestock

and livestock farms, contact with animal related products as straw, hay, soil, birth products and urine and faeces, contact with aborted animals, use of personnel protective equipment, work related wounds and accidental vaccine exposure. The third part consisted of non-occupation related questions regarding possession of animals in the last five years, consumption of raw dairy products, outdoor activities and health conditions, including smoking, tick bites during the last five years and a known history of a clinical Q fever infection, pregnancy and abortion.

This study was approved by the Medical Ethical Committee of the University Medical Centre Utrecht, Utrecht, the Netherlands (reference number 09–322). All participants received a book to express appreciation for their cooperation.

Laboratory methods

A serum sample from each participant was tested for the presence of IgG antibodies against *C. burnetii* using a Q fever indirect immunofluorescent assay (IFA; Focus Diagnostics, Cypress, CA), according to the manufacturer's protocol. Sera were screened for phase I and phase II IgG using a cut-off of 1:32. Samples with both IgG phase I and II titres of ≥1:32 were considered to be positive, while solitary IgG phase II samples were scored positive if they had a single titre of ≥1:512.

All samples were also screened for IgM using an ELISA (Focus Diagnostics), according to the manufacturer's protocol, and positive samples were confirmed with IFA. Samples with a titre of ≥1:32, both for IgM phase I and II, were considered to be positive, indicating a possibly recent infection.

Within the group of participants with a past infection, a distinction was made between serological profiles considered not likely to be compatible with a chronic infection, and serological profiles which could indicate a chronic infection. Serum samples from participants with a possibly chronic Q fever infection, having an IgG phase I titre ≥1:1024, were additionally analysed by performing a *C. burnetii* PCR.

Statistical data analysis

All individual laboratory results were merged with the self-administered questionnaires. Statistical analysis was carried out using STATA 11. The Chi square test and the two-sided proportion-test were used to estimate univariate associations between exposures and seropositivity. Analyses were carried out to calculate odds ratio's with 95% confidence intervals. The odds ratio (OR) was defined, in this context as the odds of a given exposure among veterinarians seropositive for *C. burnetii* divided by the odds of exposure among seronegative veterinarians. Veterinarians who did not completely fill in the questionnaire were excluded for the analysis of that particular question.

For the multivariable logistic regression, initially all variables with (2-sided) $p < 0.20$ and with sufficient numbers (> 10) were selected. To avoid multicollinearity, from groups of variables that had a correlation of more than 0.50 with each other, only one, the most plausible biological variable, was left in the multivariable analysis.

Stepwise backward logistic regression was carried out, starting with all data and excluding stepwise each variable that had a p-value of > 0.05. All remaining variables were considered to be risk or protective factors.

Results

Descriptive results

A total of 189 participants, being more than 90% of the attendants, completed the questionnaire and provided a blood sample during the conference. The median age of the participants was 44 years (interquartile range, 34–52 years). Of the participants, 130 (68.8%) were male and 59 (31.2%) were female (Table 9.1). One hundred and twelve of the participants worked as a livestock practitioner, 20 were non-practicing, 37 worked as livestock veterinarian at a veterinary institute (Utrecht University (UU) or Animal Health Service (GD)) and 20 were livestock veterinary students in their final year. A total of 108 (57.1%) of the participants had contact with livestock for more than 50% of working hours in their current job.

The overall seroprevalence was 65.1% (n = 189). In livestock veterinarians the seroprevalence was 69.2% (n = 169). The seroprevalence in livestock veterinary students was 30.0% (n = 20). Among the group of 169 livestock veterinarians the seroprevalence was 87.5% in practicing livestock veterinarians (n = 112), 45.0% in non-practicing livestock veterinarians (n = 20) and 27.0% in livestock veterinarians working at a veterinary institute (n = 37). IgG antibody titers against *C. burnetii* measured for both phase I and II ranged from 1:32 to 1:2048. Seven out of nine participants with a positive IgM ELISA result were confirmed with IFA, suggesting a recent infection. Four of those seven IFA positive study participants were livestock veterinary students. The other three were practicing livestock veterinarians. Seven participants with an IgG phase I titre ≥1:1024, a possible indication of a chronic Q fever infection, were followed up by performing a *C. burnetii* PCR on a blood sample, and in all cases PCR results were negative. Additionally, participants with an IgG phase I titre ≥1:512 are offered to participate in a follow-up study and are advised to be controlled for risk factors of a chronic Q fever infection.

Univariable analysis

Participants who were seropositive were likely to be male over the age of 32 years (Table 9.1). Participants living in rural or suburban areas were significantly more often seropositive than participants living in an urban area. Occupational risk

Table 9.1. Results of univariable analysis of risk factors for presence of antibodies against *Coxiella burnetii*.

	Participants							
	Seropositive[#]		Seronegative					
	No.	%	No.	%	Odds Ratio	95% confidence interval		P
Gender								
Female	24	40.7	35	59.3	1.0	.	.	.
Male	99	76.2	31	23.8	4.7	2.3	9.4	<0.001
Age								
≤32 year	19	40.4	28	59.6	1.0	.		.
33-44 year	35	71.4	14	28.6	3.7	1.6	8.6	0.003
45-52 year	37	75.5	12	24.5	4.5	1.9	10.9	0.001
53-65 year	32	72.7	12	27.3	3.9	1.6	9.5	0.002
Living region								
Urban	8	30.8	18	69.2	1.0	.	.	.
Sub-urban	21	56.8	16	43.2	3.0	1.0	8.5	0.037
Rural	94	74.6	32	25.4	6.6	2.6	16.7	<0.001
Veterinarian (years)								
Veterinarian (≤ 2)	13	27.7	34	72.3	1.0			
Veterinarian (3 - 13)	36	70.6	15	29.4	6.3	2.6	15.1	<0.001
Veterinarian (14 - 21)	33	75.0	11	25.0	7.9	3.1	20.0	<0.001
Veterinarian (≥ 22)	40	87.0	6	13.0	17.4	6.00	50.8	<0.001
Animal contact (hours/week)								
<10 hours	9	24.3	28	75.7	1.0			
10-19 hours	25	55.6	20	44.4	3.9	1.5	10.1	0.005
20-29 hours	42	80.8	10	19.2	13.1	4.7	36.2	<0.001
≥ 30 hours	43	89.6	5	10.4	26.8	8.1	88.2	<0.001
Work category								
Others	23	30.7	52	69.3	1.0			
Practicing	100	87.7	14	12.3	16.2	7.7	34.0	<0.001
Contact with cows								
No	11	31.4	24	68.6	1.0	.	.	.
Yes	112	72.7	42	27.3	5.8	2.6	12.9	<0.001
Contact with swine								
No	80	61.5	50	38.5	1.0	.		.
Yes	43	72.9	16	27.1	1.7	0.9	3.3	0.131
Contact with birth products of ruminants								
No	16	33.3	32	66.7	1.0	.	.	.
Yes	107	75.9	34	24.1	6.3	3.1	12.9	<0.001
Contact with birth products of pets								
No	101	61.2	64	38.8	1.0	.	.	.
Yes	22	91.7	2	8.3	7.0	1.5	31.9	0.004
Practice on cow farm with abortion								
No	32	43.8	41	56.2	1.0	.	.	.
Yes	91	78.4	25	21.6	4.7	2.4	9.3	<0.001

Sera were screened for phase I and phase II IgG using a cut-off of 1:32. Samples with both IgG phase I and II ≥1:32 were considered to be positive, while solitary IgG phase II samples were scored positive if they had a single titre of ≥1:512 (Focus Diagnostics, Cypress, CA).

factors in univariable analysis were: graduated as a veterinarian more than two years ago; more than 10 hours of animal contact per week; practicing as livestock veterinarian; and working with cattle, horses, dogs and cats. Participants with frequent contact with animal products, like straw, hay, roughage, raw milk, birth products of ruminants as well as of pets, urine of ruminants, practicing on cattle farms with abortion, and one or more contacts on farms with abortion problems in the last five years, were significantly more often seropositive. Accidental needle injections and cutting incidents were also found to be associated with seropositivity. Non-occupational activities like cycling and shopping were associated with seronegativity. In contrast, gardening and having dogs and (pet) birds were found to be associated with seropositivity. Consumption of dairy products, health conditions like smoking behaviour, and not wearing protective clothes during work were not found to be a significant univariate risk factor. The number of participants primarily working with sheep and goats, with a history of a clinical Q fever infection, or with pregnancy and abortion was too small for statistical analysis.

Multivariable analysis

Variables with a p-value < 0.20 in the univariable analysis were used as input for the multivariable analysis. The number of years as a veterinarian was highly correlated with age and gender; the latter two were left out of the analysis. Working category and contacts with ruminants were very highly correlated to contact with hay/straw, roughage, raw milk, birth products of ruminants and with urine of ruminants; the latter 5 were left out of the analysis.

Table 9.2. Final multivariable model for risk factors associated with presence of antibodies against *Coxiella burnetii* in 189 veterinarians.

Variable	Category	No.	OR	[95% CI]		P
Animal contacts	< 10 hours	37	1.0			
(hours/week)	10-19 hours	45	12.0	2.5	57.1	0.002
	20-29 hours	52	1.2	0.2	7.6	0.869
	≥ 30 hours	48	16.0	1.8	141.8	0.013
Veterinarian (years)	≤ 2	47	1.0			
	3-13	51	17.5	4.0	77.4	<0.001
	14-21	44	26.5	4.8	145.9	<0.001
	≥ 22	46	58.1	10.3	328.0	<0.001
Living region	Urban	26	1.0			
	Sub-urban	37	11.9	2.1	68.5	0.005
	Rural	126	17.9	3.6	88.1	<0.001
Work category	Others	75	1.0			
	Practicing	114	15.8	2.9	87.2	0.002
Contact with swine	No	130	1.0			
	Yes	59	3.4	1.1	10.2	0.029

In this group of livestock veterinarians, risk factors for *C. burnetii* seropositivity in the multivariable analysis (Table 9.2) were: number of hours with animal contact per week, number of years graduated as veterinarian, living in a rural (OR, 17.9 (95% CI: 3.6–88.1)) or semi urban area (OR, 11.9 (95% CI: 2.1–68.5)), working as practicing livestock veterinarian (OR, 15.8 (95% CI: 2.9–87.2)), and occupational contact with swine (OR, 3.4 (95% CI: 1.1–10.2)).

Discussion

In this cross-sectional study, an overall *C. burnetii* seroprevalence of 65.1% among Dutch livestock veterinarians was found. The number of hours with animal contact per week, the number of years the participants were graduated and practicing as a veterinarian, were the main independent risk factors in this study. These risk factors suggest a high dose-effect relation for seropositivity in Dutch livestock veterinarians. In 1984, 84% of 222 Dutch livestock veterinarians were seropositive for IgG antibodies against *C. burnetii* (Richardus et al., 1987). The use of a different laboratory test and cut-offs, differences in study population and different infection rates of livestock over time could be possible explanations for other seroprevalence estimates.

Dutch livestock veterinarians have a high risk of getting *C. burnetii* seropositive because of intensive contact with potentially infected livestock, and the immune system can be boosted frequently because of a high prevalence in Dutch livestock (Muskens et al., 2007; Van den Brom et al., 2012a). Contact with swine was found to be an independent risk factor, but the group of veterinarians involved was also exposed to cattle. Further, the main geographical areas where pigs are kept in the Netherlands corresponds with the high-incidence areas where the human Q fever epidemic related to dairy goats was situated and where high seroprevalences were found in the rural population. On the other hand, treatment of swine has previously been described as a risk factor for seropositivity for veterinarians (Whitney et al., 2009). The natural susceptibility of swine to *C. burnetii* was demonstrated during a Q fever epidemic in Uruguay. A seroprevalence of 21.4% was measured in 391 healthy slaughter pigs (Somma-Moreira et al., 1987). No information about Q fever prevalences in swine in the Netherlands is available.

In this survey, 20 veterinary students participated, and the seroprevalence was 30%. In a survey in Spain, a seroprevalence of 11% among veterinary students was found. First course students showed a significant lower seroprevalence. Multiple risk factors were associated with *C. burnetii*: study course, contact with live animals especially ruminants and contact with persons working with animals (Valencia et al., 2000). A large serological survey (n = 674) was already carried out in the Netherlands in 2006. At that time 18.7% of the veterinary students were seropositive. Students in their final year with the livestock study direction had a seroprevalence of 37.3%. The main risk factors were a study direction focusing on

large animals, advanced year of study, having had a zoonosis during study and having ever lived on a farm with ruminants (De Rooij et al., 2012). To detect possible recent exposure to *C. burnetii*, testing was also performed by ELISA IgM, and it is not remarkable that four out of seven possible recent infections occurred in veterinary livestock students, indicating this group is susceptible for the infection during the practical rotations during their study. The lower prevalence in veterinary students, an indication for recent infection in seven of whom four were students, and the main risk factors we found, are another indication for a high dose-effect relation for seropositivity.

Our study clearly indicates that livestock veterinarians are an occupational risk group. The prevalence found in this study was much higher than described in several international sero-epidemical studies among livestock veterinarians (Marrie and Fraser, 1985; Valencia et al., 2000; Abe et al., 2001; Ergonul et al., 2006; Dorko et al., 2008; Whitney et al., 2009; Chang et al., 2010), with the exception of a small survey among 12 veterinarians in southern Italy, which revealed a seroprevalence of 100% (Monno et al., 2009). In other studies, contact with livestock is described as an important risk factor for seropositivity (Dorko et al., 2008; Whitney et al., 2009; Chang et al., 2010), and exposure to goats was the most important risk factor associated with *C. burnetii* infection in Southern Taiwan (Chang et al., 2010). Treatment of cattle, swine or wildlife were main risk factors associated with *C. burnetii* seropositivity in US veterinarians (Whitney et al., 2009). In Slovakia and Nova Scotia, professional orientation and regular contact with farm animals and pets (Dorko et al., 2008), and exposure to sheep placentas (Marrie and Fraser, 1985) were described as important risk factors, respectively. In contrast, in Japan, no significant correlation was found between years of occupational experience and *C. burnetii* seropositivity (Abe et al., 2001).

The final independent risk factor was living in a rural or sub-urban area. Participants living in these areas were significantly more often seropositive than participants living in an urban area. Rural and sub-urban living areas have been described before as a risk factor (Lyytikäinen et al., 1998; Stein and Raoult, 1999; Gardon et al., 2001; Nebreda et al., 20010; Karagiannis et al., 2009), although urban outbreaks also have been described, but could mostly be related to exposure to animals or animal products (Langley et al., 1988; Oren et al., 2005; Schimmer et al., 2010). In the Netherlands, livestock farms are mainly situated in rural or sub-urban areas. The knowledge that ruminants are the main reservoir for *C. burnetii* (Arricau-Bouvery and Rodolakis, 2005; Raoult et al., 2005) and the fact that *C. burnetii* can easily be spread by aerosols (Marrie, 1990a; Maurin and Raoult, 1999; Schimmer et al., 2009), presumably explains why living in rural or sub-urban area is a risk factor for seropositivity.

In the univariable analysis, age and gender were risk factors for seropositivity. Nevertheless, both were left out of the multivariable analysis because they were highly correlated with the number of years participants were graduated as veterinarian. The higher incidence in males than in females has been reported in several sero-epidemical studies among veterinarians, but without a clear explanation (Marrie and Fraser, 1985; Richardus et al., 1987; Valencia et al., 2000; Whitney et al., 2009). Also a Spanish study among veterinary students revealed that male students in the fifth study year had a significantly higher risk to be seropositive than female students (Valencia et al., 2000). A higher clinical incidence in males and persons aged between 40–60 years in the Dutch population has been described during the Q fever outbreaks between 2007–2010 (Dijkstra et al., 2012). Age above 46 years, was also previously described as a risk factor for seropositivity in veterinarians (Whitney et al., 2009).

To differentiate in the group of practicing veterinarians, all analyses were repeated in the multivariable analysis for the subset of practicing veterinarians only, mainly working with cattle, swine and poultry, or individual housed animals. The analysis on the subset of practicing veterinarians did not result in additional significant results (data are not shown), and was less robust than the multivariable analysis based on the full data set.

In conclusion, Dutch livestock veterinarians are an occupational risk group with increased risk for *C. burnetii* infection presumably because of their direct contact with infected livestock. Dutch livestock veterinarians should be aware of this risk and be extra alert regarding symptoms of Q fever. Most of the infections are not notified, as they remain asymptomatic or result in only mild flu-like symptoms. Serious infections leading to pneumonia or hepatitis may occur. A *C. burnetii* infection can cause serious complications during pregnancy and in those with underlying disease, therefore these groups should be monitored properly. Vaccination of occupational groups at risk is common in Australia (Marmion, 2007; Gidding et al., 2009). In the Netherlands, vaccination has been made available in the first half of 2011, but only for specific risk groups, as those patients with heart valve and vascular disorders. During the community Q fever outbreaks between 2007 and 2009 in the Netherlands, few patients reported occupational exposure (Dijkstra et al., 2012). Most veterinarians are not eligible for vaccination because the presence of antibodies is an absolute contraindication for administering the currently available Australian vaccine. However, vaccination could be considered for seronegative veterinary students at the beginning of their study (Gidding et al., 2009). Routine serological follow-up is useful as well as basic safety rules, like hygiene measures and the use of protection clothes (Valencia et al., 2000; Dorko et al, 2008; Henning et al., 2009; Whitney et al., 2009), although in this study disregard of protective measures was not found to be an independent risk factor. Occupational exposure to several zoonotic diseases makes basic safety rules useful for protecting the livestock veterinarian.

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Author contributions

Revised the manuscript: BS PV. Read and approved the final manuscript: RV BS PS WS Wvdh PV. Conceived and designed the experiments: RV BS WvdH PV. Performed the experiments: PS. Analyzed the data: WS RV BS. Wrote the paper: RV.

Chapter 10

Coxiella burnetii infections in sheep and goats: an opinionated review

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Abstract

Q fever is an almost ubiquitous zoonosis caused by *Coxiella burnetii*, which is able to infect several animal species, as well as humans. Cattle, sheep and goats are the primary animal reservoirs. In small ruminants, infections are mostly without clinical symptoms, however, abortions and stillbirths can occur, mainly during late pregnancy. Shedding of *C. burnetii* occurs in faeces, milk and, mostly, in placental membranes and birth fluids. During parturition of infected small ruminants, bacteria from birth products become aerosolised. Transmission to humans mainly happens through inhalation of contaminated aerosols. In the last decade, there have been several, sometimes large, human Q fever outbreaks related to sheep and goats. In this review, we describe *C. burnetii* infections in sheep and goats, including both advantages and disadvantages of available laboratory techniques, as pathology, different serological tests, PCR and culture to detect *C. burnetii*. Moreover, worldwide prevalences of *C. burnetii* in small ruminants are described, as well as possibilities for treatment and prevention. Prevention of shedding and subsequent environmental contamination by vaccination of sheep and goats with a phase I vaccine are possible. In addition, compulsory surveillance of *C. burnetii* in small ruminant farms raises awareness and hygiene measures in farms help to decrease exposure of people to the organism. Finally, this review challenges how to contain an infection of *C. burnetii* in small ruminants, bearing in mind possible consequences for the human population and probable interference of veterinary strategies, human risk perception and political considerations.

Introduction

Q fever is an almost ubiquitous zoonosis caused by *Coxiella burnetii*. This organism infects several animal species, as well as humans (Babudieri and Moscovici, 1952; Arricau-Bouvery and Rodolakis, 2005). Domestic ruminants are the primary animal reservoir of *C. burnetii*, but infections are also found in rodents, birds and arthropods (Babudieri and Moscovici, 1952). In addition to ruminants, cats and dogs are also able to shed the organism and are able to infect humans (Marrie et al., 1988; Buhariwalla et al., 1996). The main symptom in infected sheep and goats is abortion during late pregnancy. Infected animals can shed the organism in faeces, milk and, mostly, in placental membranes and birth fluids (Maurin and Raoult, 1999; Arricau-Bouvery et al., 2003; Wouda and Dercksen, 2007). Placental membranes may contain up to 10^9 hamster infective doses of *C. burnetii* per gram of tissue (Babudieri, 1959). During parturition, billions of bacteria are excreted in birth products of infected small ruminants; then, as well as afterwards, after drying, bacteria can easily be aerosolised and infect humans by inhalation (Marrie, 1990a; Maurin and Raoult, 1999; Schimmer et al., 2010).

Q fever was first described in 1933 as a febrile illness in abattoir workers in Brisbane, Australia, (Derrick, 1937). Later, it was demonstrated to be caused by *Rickettsia burneti* (Maurin and Raoult, 1999), later renamed as *C. burneti* (Philip, 1948) and finally *C. burnetii*. The existence of *C. burnetii* has been described worldwide, except New Zealand (Woldehiwet, 2004). Nowadays, Q fever is an endemic, often occupational, disease occurring in many countries, although also epidemic patterns with rural outbreaks affecting large numbers of non-occupational related people have been described (Vellema and Van den Brom, 2014).

Q fever is an emerging zoonosis and several human Q fever outbreaks have been related to sheep and goats. This review describes *C. burnetii* infections in sheep and goats, as well as small ruminant related human Q fever outbreaks. Finally, it discusses how to contain *C. burnetii* abortion outbreaks in small ruminant farms, to prevent shedding of *C. burnetii* and in that way environmental contamination, trying to prevent humans to acquire Q fever.

Coxiella burnetii

C. burnetii is able to affect humans and several animal species. The causal agent is a highly osmotic resistant, Gram-negative and obligate intracellular bacterium. The genus *Coxiella* is classified, based on gene-sequence analysis, in the order of *Legionellales*, family *Coxiellaceae*, together with *Rickettsiella* and *Aquicella* (Seshadri et al., 2003). *Coxiella burnetii* can exist in a virulent phase I and an avirulent phase II. Antigenic variation between these phases is based on a change from smooth to rough lipopolysaccharide (LPS). The smooth LPS of *C. burnetii* in

phase I disturbs an effective immune response, giving the phase I bacterium the opportunity to survive and multiply in the host. Therefore, in phase I *C. burnetii* is highly infectious. The less virulent form, phase II, has still not been isolated from the host (Babudieri, 1959), but can only be seen after culturing in non immunocompetent cell cultures or hen eggs (Arricau-Bouvery and Rodolakis, 2005). The relevance of this second phase of the bacterium in natural infections is questionable.

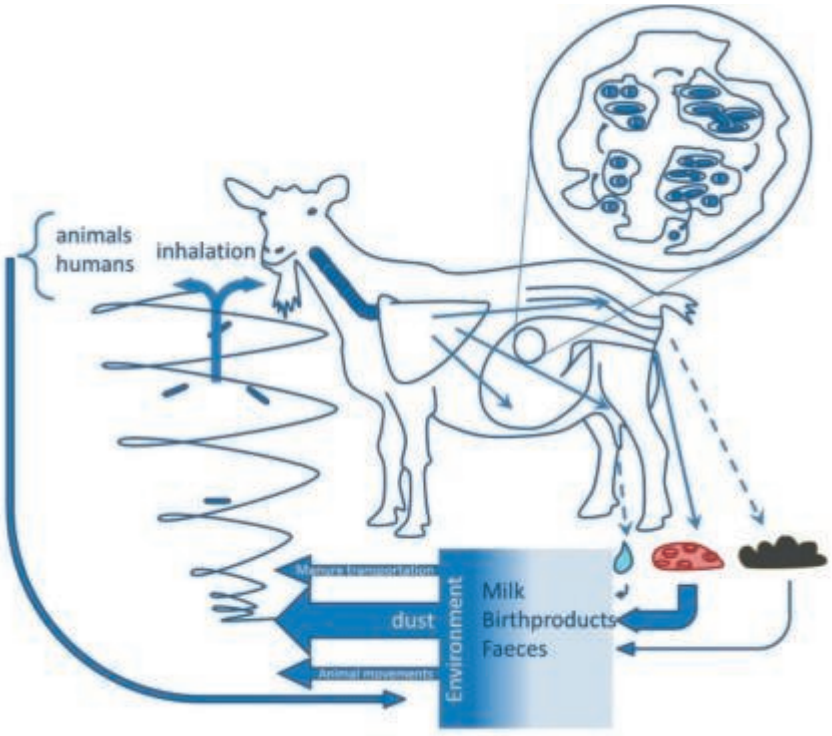

Figure 10.1. Infection routes of *C. burnetii* and its replication cycle in the host.

Domestic ruminants are the primary animal reservoir of *C. burnetii*. Replication occurs in the host and after entering an eucaryotic host cell, in the phagosome the phase I small-cell variants transfers into large-cell variants. This large-cell variant of the organism multiplies and persists. The small-cell variants and small dense cells are released by the cell. Within the host, the organism is present in the various host cells in different forms, although trophoblast cells of the allantochorion are the main target cells of *C. burnetii*. Shedding of the organism occurs mainly in birth products, but also in milk and faeces. Dust particles contaminated with *C. burnetii* are the main route of infection for animals and humans. In addition to shedding products from domestic ruminants, other animals can be a source of infection with *C. burnetii*.

Morphologically, three different forms of the organism can be distinguished: large-cell variants (LCV), small-cell variants (SCV) and small dense cells (SDC). These forms differ with regard to morphologic and antigenic composition and physical and chemical resistance (Heinzen et al., 1999). After entering an eucaryotic host cell, in the phagosome the phase I small-cell variants transfer into large-cell variants. These large-cell variants of the bacterium multiply and persist. The small-cell variants and small dense cells are released by the cell (Figure 10.1). They are considered as the persistent forms in the host and there is evidence that they are the resistant manifestations of the organism in the environment (Arricau-Bouvery and Rodolakis, 2005).

Pathogenesis, clinical features and epidemiology of *Coxiella burnetii* infections in small ruminants

Knowledge regarding pathogenesis of Q fever in small ruminants can help to understand the course of the disease and its epidemiology. However, only a few studies have been published that provide information about the pathogenesis in sheep and goats (Lennette et al., 1952; Martinov et al., 1989a; Sanchez et al., 2006; Roest et al., 2012). After inoculation of pregnant goats with *C. burnetii*, the trophoblast cells of the allantochorion are primary target cells for the organism. Although it is unknown how trophoblasts become initially infected, gradually more trophoblasts become infected and the allantochorion shows increasingly more severe signs of inflammation. In contrast, in the trophoblasts covering the cotyledonary villi, which are involved in the exchange of gasses and nutrients, *C. burnetii* was not detected (Roest et al., 2012); this could explain the absence of premature foetal death. Instead, foetuses may either die shortly before or during the inflammation induced abortion, or may be born alive. This pattern differs from findings in caprine brucellosis and chlamydiosis, in which alterations in the foetoplacental binding lead to foetal death culminating to abortion (Anderson et al., 1986a; Anderson et al., 1986b; Buxton et al., 1990; Rodolakis et al., 1998).

The immune response to *C. burnetii* infection is also better studied in goats than in sheep (Roest et al., 2013b), but results from work in goats could also be applicable in sheep. After inoculation, *C. burnetii* phase II specific antibodies, both IgM and IgG, can be detected after two weeks and remain increased for up to 13 weeks post-infection. Antibodies directed against *C. burnetii* phase I increase as well, but about four weeks later compared to the phase II antibodies (Roest et al., 2013b). Duration of antibody response is not exactly determined. However, it can be concluded from field studies that the immune response can last for several months up to years. The role of cellular immunity after infection is not clear (Roest et al., 2013b).

Even though *C. burnetii* can infect many animal species, infections remain usually asymptomatic. In small ruminants during the acute phase, presence of the organism

can be demonstrated in blood, lungs, spleen and liver (Maurin and Raoult, 1999). It is not clear if presence of *C. burnetii* in organs other than the placenta affects the function of those organs, since for sheep and goats only mild lesions have been described (Lennette et al., 1952; Sanchez et al., 2006; Roest et al., 2012). In non-pregnant animals, *C. burnetii* infection is asymptomatic. In dairy goat herds that experience abortions caused by *C. burnetii,* increased incidence of metritis can be present. Birth of kids with suboptimal bodyweight and increased mortality rate have also been reported. Rearing of seemingly healthy kids can be complicated by respiratory and digestive tract disorders (Wouda and Dercksen, 2007). The most important clinical presentations of infection with *C. burnetii* in pregnant small ruminants are abortion and stillbirth.

Abortion occurs most frequently with no preceding clinical symptoms, at the end of pregnancy, (Arricau-Bouvery and Rodolakis, 2005). Increased abortion rates as a consequence of infection with *C. burnetii* are rare, however, they have been described for goat herds, where up to 90% of pregnant animals aborted (Palmer et al., 1983; Sanford et al., 1994; Hatchette et al., 2003; Arricau-Bouvery and Rodolakis, 2005; Wouda and Dercksen, 2007). In the season that follows an abortion storm, reproductive problems are often less prominent (Berri et al., 2007; Wouda and Dercksen, 2007; Van den Brom and Vellema, 2009). Not all infections of pregnant sheep and goats result in abortion (Martinov et al., 1989a; Welsh et al., 1958). In line with this, in several human Q fever outbreaks related to sheep, no ovine abortion was observed (Porten et al., 2006; Gilsdorf et al., 2008). After experimental infection, some goats aborted, whilst others gave birth to healthy and liveborn kids, with no differences in *C. burnetii* excretion between such animals (Roest et al., 2012).

In abortion associated with *C. burnetii* infection, foetuses appear to be normal, are mainly fresh, although occasionally autolytic. Macroscopically, placentitis can be present, usually characterised by presence of a purulent yellow-brownish exudate, covering mainly the severely thickened inter-cotyledonary areas. Microscopically in almost all cases, the trophoblasts of the inter-cotyledonary allantochorion and the base of the cotyledonary villi are affected. The severity of inflammation varies from mild mononuclear infiltration to severe necrosis and purulent exudation. Chorionic epithelial cells, especially at the base of the cotyledonary villi, often have a foamy vacuolated cytoplasm and contain basophilic intra-cytoplasmatic granulation, findings highly suggestive of *C. burnetii* infection (Van den Brom et al., 2012b). At histopathological examination, the liver of some foetuses may show mild granulomatous hepatitis, usually with no abnormalities present in other organs (Wouda and Dercksen, 2007; Roest et al., 2012; Van den Brom et al., 2012b). Parturitions of *C. burnetii* infected pregnant small ruminants are accompanied by massive excretion of bacteria and shedding into the environment. This is the most important excretion route, which by far exceeds others. Bacteria are also excreted

with milk (Roest et al., 2012; Van den Brom et al., 2012a), but excretion via faeces and vaginal mucus is questionable, as possible contamination from the environment cannot be ruled out (Welsh et al., 1958; Roest et al., 2012) (Figure 10.1).

Inhalation of aerosolised *C. burnetii* bacteria is the most probable route of introduction of the organism in a farm (Welsh et al., 1958; Berri et al., 2005a; Roest et al., 2012). Depending on factors as immune status of the animals, flock/herd size and virulence of *C. burnetii*, eventually, introduction of *C. burnetii* in a farm leads to spreading of the infection within the flock/herd and, possibly, results in abortion storms, as has occurred in the Netherlands between 2005 and 2009 (Wouda and Dercksen, 2007; Van den Brom and Vellema, 2009; Roest et al., 2011a).

Diagnostics

Post mortem examinations

At post-mortem examination, sections of tissues can be used for immunohistochemical analysis using antisera against *C. burnetii*. Immunohistochemical procedures can be performed according to the DAKO EnVision + System (DAKO Corporation, California, USA) (Wouda and Dercksen, 2007; Van den Brom et al., 2012b).

Serological examination

Demonstration of presence of antibodies directed against *C. burnetii* is possible with several serological tests, e.g. microagglutination (MAT), complement fixation test (CFT), immunofluorescence assay (IFA) and enzyme-linked immunosorbent assay (ELISA) (Table 10.1). Microagglutination and immunofluorescence assay are currently less frequently used, with immunofluorescence assay not being nowadays commercially available for small ruminants.

ELISAs are mainly performed to detect IgG antibodies. Antibodies against *C. burnetii* phase I and phase II can be discriminated, depending on the antigen used (Kovácová et al., 1998). Most commercially available tests detect both types of antibodies, although the ratio between both types may differ between the various tests (Horigan et al., 2011), this is also being influenced by the antigen extraction procedure (Lang, 1988). Some in-house-made or commercially available ELISAs can be used for specifically detecting antibodies directed against one of both phases. After infection, phase I antibodies can be demonstrated for a longer period than phase II antibodies. IgM ELISAs are currently not commercially available (Kittelberger et al., 2009). Since the 1980's, ELISAs are the most frequently used tests. Compared to immunofluorescence assay, ELISAs have a sensitivity for small ruminants of 82% to 100% and a specificity of 93% to 96% (Jaspers et al., 1994). Complement fixation test and ELISAs are widely used for detecting antibodies against *C. burnetii* (Van den Brom et al., 2013a). Both tests have been reported to be highly specific, with ELISAs being

more sensitive than complement fixation test (Rousset et al., 2007; 2009a; Kittelberger et al., 2009; Ruiz-Fons et al., 2010; Horrigan et al., 2011; Natale et al., 2012) and comparable to immunofluorescence assay (Rousset et al., 2007). However, it has also been found that ELISA tests show a negative result in an increasing proportion of samples detected positive by means of complement fixation test. It has been suggested that this was caused by the presence of IgM (Emery et al., 2012). In practice, ELISAs are widely used in serum samples from individual animals, used for seroepidemiological studies (Kovácová and Kazár, 2000). Antibody ELISA for samples collected from a farm's bulk tank milk has been developed for goats in Norway (Kampen et al., 2012), for sheep in Spain (Ruiz-Fons et al., 2011) and for both sheep and goats in the Netherlands (Van den Brom et al., 2012a) and have been shown to be useful.

Table 10.1. Veterinary diagnosis of *Coxiella burnetii* infection.

Materials	Method	Advantages	Drawbacks
Humoral immunity			
Blood serum, plasma	Indirect immunofluorescence assay		Not commercially available for small ruminants
	Complement fixation test	Detects both acute and chronic infection	Insensitive
	Enzyme-linked immunosorbent assay	Widely used; commercially available; cost efficient	May miss the acute phase
Milk	Enzyme-linked immunosorbent assay	Cost efficient Applicable to bulk tank milk	Mainly IgG1
Cellular immunity			
Live animal	Skin test	Sensitive; inexpensive	Labourous; poorly validated
Whole blood	Interferon-γ test	Sensitive	Requires fresh whole blood
Pathogen detection			
Infected tissue (placenta)	Immunohistochemistry	Specific	Possibly not very sensitive
Infected material (placenta, milk, manure, foetus, uterus)	PCR	Sensitive	No distinction between viable and non-viable organisms; contaminations
	Inoculation into guinea pigs or embryonated eggs		Expensive, zoonotic risks, use of laboratory animals; insensitive
	In vitro culture		Expensive; zoonotic risk; insensitive

PCR

Several PCR techniques, including conventional PCR, real-time PCR (RT-PCR), multiplex PCR and nested PCR, can be used to demonstrate presence of *C. burnetii* DNA in samples of different origin. These samples include clinical specimens (e.g., tissues from animals after post-mortem examination, birth products, vaginal swabs, faeces, blood serum, milk), processed foods of animal origin (e.g., cheese,

yoghurt, pasteurised milk) or environmental samples (e.g., soil, dust, air samples) (Table 10.1). PCR tests can target different regions of the bacterial genome. In general, PCR tests based on multicopy genes like IS1111 are more sensitive than those based on single copy genes. Real-time PCR tests using multicopy genes, can only roughly quantify the amount of *C. burnetii* DNA in samples since the number of IS1111 elements can vary from 7 to 110 between different strains (Klee et al., 2006). A disadvantage of PCR techniques can be the lack of differentiation between viable and non-viable bacteria, although, recently, a PCR methodology has been published for *C. burnetii* that circumvents this issue (Mori et al., 2013). Due to the wide distribution of DNA of *C. burnetii* in the environment and the ease that samples can be contaminated during collection, it is important to take into account the relative amount of DNA that is detected, before conclusions would be drawn. For use in not advanced laboratory settings, loop mediated isothermal amplification (LAMP) assays have been developed with reported reliable results (Pan et al, 2013).

Culture and typing

Successful isolation and cultivation of *C. burnetii* in laboratory animals, embryonated eggs and cell culture was already achieved in the first studies on the Q fever agent (Cox, 1938; Davis and Cox, 1938; Dyer, 1938; Parker and Davis, 1938; Burnet and Freeman, 1983). These techniques are still used nowadays (Table 10.1). Inoculation of mice can be used for isolation of *C. burnetii* from samples with low bacterial load and/or when tissues are probably contaminated with other bacteria. This is an advantage of using live animals. Culturing in eggs and cell cultures should preferably be done without antibiotics for an optimal growth of *C. burnetii*. To avoid bacterial contamination, filtering is necessary, but this reduces the sensitivity of the test.

In embryonated eggs, *C. burnetii* grows almost exclusively in the yolk sac endoderm cells. A disadvantage of this culture system is that growth of *C. burnetii* cannot be monitored by visual inspection. Compared to other techniques, the cell culture system has several advantages, therefore the cell culture system is currently the most widely used *in vitro* system to isolate and cultivate *C. burnetii*, and several cell lines can be used (Voth and Heinzen, 2007). A recent major improvement in the methods of isolation and cultivation of *C. burnetii* is the introduction of a host cell free medium, which mimics the composition of the acidic environment in which *C. burnetii* replicates (Omsland et al., 2009; Omsland, 2012). 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*, and probably in future developments of vaccine production.

The genetic heterogeneity of *C. burnetii* can be assessed with a number of molecular techniques. Multiple locus variable number tandem repeats analysis (MLVA), multispacer sequence typing (MST) and single nucleotide polymorphism (SNP)

typing are most widely used. An important characteristic for typing systems is discriminatory power, i.e. the ability to distinguish between unrelated strains. The published MLVA typing method for *C. burnetii* is assumed to be more discriminatory than MST (Svraka et al., 2006; Chimielewski et al., 2009). In the Dutch Q fever outbreak, MLVA and MST were used to confirm the epidemiological link between dairy goats and humans (Roest et al., 2011b; Tilburg et al., 2012a). This was the first time that typing techniques were used to identify the source of a human Q fever outbreak. An overview of published genotyping techniques for *C. burnetii* is given in Table 10.2 (Roest et al., 2013a).

Table 10.2. Overview of techniques available for genotyping *C. burnetii.*

Abbreviation	Full name	Principle	Year*	Reference
RFLP typing	restriction fragment length poly-morphism typing	Analysis of fragments after digestion with specific restriction enzymes	1990	Heinzen et al. (1990); Jager et al. (1998)
Com1 typing	Com1 encoding genes sequencing	Sequence analysis of the Com1 encoding genes	1997	Zhang et al. (1997)
Com1/MucZ typing	Com1 and MucZ encoding genes sequencing	Sequence analysis of the Com1 and MucZ encoding genes	1999	Sekeyova et al. (1999)
MST	multispacer sequence typing	DNA sequence variation in short intergenic regions in the genome	2005	Glazunova et al. (2005)
MLVA	multiple locus variable number tandem repeats analysis	Variation in repeat number in tandemly repeated DNA elements on multiple loci in the genome	2006	Arricau-Bouvery et al., (2006); Svraka et al. (2006)
IS1111 typing	IS1111 repetitive element PCR-based differentiation typing	Identification of different IS1111 insertion elements	2007	Denison et al. (2007)
RAPD	randomly amplified polymorphic DNA	Analysis of randomly amplified DNA fragments of the genome	2009	Sidi-Boumedine et al. (2009)
SNP typing	single nucleotide polymorphism typing	Differentiating a single nucleotide difference on a locus in the genome by probes	2011	Huijsmans et al. (2011)

* Year of first description of the technique for *C. burnetii.*

Prevalence of *C. burnetii* in different body fluids and tissues from sheep or goats

Prevalences of *C. burnetii* in sheep or goats have been described based on the analysis of different body fluids and tissues. Seroprevalences have been described for many countries (Table 10.3), but these data are difficult to compare, because of the differences in methodology of the study, composition of the study populations and use of different tests on each occasion (Guatteo et al., 2011; Van den Brom et al., 2013a). In most surveys, the prevalences in goats are significantly higher than in sheep, indicating goats being more sensitive to *C. burnetii* infection.

Table 10.3. Prevalence of antibodies against *C. burnetii* in blood serum in published studies in small ruminants around the world.

Country	Prevalence % (n)		Test employed	Reference
	animal based	farm based		
Sheep				
Canada	24 (34)	ns	IFA	Hatchette et al. (2002)
	41 (334)	89 (46)	CFT	Dolcé et al. (2003)
Chad	11 (142)	43 (28)	ELISA	Schelling et al. (2003)
Croatia	11 (182)	ns	CFT	Morovic et al. (2008)
	5 (920)	ns	CFT	Morovic et al (2008)
Cyprus	19 (481)	ns	IFA	Psaroulaki et al. (2006)
Greece	10 (554)	100 (ns)	IFA	Pape et al. (2009)
Iran	20 (1,100)	100 (ns)	ELISA	Asadi et al. (2013)
Italy	9 (7,194)	38 (675)	ELISA	Masala et al. (2004)
Kenya	18 (159)	ns	ELISA	Knobel et al. (2013)
Netherlands	2 (12,052)	15 (1,208)	ELISA	Van den Brom et al. (2013a)
Norway	0 (590)	0 (118)	ELISA	Kampen et al. (2012)
Portugal	9 (ns)	38 (24)	ELISA	Anastacio et al. (2013)
Spain	9 (1,001)	68 (34)	ELISA	Garcia-Perez et al. (2009)
	12 (1,379)	74 (46)	ELISA	Ruiz-Fons et al. (2010)
Turkey	20 (743)	81 (42)	ELISA	Kennerman et al. (2010)
United Kingdom	12 (1,022)	62 (58)	IFA	McCaughey et al. (2010)
USA	17 (3,642)	ns	CFT/MA	McQuiston and Childs (2002)
Goats				
Canada	16 (64)	ns	IFA	Hatchette et al. (2002)
Chad	13 (134)	46 (28)	ELISA	Schelling et al. (2003)
Croatia	31 (276)	ns	CFT	Morovic et al. (2008)
Cyprus	48 (420)	ns	IFA	Psaroulaki et al. (2006)
Greece	7 (61)	ns	IFA	Pape et al. (2009)
Iran	66 (76)	100 (9)	ELISA	Khalili and Sakhaee (2009)
	27 (180)	100 (ns)	ELISA	Asadi et al. (2013)
Italy	13 (2,155)	47 (82)	ELISA	Masala et al. (2004)
Kenya	32 (378)	ns	ELISA	Knobel et al. (2013)
Netherlands	21 (2,828)	47 (123)	ELISA	Schimmer et al. (2011)
	8 (3,134)	18 (442)	ELISA	Van den Brom et al. (2013a)
Poland	0 (918)	0 (48)	ELISA	Czopowicz et al. (2010)
Portugal	10 (ns)	29 (52)	ELISA	Anastacio et al. (2013)
Spain	9 (115)	45 (11)	ELISA	Ruiz-Fons et al. (2010)
United Kingdom	9 (54)	43 (7)	IFA	McCaughey et al. (2010)
USA	42 (2,624)	ns	CFT/MA	McQuiston and Childs (2002)

CFT: complement fixation test; ELISA: enzyme-linked immunosorbent assay; IFA: immunofluorescence assay; MA: microagglutination.
ns: not specified.

Table 10.4. Prevalence of *C. burnetii* in bulk milk tank in published studies in small ruminants around the world.

Country	Prevalence % (n)		Reference
	by employing PCR	by employing ELISA	
Sheep			
Iran	0 (110)	np	Rahimi et al. (2010)
	6 (140)	np	Rahimi et al. (2011)
Netherlands	0 (16)	19 (16)	Van den Brom et al. (2012a)
Switzerland	0 (81)	np	Fretz et al. (2007)
Spain	22 (154)	np	Garcia-Perez et al. (2009)
	np	40 (154)	Ruiz-Fons et al. (2011)
Goats			
Iran	2 (56)	np	Rahimi et al. (2010)
Iran	5 (110)	np	Rahimi et al. (2011)
Netherlands	33 (292)	30 (292)	Van den Brom et al. (2012a)
Norway	np	0 (348)	Kampen et al. (2012)
Switzerland	0 (39)	ns	Fretz et al. (2007)

np: not performed

Table 10.5. Prevalence of *C. burnetii*-associated abortion in submissions of material from abortion cases in published studies in small ruminants around the world.

Country	Prevalence % (n)	Test employed	Reference
Sheep			
Germany	4 (1,153)	ns	Plagemann (1989)
Hungary	2 (246)	IHC	Szeredi et al. (2006)
Italy	11 (366)	PCR	Masala et al. (2007)
Netherlands	0 (98)	IHC	Van Engelen et al. (2014)
	2 (272)	IHC	Van den Brom et al. (2012b)
Switzerland	1 (86)	ns	Chanton-Greutmann et al. (2002)
USA	<1 (1,784)	ns	Kirkbride (1993)
Goats			
Hungary	1 (75)	IHC	Szeredi et al. (2006)
Italy	0 (31)	PCR	Masala et al. (2007)
Netherlands	0 (43)	IHC	Van Engelen, et al. (2014)
	10.6 (170)	IHC	Van den Brom et al. (2012b)
Switzerland	10 (144)	ns	Chanton-Greutmann et al. (2002)
USA	9 (211)	IHC	Moeller (2001)

IHC: immunohistochemical methods; PCR: polymerase-chain reaction.
ns: not specified.

Testing of bulk tank milk (BTM) samples for *C. burnetii* is relatively new in sheep and goats and has been described for only five countries (Table 10.4). Bulk tank milk samples can also be used for ELISA testing, thus measuring immunological flock/herd responses (Van den Brom et al., 2012a).

C. burnetii is diagnosed in abortion materials usually by histological examination and additional immunohistochemistry and less often by real-time PCR (Masala, et al., 2007; Wouda and Dercksen, 2007; Van den Brom et al., 2012a). Analysis of abortion materials have been described for several countries (Table 10.5). Prevalences of *C. burnetii* in abortion submissions are relatively small compared to the prevalences that are reported in some surveys (Table 10.3), although in Italy a relatively high number of *C. burnetii* abortions has been found in sheep by real-time PCR (Masala et al., 2007). In the Netherlands, since the start of the compulsory vaccination campaign of dairy sheep/goats in 2010, abortion caused by *C. burnetii* has not been diagnosed (Van Engelen et al., 2014); it is noteworthy that in previous years (2006-2011), *C. burnetii* was the main abortifacient agent in goats (Van den Brom et al., 2012b). This reduction in the number of confirmed *C. burnetii* abortions suggests that vaccination with a phase I vaccine is an effective measure to prevent abortion caused by *C. burnetii* in small ruminants.

Treatment, preventive measures and surveillance in small ruminants

Therapeutic and preventive measures in small ruminants are aimed to reduce abortion rates and bacterial shedding, thereby aiming to reduce environmental contamination. Specific precautionary measures should be taken when an animal is introduced into a *C. burnetii* free farm, in order to prevent introduction of infection (Arricau-Bouvery and Rodolakis, 2005). Recently, a detailed document regarding control of Q fever has been produced by the European Food Safety Authority (2010) and describes in detail all necessary steps and actions for effective control of the disease.

It is difficult to determine susceptibility of *C. burnetii* to antibiotics, because it is an obligate intracellular organism. In suspected cases of *C. burnetii* abortion in small ruminants, antibiotic treatments with two successive injections of oxytetracyclin (dose rate: 20 mg kg^{-1} bodyweight) during the last month of pregnancy has been recommended (Berri et al., 2002; Arricau-Bouvery and Rodolakis, 2005; Angelakis and Raoult, 2010), although, in sheep, no beneficial effect of the antibiotic treatment has been recorded in level or duration of bacterial shedding. Nevertheless, abortion rate decreased in the same study (Astobiza et al., 2013). Wouda and Dercksen (2007) expressed doubts whether antibiotics decreased abortion rates in goat herds in the Netherlands. Berri et al. (2005a) have suggested that antibiotic treatment resulted in a long-term effect in sheep at flock level, by preventing spread of the organism to unaffected animals in the same flock, a hypothesis that could not be confirmed in subsequent studies (Astobiza et al., 2013).

Prevention of abortion and shedding in small ruminants can be best established by vaccination. Q fever vaccines vary with regard to their preparation processes for the organism strain that they contain. Most effective vaccines are composed of *C. burnetii* in phase I stage (Arricau-Bouvery et al., 2005). Such currently available vaccines claim to preventing abortion and to contributing to reduction of shedding in vaginal charges, faeces and milk. Vaccination seems to be most effective when administered in non-infected small ruminants before their first pregnancy (Hogerwerf et al., 2011; Van den Brom et al., 2013b). In line with the situation in cows (Guatteo et al., 2008), vaccination of naturally infected small ruminants does not stop abortion, often shedding also continues, probably caused by persistent infection of the mammary gland and the uterus (Arricau-Bouvery and Rodolakis, 2005; Stuen and Longbottom, 2011).

Pasteurisation of milk from *C. burnetii* infected farms is recommended to prevent oral infection of humans, although this route is not a major infection route for humans (Arricau-Bouvery and Rodolakis, 2005).

General hygiene measures in farms aim to reduce exposure of people to *C. burnetii*. Since shedding during parturition is the main source of transmission, lambing/kidding in infected farms should take place under strict hygiene conditions. Placentas and foetuses should be collected, properly stored and destroyed. Occupationally exposed persons, e.g., farmers or veterinarians, should wear protective clothing, although it has been demonstrated that this does not completely prevent infections of humans (Whelan et al., 2011). Manure should be properly composted. Based on temperature measurements in a dunghill combined with data on the heat resistance of *C. burnetii*, it can be concluded that survival of *C. burnetii* in a well composted dunghill is unlikely. Other described options to reduce risk of manure are treatment of the manure with lime or calcium cyanide (0.4%) (Arricau-Bouvery et al., 2001). Spread of manure from infected farms on fields should not take place under windy circumstances (Arricau-Bouvery and Rodolakis, 2005). In specific cases, further reduction of environmental contamination can be established by appropriate tick and vermin control measures (Angelakis and Raoult, 2010). Pregnant women, young children, elderly people and immunocompromised individuals should avoid any contact with *C. burnetii* shedding animals and their unpasteurised products.

Surveillance of *C. burnetii* shedding on farms can be performed in several ways. Compulsory or voluntary investigation of aborted foetuses and placentas and/or stillborn lambs is a proper diagnostic tool, but depends on the awareness and willingness of farmers to submit these materials for necropsy. Additionally, shedding of small ruminants during normal parturitions can easily be missed, as not all *C. burnetii* infections in small ruminants result in abortion and/or stillbirth. Individual serology is sensitive, but gives no proper information on shedding. PCR testing of vaginal swabs sometimes can give information on shedding of individual animals, but

these samples can easily be contaminated in a contaminated environment (Roest et al., 2012). In dairy farms, bulk tank milk monitoring by using PCR has been described to be useful in detecting shedding of *C. burnetii* at farm level (Van den Brom et al., 2012a). In addition, detection and successful removal of individual shedders, resulted in *C. burnetii* negative PCR bulk tank milk samples (Van den Brom et al., 2013b). By using this technique, surveillance on shedding of *C. burnetii* and removal of chronically infected small ruminants is easier to establish in dairy farms.

In the Netherlands, a large human Q fever outbreak has led to implementation of a large number of preventive and control measures for small ruminant dairy farms. As a consequence of the precautionary principle, all pregnant sheep and goats were culled based on *C. burnetii* bulk tank milk PCR positive results (Vellema and Van den Brom, 2014), a measure that had not been described before. Implemented control measures and acquired protection reflected in an increase of seroprevalence to *C. burnetii* in the human population, most likely resulted in a control of these outbreaks (Dijkstra et al., 2012).

Human Q fever outbreaks related to small ruminants

The manifestation of Q fever in humans is highly variable, from a non-specific febrile illness to hepatitis, pneumonia and even long-standing infection. It has been suggested that inoculum size can affect expression of *C. burnetii* infection (Angelakis and Raoult, 2010). In this hypothesis, a low bacterial load could lead to subclinical infection or mild, influenza-like illness, while a high bacterial burden could be associated with a severe clinical expression in the acute phase, such as pneumonia. The median incubation period of acute Q fever has been reported to be 21 days in an outbreak situation, but this may also depend on the initial bacterial load (Porten et al., 2006).

Acute Q fever can develop into a long-standing disease in about 2% of patients (ECDC, 2010). This is a serious condition with high morbidity and mortality with endocarditis and vascular infections as main presentations. Case series, mainly from France, suggest very high risk for adverse pregnancy outcome as spontaneous abortion, intrauterine foetal death, premature delivery or decreased birth bodyweight after symptomatic and asymptomatic infection in early pregnancy (Langley et al. 2003; Carcopino et al. 2007). This has not been confirmed in community-based studies in the Netherlands and Denmark (van der Hoek et al. 2011a; Munster et al. 2013; Nielsen et al. 2013a). Apart from differences in the human study populations, also differences in the bacterial properties, could play a role, since the strains that were responsible for the outbreaks in France and the Netherlands differed with regard to plasmid sequences (Angelakis et al. 2013).

Transmission of *C. burnetii* from animals to humans mainly occurs by inhalation of aerosols (Benenson and Tigertt, 1956; Maurin and Raoult, 1999). The importance

of parturition of sheep or goats in the dissemination of *C. burnetii* from animal to humans has been demonstrated already in the 1950's (Abinanti et al., 1953). During parturition, when billions of organisms are aerosolised from the amniotic fluid of infected small ruminants, those nearby, e.g. farmers or veterinarians, will be exposed most. Fortunately, often these persons likely already are partially protected from previous (asymptomatic) infections (Schimmer et al., 2012a; Van den Brom et al., 2013c). Whether serologically naïve people in the wider environment would be exposed, depends upon the prevailing weather and environmental conditions during the parturition period (van der Hoek et al., 2011b).

People working with farm animals generally have a high seroprevalence of antibodies against *C. burnetii*, even in countries where acute Q fever notifications are rare, e.g. USA (Whitney et al, 2009), or where outbreaks of the disease have never been described, e.g. Denmark (Nielsen et al., 2013b).

Outbreaks of acute Q fever mostly occur among people who are not occupationally exposed, but who live close to *C. burnetii* shedding goats or sheep. This is the main explanation for the large seasonal outbreaks in the Netherlands in 2007, 2008 and 2009 (van der Hoek et al., 2012a). The average herd size of Dutch dairy goat farms was approximately 900 in 2008 (Van den Brom and Vellema, 2009). As a consequence, primarily infected dairy goat farms were able to shed large numbers of *C. burnetii* and subsequently caused massive environmental contamination. Nevertheless, the risk of acquiring human Q fever not only depends on farm size, but is also influenced by other factors, e.g. environmental conditions and seroprevalences among people and animals (Van der Hoek et al., 2011b; Dijkstra et al., 2012). People living within 2 km from a dairy goat farm that experienced *C. burnetii*-induced abortion waves, had a 30-times higher risk for acute Q fever than those living more than 5 km away (Schimmer et al., 2010). The majority of these people had never visited the farm. In other reported outbreaks, acute Q fever patients from the general population had been in close contact with infected small ruminants, e.g. in farming markets (Gilsdorf et al., 2008) or in farms during 'viewing days' (Whelan et al., 2012). Subsequently, it was demonstrated that, during the outbreaks in the Netherlands, infected sheep were a source of infection to humans only in case of direct contact with the sheep, but not for the surrounding population (van der Hoek et al., 2012b).

While dairy goat farms with *C. burnetii* induced abortions were clearly the major sources of infection for humans during the large outbreaks in the Netherlands, most outbreaks of Q fever in humans in other countries are related to sheep (Table 10.6). Of 29 human Q fever outbreaks reported in four countries in Europe (Bulgaria, France, Germany, the Netherlands) between 1982 and 2010, 17 were associated with sheep, whilst only three with goats, including the large outbreaks in the Netherlands (Georgiev et al., 2013). All these countries have many more

Table 10.6. Large (>50 confirmed cases) human Q fever outbreaks related to small ruminants in published studies around the world.

Country	Likely source	Total confirmed cases (n)	Reference
Bulgaria	sheep, goats	220	Panaiotov et al. (2009)
Canada	goats	66	Hatchette et al. (2001)
Croatia	sheep, goats	97	Morovic et al. (2008)
	sheep, goats	100	Morovic et al. (2008)
France	sheep	99	Tissot-Dupont et al. (2007)
Germany	sheep	167	Porten et al. (2006)
	sheep	160	Gilsdorf et al (2008)
Italy	sheep	133	Santoro et al. (2004)
Netherlands	dairy goats	168	National infectious diseases surveillance database 'Osiris'
	dairy goats	1,000	
	dairy goats	2,354	
Switzerland	sheep	415	Dupuis et al. (1987)

sheep than goats. However, goat-associated outbreaks in Canada, Bulgaria and the Netherlands followed increases in numbers of goats.

Concluding remarks

C. burnetii infections in small ruminants usually pass unnoticed, but may result in severe abortion outbreaks. During abortion or normal lambing, large numbers of *C. burnetii* are aerosolised, possibly exposing humans, in the surrounding up to some kilometres distance from the source, depending on climatic and environmental conditions. Dealing with a recently confirmed infection of *C. burnetii* in small ruminants, requires a tailor-made approach taking into account many factors, not only with regard to the small ruminant and human population but also to the local environment.

Diagnosing a recent infection of *C. burnetii* in small ruminants is not always easy. In case of abortion, we advise farmers to submit aborted fetuses and placental membranes to GD Animal Health where necropsy is performed according to standard procedures including gross examination of thoracic and abdominal organs and placental membranes. In case of an infection with *C. burnetii,* frequently an inter-cotyledonary thickening of the placenta with an exudate is found. Histopathological examination reveals diffuse suppurative inflammation in the chorion with many organisms in cytoplasmic vacuoles in the chorionic epithelial cells. Immunohistochemistry is a very specific mean to confirm *C. burnetii* as the cause of abortion, although sensitivity of immunohistochemistry may be too low to diagnose all infections. PCR type techniques on the other hand are very sensitive and specific, but should be linked to histological changes in order to mitigate

against false positive results (Borel et al., 2014). Nevertheless, not all *C. burnetii* infections in small ruminants result in abortion, while shedding of the organism can still be present. In case that infections in small ruminants do not result in abortions, there is no 'gold standard' for diagnosing the infection and no clear definition of infection. Serological testing does not provide information on shedding, PCR test results of vaginal swabs might be not specific enough (Roest et al., 2012) and should therefore only be used within a few days after parturition or abortion with a cut-off level of at least 10^4 bacteria mL^{-1} (Rodolakis, 2010). When sufficient quantities of *C. burnetii* are present in a sample, culture is possible and specific, but expensive and only possible to perform under bio safety level-3 conditions.

Epidemiological links between *C. burnetii* infected humans and possible sources should be confirmed by genotyping evidence. In the Dutch Q fever outbreak one predominant genotype of *C. burnetii* was present among dairy goats and dairy sheep and this genotype could also be identified in humans cases (Tilburg et al., 2012b). Genotyping also revealed that in cattle from different geographic areas a distinct cluster of genotypes was present (Tilburg et al., 2012c; Pearson et al., 2014). This genotype is hardly detected in human Q fever patients which may explain why cattle are not considered as a source for human Q fever outbreaks. Genotyping is useful in identifying differences in the genetic background of strains and will help to understand the epidemiology of Q fever.

Whether or not *C. burnetii* infections in small ruminants should be treated with antibiotics can be discussed. *In vitro*, *C. burnetii* seems sensitive to oxytetracyclin and application has been demonstrated to reduce abortion rates and shedding (Berri et al., 2005a), but in other studies no effect on reducing the number of abortions was found (Sanford et al., 1994; Wouda and Dercksen, 2007), and antibiotic treatment neither seems to prevent shedding of the organism nor to limit duration of bacterial excretion (Astobiza et al., 2013). Nowadays, where excessive antibiotic use in livestock is under discussion because of development of resistance, a doubtful therapy with antibiotics to prevent abortions caused by *C. burnetii* cannot be justified.

Vaccination of non-infected small ruminants before their first mating with a phase I vaccine (Coxevac®) leads to significant reduction of abortion rates and subsequently shedding of bacteria (Arricau-Bouvery et al., 2005; Hogerwerf et al., 2011). There are even indications that vaccination during pregnancy reduces shedding (Eibach et al., 2013), although this vaccine is not licenced for application in pregnant goats. Since the start of the compulsory vaccination campaign of all dairy sheep and dairy goats in the Netherland in 2010, no abortions caused by *C. burnetii* have been noticed anymore (van Engelen et al., 2014). Moreover, the number of bulk tank milk *C. burnetii* PCR positive farms decreased gradually since the start of the

compulsory monitoring in October 2009. In 2013, only bulk tank milk of some dairy goat farms were still *C. burnetii* PCR positive, possibly caused by intermittently shedding by a low number of animals that had been infected before their first vaccination (Van den Brom et al., 2013b). This may be due to a persistent infection of the mammary gland and uterus in small ruminants (Arricau-Bouvery and Rodolakis, 2005; Stuen and Longbottom, 2011). During the Dutch human Q fever outbreaks, small numbers of human patients could be related to contact with *C. burnetii* infected small ruminants (Koene et al., 2011; Whelan et al., 2011; 2012). Several studies have shown that living near a farm with a history of *C. burnetii* abortions was a risk factor for acquiring human Q fever (Schimmer et al., 2010) and prevention of human cases should mainly focus on hygiene measures in combination with prevention and reduction of shedding. After the start of the human Q fever outbreaks in the Netherlands in 2007, compulsory measures, like vaccination, hygiene measures, bulk tank milk monitoring, and, because of the precautionary principle, culling of pregnant animals on infected farms were implemented. Culling of pregnant animals as a measure to prevent human Q fever is under discussion, since environmental contamination is already established at that time (Stuen and Longbottom, 2011). Nevertheless, even when shedding has already started, further environmental contamination should be prevented, therefore, culling of pregnant non-vaccinated animals on a farm with confirmed *C. burnetii* induced abortion should be seriously considered, as a precautionary measure to prevent occurrence of human Q fever. Since 2009, there has been a sharp decline in the number of notified human Q fever cases in the Netherlands, most likely as a consequence of implemented control measures in combination with a rise in seroprevalence in the human population (Dijkstra et al., 2012). In December 2012, it was officially announced that the Dutch Q fever outbreak had ended; in 2013, numbers of recent human Q fever cases were found to be on a pre-outbreak level, although the number of tested persons still was much higher than before the start of the outbreak in 2007, due to an increased awareness among physicians.

Conflict of interest statement

None of the authors declares to have a conflict of interest which may arise from being an author of this review. There has been no financial support for this work that could have influenced its outcome.

Chapter 11

The rise and control of the 2007-2012 human Q fever outbreaks in the Netherlands

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Abstract

Q fever is an almost ubiquitous zoonosis caused by *Coxiella burnetii*, which is able to infect several animal species, as well as people. Cattle, sheep and goats are the primary animal reservoirs. In small ruminants, an infection may result in abortion and stillbirth. Infected animals can shed the organism in faeces, milk and mainly in foetal membranes and foetal fluids. Transmission to humans mainly occurs through the aerosol route.

Q fever was described as a febrile illness, which had started to occur in 1933 in abattoir workers in Brisbane, Australia. Since the first documented outbreaks, Q fever has been described in many countries all over the world, and in 1955 its existence was reported in 51 countries on five continents. In the Netherlands, Q fever was diagnosed for the first time in humans in 1956, and became a notifiable disease in 1978. Between 1978 and 2006, the average number of notifications per annum was seventeen. In 2007, the first year of what later turned out to be one of the largest recorded community outbreaks of Q fever, an outbreak occurred with 168 human patients notified, and in 2008 and 2009, 1,000 and 2,354 human Q fever patients were notified, respectively, and dairy goats were suspected to be the source.

In 2005, *C. burnetii* was diagnosed for the first time as a cause of abortion at two dairy goat farms in the Netherlands. In 2006, 2007, 2008, and 2009, six, seven, seven, and six new abortion waves at dairy goat farms were confirmed, respectively. The infected dairy goat farms were mainly located in the same area where human cases occurred and they were considered the most plausible source of human infection. In the same period, cases of abortion caused by *C. burnetii* were confirmed at two dairy sheep farms.

Since 2007, a large multidisciplinary research portfolio has started, aimed at generating better knowledge about this disease. In June 2008, Q fever in small ruminants kept for milk production became notifiable in the Netherlands for farms with an abortion rate of more than five per cent. In the autumn of 2008, a voluntary vaccination campaign in goats was made possible in the high-risk Q fever area in the south of the Netherlands with the so far unregistered phase I vaccine containing inactivated *C. burnetii* (Coxevac® , CEVA Santé Animale). From 2009 onwards, vaccination became compulsory for dairy sheep and dairy goat farms in the south of the country, and was compulsory in the whole country from January 2010 onwards for dairy sheep and dairy goat farms, and for small ruminant farms offering recreational activity. Since February 2009, a stringent hygiene protocol became mandatory for all dairy goat and dairy sheep farms, and on 1 October 2009, bulk milk monitoring became mandatory

on farms with more than fifty dairy goats or dairy sheep, and *C. burnetii* PCR positive bulk milk has since been used as an additional criterion for veterinary notification of Q fever. At the end of 2009, it was decided to cull all pregnant animals on farms with a *C. burnetii* PCR positive bulk tank milk. Since 2010, there was a sharp decline in the number of notified human cases with 504, 81, and 66 cases notified in 2010, 2011, and 2012, respectively. In combination with a rise in the human population with antibodies against *C. burnetii*, the implemented control measures most likely have ended this large outbreak.

Introduction

Q fever is an almost ubiquitous zoonosis caused by *Coxiella burnetii*, an aerobic Gram-negative highly resistant bacterium which is able to infect several animal species, as well as people. Cattle, sheep and goats are the primary animal reservoirs (Zeman et al., 1989; Damoser et al., 1993; Maurin and Raoult, 1999; Hatchette et al., 2001). In small ruminants, an infection may result in abortion, mainly in late pregnancy, and stillbirth. Infected animals can shed the bacterium in faeces, milk and, in particularly high concentrations, mainly in foetal membranes and foetal fluids (Maurin and Raoult, 1999; Wouda and Dercksen, 2007). Placentas of infected small ruminants can contain over 10^9 hamster infective doses or bacteria per gram of tissue (Babudieri, 1959; Fournier et al., 1998). Transmission to humans mainly occurs through the aerosol route (Marrie, 1990b; Maurin and Raoult, 1999; Schimmer et al., 2009, 2010).

Q fever was described as a febrile illness which had started to occur early 1933 in abattoir workers in Brisbane, Queensland, Australia (Derrick, 1937). Burnet and Freeman (1937) reproduced the disease in guinea pigs, mice, monkeys and albino rats with an emulsion of infectious guinea pig liver received from Derrick, and demonstrated rickettsial organisms in spleen sections from infected mice. In the same period, Davis and Cox (1938), working on the possible vectors of Rocky Mountain spotted fever, allowed *Dermacentor andersoni* ticks collected near Nine Mile Creek, Montana, to feed on guinea pigs and found that some guinea pigs developed a febrile illness with enlarged spleens. The 'Nine Mile agent' was demonstrated intravacuolarly in infected tissue culture (Cox, 1938, 1939) and was able to cause an infection in man (Dyer, 1938). In 1938, *Rickettsia diaporica*, the proposed name for the organism (Cox, 1939) incorporating both rickettsial features and the ability of the organism to pass a bacteriological filter, was propagated in tissue cultures and in developing chicken embryos (Cox, 1939; Cox and Bell, 1939). Derrick (1937) proposed the name Q fever or query fever for this disease.

The American and Australian groups started working together and demonstrated that the Australian Q fever agent, the zoonotic agent, and the Nine Mile agent were in fact isolates of the same microorganism, *Rickettsia burnetii* (Derrick, 1939; Maurin and Raoult, 1999), later renamed as *C. burnetii* (Philip, 1948), a name which honours both Cox and Burnet as Q fever pioneers.

Since the first documented outbreaks, Q fever has been described in many other countries all over the world. Kaplan and Bertagna (1955) reported its existence in 51 countries on five continents, mainly in cattle, sheep, goats, and man. In New Zealand, Poland, the Scandinavian countries, and the Netherlands no confirmed cases had been found at that time.

This article describes abortion waves in sheep and goats in the Netherlands caused by *C. burnetii* which started to occur in 2005, causing environmental contamination and a subsequent rise in human Q fever cases. A large multidisciplinary research portfolio was developed and implemented aimed at generating better knowledge about this disease to be able to take adequate control measures. Finally, this article presents and discusses measures taken which, in combination with a rise in the human population with antibodies against *C. burnetii*, most likely resulted in a control of this outbreak at the end of 2012.

Abortion waves in sheep and goats

C. burnetii can infect several animal species, as well as humans (Babudieri and Moscovici, 1952; Babudieri, 1959; Marrie, 1990a; Maurin and Raoult, 1999; Arricau-Bouvery and Rodolakis, 2005; Muskens et al., 2007). Cattle, sheep and goats are the primary animal reservoir (Zeman et al., 1989; Damoser et al., 1993; Maurin and Raoult, 1999; Hatchette et al., 2001; Wouda and Dercksen, 2007) although cats have also been described as a major source of infection (Marrie et al., 1988). In cattle, an infection is usually asymptomatic but may occasionally result in abortion, fertility problems and metritis (Arricau-Bouvery and Rodolakis, 2005). Infected small ruminants may deliver live or dead lambs but infection may also result in large abortion waves, mainly at the end of gestation (Arricau-Bouvery and Rodolakis, 2005; Wouda and Dercksen, 2007; Roest et al., 2012).

Historically, the seroprevalence of Q fever in ruminants in the Netherlands was considered to be low, and in a survey held between 1951 and 1953, and in 1954, all 524 (Wolff and Kouwenaar, 1954) and 745 ruminants tested (Dekking and Zanen, 1958), respectively, were seronegative. In a survey in 1987, using an indirect ELISA, antibodies against *C. burnetii* were demonstrated in 3.5% of 3,603 sheep from 191 flocks. A total of 52 flocks (27.2%) had one or more seropositive sheep. This limited survey also included 498 goats of 0.5–1 year old, and 96 adult goats, and showed that less than 1% of goats had antibodies against *C. burnetii* (Houwers and Richardus, 1987).

The sheep industry in the Netherlands has been more or less stable in recent decades, with a little less than one million breeding ewes. In recent years, commercial dairy sheep are kept on forty farms, and the number of animals per farm differs widely from less than fifty to almost a thousand. The dairy goat industry started after the introduction of the milk quota system in the dairy cattle industry in 1984, and on 350 farms, on average almost thousand dairy goats per farm are kept and the number of animals per farm is still rising (Santman-Berends et al., 2013; Van den Brom and Vellema, 2009).

In 2005, *C. burnetii* was diagnosed for the first time in the Netherlands as a cause of abortion on a dairy goat farm, using a recently developed immunohistochemistry

on sections of placenta (Wouda and Dercksen, 2007). A second case was confirmed later in 2005. In 2006, 2007, 2008, and 2009, six, seven, seven, and six new cases were confirmed on dairy goat farms, respectively, mainly in the southern part of the country, making *C. burnetii* the main abortifacient agent in goats in the Netherlands between 2006 and 2009 (Van den Brom and Vellema, 2009; Van den Brom et al., 2012b). In the same period, two cases of abortion caused by *C. burnetii* were found on dairy sheep farms, one in 2006 in the southern part of the country and another in 2008 in the northern part of the country (Van den Brom and Vellema, 2009; Roest et al., 2011a, 2011b). The average number of goats per infected farm was 900, and on average 20% of the pregnant animals aborted. The average number of sheep for the two infected sheep farms was 400 and the abortion rate was around 5% (Van den Brom and Vellema, 2009; Roest et al., 2011b).

Although the average abortion rate on the above described dairy goat farms was around 20%, differences between farms ranged from ten to sixty per cent of the pregnant animals aborting (Wouda and Dercksen, 2007; Van den Brom and Vellema, 2009). High abortion rates as a consequence of an infection with *C. burnetii* are rare but have been described previously to occur in goat herds, where up to 90% of pregnant animals may abort (Palmer et al., 1983; Hatchette et al., 2003; Arricau-Bouvery and Rodolakis, 2005). In the lambing season following an abortion wave, the reproductive problems are often much less prominent (Berri et al., 2007; Wouda and Dercksen, 2007; Van den Brom and Vellema, 2009).

Infected animals can shed the bacterium mainly in birth products, but also in milk and faeces. Shedding can last for months and is longer in goats than in sheep (Arricau-Bouvery and Rodolakis, 2005) and differs between ruminant species (Rodolakis et al., 2007). Placentas of infected small ruminants can contain over 10^9 hamster infective doses or bacteria per gram of tissue (Babudieri, 1959; Fournier et al., 1998). Transmission to humans mainly occurs through the aerosol route (Maurin and Raoult, 1999; Marrie, 1990b; Schimmer et al., 2009, 2010; Van der Hoek et al., 2011b, 2012b).

History of human Q fever outbreaks in Europe

In Europe, cases of Q fever in humans were first reported from soldiers in the Balkan region including Bulgaria in 1940, and during the second World War, in 1944 and 1945, German and American troops, in the Balkans and in Italy, respectively, suffered from an influenza like disease called “Balkangrippe”, later identified as Q fever (Caminopetros, 1948; Wolff and Kouwenaar, 1954; Georgiev et al., 2013). After the second World War, cases of Q fever in humans were reported in Germany and the Mediterranean area, and in 1950 and 1951 more than 20,000 human cases were estimated to have occurred in Italy alone (Babudieri, 1953; Wolff and Kouwenaar, 1954; Georgiev et al., 2013).

An extensive study of the European situation has been published by Georgiev et al. (2013). In a survey carried out between 1951 and 1953, in all 2,411 suspected Dutch human cases no evidence for Q fever was found (Wolff and Kouwenaar, 1954), and between 1954 and 1956, the investigation of 6,000 blood samples from people in the Netherlands with an atypical pneumonia found no antibodies against *C. burnetii* (Dekking and Zanen,1958).

Human Q fever in the Netherlands

Q fever was first diagnosed in the Netherlands in three human patients in 1956. All three cases occurred in the Rotterdam area, and no epidemiological association between these three males could be established (Dekking and Zanen, 1958; Westra et al., 1958).

In a sero-epidemiological study performed on sera collected in 1982 and 1983, seroprevalences in high-risk groups of veterinarians, taxidermists, and female wool spinners were found to be on average 75.9%. In large animal veterinary practitioners, 186 out of 222 (83.8%) were seropositive compared to 390 out of 857 (45.5%) blood donors, blood sampled in 1983 and living in the cities of Rotterdam, Groningen, and Maastricht with a mean seroprevalence of 24%, 60.4%, and 61.7%, respectively. The seropositive results of the veterinarians were equally distributed over all age groups, suggesting most infections had occurred in early childhood. Males were more often infected than females. It was concluded that from 1968 onwards, seroprevalence in controls had remained fairly constant (Richardus et al., 1984, 1987; Houwers and Richardus, 1987).

In 1978, Q fever became a notifiable disease in humans in the Netherlands. The number of notifications between 1978 and 2006 ranged between 1 and 32 cases annually, with an average of 17 cases per year. These cases predominantly involved patients with occupational risk. The total number of hospitalized Q fever patients between 1994 and 2001 was 49 (Delsing and Kullberg, 2008; Schimmer et al., 2009).

In May 2007, a medical microbiologist reported several cases of atypical pneumonia to the municipal health service in Noord-Brabant, a province in the south of the Netherlands. In the same month, a physician in Herpen, a village in the same province, reported an increase in cases of atypical pneumonia in his practice. A few weeks later, another physician in the same region also reported an increase of atypical pneumonias in his practice (Steenbergen et al., 2007). Retrospective investigation proved that *C. burnetii* was the causal agent and in 2007, a total of 168 confirmed human cases were reported. The onset of the majority of cases was between week 18 and 24. The age of the patients ranged from 7–87 years, the female to male ratio was 1:1.7, and the preliminary hospitalization rate was 43%

(Schimmer et al., 2008). Many patients suffered from persisting fatigue for several months after the onset of the disease (Nabuurs-Franssen et al., 2009).

In 2008, Q fever returned and at the end of the year, 1,000 human cases had been notified, making it one of the largest recorded community outbreaks of Q fever. In 2009, 2010, 2011, and 2012, another 2,354, 504, 81, and 66 human Q fever cases were notified, respectively. At the start of the epidemic, fever, fatigue, night sweating, headache and general malaise were the main symptoms, and in 65% of the human cases, a pneumonia was reported (Delsing and Kullberg, 2008; Schimmer et al., 2008, 2009; Dijkstra et al., 2012).

Q fever is often an occupational hazard. People working with farm animals, such as livestock handlers, farmers, veterinarians, slaughterhouse and laboratory personnel are at higher risk of being infected. Nevertheless, community outbreaks have been described before (Derrick, 1937; Tselentis et al., 1995; Armengaud et al., 1997; Lyytikäinen et al., 1998; Nabuurs-Franssen et al., 2008).

The manifestation of Q fever in humans is highly variable. In the first described cases, fever and headache were the most prominent symptoms (Derrick, 1937). The onset of disease was acute and course and duration of the fever varied. Headache was often severe and persistent and in many cases the main complaint. In comparison with the high fever, the pulse rate of the patients was slow (Derrick, 1937). Nowadays, it is clear that the clinical presentation can differ from asymptomatic to fatal chronic infections (Arricau-Bouvery and Rodolakis, 2005; Muskens et al., 2007; Nabuurs-Franssen et al., 2008). The incubation period for acute Q fever varies from one to four weeks and in some cases even up to six weeks or longer (Maurin and Raoult, 1999; Steenbergen et al., 2007; Delsing and Kullberg, 2008) and depends in part on the inoculation dose of *C. burnetii* (Maurin and Raoult, 1999).

In an analysis of 3,264 notified human cases of acute Q fever in the Dutch epidemic, the patients most affected were men, smokers and persons aged 40–60 years. Pneumonia was the most common clinical presentation. Only 3.2% of the patients was working in the agriculture sector and 0.5% in the meat-processing industry including abattoirs (Dijkstra et al., 2012).

In about 1–5% of the cases, an acute infection may lead to a chronic infection (Nabuurs-Franssen et al., 2008) although the definition of this state of infection could be debated (Maurin and Raoult, 1999; Nabuurs-Franssen et al., 2009). The chronic infection can manifest itself as endocarditis, chronic fatigue syndrome and problems related to pregnancy. Endocarditis is the main presentation of chronic Q fever (Maurin and Raoult, 1999; Nabuurs-Franssen et al., 2008; Schimmer et al., 2008), and can occur months, even years after an acute infection (Arricau- Bouvery and Rodolakis, 2005). Chronic fatigue syndrome is characterized by inappropriate

fatigue. Other symptoms are night sweats, myalgia, arthralgia, mood swings and changes in sleeping pattern. The syndrome can occur after an acute infection and can last for months or years (Arricau- Bouvery and Rodolakis, 2005; Delsing and Kullberg, 2008). About 1–5% of the chronic cases lead to fatal complications (Arricau-Bouvery and Rodolakis, 2005). Q fever infections during pregnancy are almost always asymptomatic (Tissot-Dupont et al., 2007; Nabuurs-Franssen et al., 2008), but serious obstetric complications have been described, such as placentitis, spontaneous abortion, intrauterine growth retardation, intrauterine foetal death, premature delivery and low birth weight (Jover-Diaz et al., 2001; Raoult et al., 2002; Langley et al., 2003; Carcopino et al., 2007; Delsing and Kullberg, 2008). Infections during pregnancy may lead to repeated abortions in following pregnancies (Arricau-Bouvery and Rodolakis, 2005), caused by the fact that latent infections in women can be reactivated during following pregnancies (Delsing and Kullberg, 2008; Nabuurs-Franssen et al., 2008). However, a recent study, aiming at quantifying the consequences of an infection with *C. burnetii* during pregnancy, did not show evidence of adverse pregnancy outcome among 1,174 women, living in the area with highest incidence of Q fever in the epidemic in the Netherlands, and who had IgM and IgG antibodies to *C. burnetii* during early pregnancy (Van der Hoek et al., 2011a).

Because in goats high numbers of *C. burnetii* bacteria are shed after an infection which results in an abortion wave, the high abortion rates, and the fact that abortion waves in goats and human cases were mainly located in the same area, dairy goat farms were considered the most plausible source of human infection in the Dutch epidemic, although real evidence for such a connection is difficult (Steenbergen et al., 2007; Delsing and Kullberg, 2008; Schimmer et al., 2009).

Multidisciplinary research in the Netherlands

The human Q fever outbreak in the Netherlands stimulated the development and implementation of a large multidisciplinary research portfolio, aimed at generating better knowledge about the background and transmission of *C. burnetii*, to be able to take adequate control measures.

Q fever research in sheep and goats

Research started with the analysis of the 2005–2007 abortion waves, and demonstrated that on the first thirteen farms with an abortion outbreak, 20% of the pregnant goats had aborted. The abortion rate on the two infected dairy sheep farms was 5% (Van den Brom and Vellema, 2009).

In 2008, a serological survey, using an indirect ELISA (Ruminants Serum Q Fever LSI Kit, LSI, Lissieu, France), was carried out in 15,186 sheep and goats in the Netherlands. In total, 2.4% (95% CI: 2.2–2.7) of the sheep and 7.8% (95% CI:

6.9–8.8) of the goats was seropositive for antibodies against *C. burnetii*. In 14.5% (95% CI: 12.5–16.5) of the sheep flocks and 17.9% (95% CI: 14.2–21.5) of the goat herds at least one seropositive animal was found. The herd prevalence in dairy goat farms was 44.7% (95% CI: 35.4–54.3). In sheep flocks with at least one seropositive sheep, the within flock seroprevalence was 14.8% (95% CI: 12.6–17.0). In goat herds with at least one seropositive goat, the within herd seroprevalence was 29.0% (95% CI: 24.6–33.3). The seropositive sheep were equally distributed across the country. Dairy sheep and dairy goats had a significantly higher chance of being seropositive than non-dairy sheep and goats. The Q fever seroprevalence was highest in pregnant and periparturient dairy goats in the south-eastern part of the Netherlands, which coincides with the region with the highest human incidence of Q fever (Van den Brom et al., 2013a). In 2009–2010, before being vaccinated, farm prevalence of *C. burnetii* in dairy goat farms was 43.1% (95% CI: 34.3–51.8). Overall goat seroprevalence was 21.4% (95% CI: 19.9–22.9), and among the 53 positive farms 46.6% (95% CI: 43.8–49.3). Possible risk factors for farm and goat seropositivity were spread of *C. burnetii* from relatively closely located bulk tank milk PCR positive small ruminant farms, next to introduction and spread from companion animals, imported straw and use of artificial insemination (Schimmer et al., 2011).

Dairy sheep and dairy goat farmers were also given the opportunity to test bulk milk samples using a PCR (TaqvetTM *C. burnetii*, TaqMan Quantitative PCR, LSI, Lissieu, France) and an indirect ELISA. In 2008, there were 392 farms with more than 200 dairy goats, of which 292 voluntarily submitted a bulk tank milk sample. Of these samples, 96 (32.9%) were PCR positive and 87 (29.8%) were *C. burnetii* ELISA positive. None of the bulk tank milk samples from dairy sheep farms (n = 16) were *C. burnetii* PCR positive but three of these farms were *C. burnetii* ELISA positive (Van den Brom et al., 2012a). Using this PCR on pooled and individual milk samples on a bulk tank milk *C. burnetii* PCR positive farm, it was possible to detect PCR positive animals, and after culling of these animals, the bulk tank milk remained negative in *C. burnetii* PCR until the end of the observation period (Van den Brom et al., 2013b).

After the rise in human Q fever cases in 2007 and 2008, a vaccination campaign started in dairy goats and dairy sheep, aiming at reducing environmental contamination. However, it was complicated to measure the effect of vaccination on shedding of *C. burnetii* under field conditions as a PCR on vaginal swabs, the hitherto used way to measure shedding, did not seem to give reliable results, probably due to environmental contamination of the samples collected. After it was decided to kill all pregnant small ruminants on bulk tank milk PCR positive farms, the effect of vaccination on bacterial shedding was studied in culled animals. On the day of culling, samples of uterine fluid, vaginal mucus, and milk were obtained from 957 pregnant animals in thirteen herds, and it was demonstrated

that prevalence and bacterial load were reduced in vaccinated animals compared with unvaccinated animals, and these effects were most pronounced in animals during their first pregnancy (Hogerwerf et al., 2011).

After the Central Veterinary Institute (CVI, Wageningen-UR, Lelystad) in the Netherlands succeeded in culturing *C. burnetii*, research started on molecular characterization of *C. burnetii*, using multilocus variable-number tandem-repeat analyses (MLVA-typing), and 126 *C. burnetii*-positive samples from ruminants were genotyped (Roest et al., 2011c). One unique genotype seemed to predominate in dairy goat herds, and this genotype was similar to a human genotype from the Netherlands, strengthening the probability that this genotype of *C. burnetii* played a predominant role in the human Q fever epidemic in the Netherlands (Roest et al., 2011c; Tilburg et al., 2012c). Using a more stable, sequence-based typing method, multispacer sequence typing (MST), on samples from humans and a group of ruminants, a firmer correlation between Q fever cases in humans and animals was established (Tilburg et al., 2012a).

Transmission of *C. burnetii* to humans is thought to occur primarily through the aerosol route, although data on *C. burnetii* in aerosols and other environmental matrices were limited. During the outbreak of 2009, relatively high levels of *C. burnetii* DNA in surface area swabs and aerosols were demonstrated on bulk milk-positive farms, including farms with a *C. burnetii* related abortion history, supporting the hypothesis that these farms can pose a risk for the transmission of *C. burnetii* to humans (de Bruin et al., 2012).

In a recent study on Q fever pathogenesis, replication of *C. burnetii* in intranasally inoculated pregnant goats seemed to occur predominantly in the trophoblasts of the foetal part of the placenta. High numbers of *C. burnetii* were excreted during abortion, but also during parturition of live born kids. *C. burnetii* was not detected in faeces or vaginal mucus before parturition, as long as no contamination of the environment had taken place (Roest et al., 2012). This finding indicates that the presence of *C. burnetii* in faecal and vaginal samples does not reflect the infection status of the animal, and probably has consequences for conclusions of earlier Q fever publications (Arricau Bouvery et al., 2003; Guatteo et al., 2006, 2007).

Q fever research in humans

Community

After the 2007 human Q fever outbreak in the Netherlands, living in the east of the cluster area, smoking, and contact with agricultural products were found to be risk factors for acquiring a recent infection (Karagiannis et al., 2009). In 2008 and 2009, a sharp increase in Q fever notifications was found, mainly in the province of Noord-Brabant, and large dairy goat farms with abortion waves had been

incriminated (Schimmer et al., 2009). In the same area, relatively high seroprevalences in dairy goats were found, and a significantly larger proportion of bulk tank milk samples was PCR and ELISA positive, compared to the rest of the country (Van den Brom et al., 2012a, 2013a). In May 2008, an isolated Q fever outbreak occurred in an urban area in the south of the Netherlands, and distribution and timing of cases suggested a common source. Using a generic geographic information system, a method for source detection was developed, and a single dairy goat farm was demonstrated to be the most plausible source. People living within two kilometres of this farm, on which dairy goats had experienced abortions since mid April 2008, had a much higher risk for Q fever than those living five kilometres away (Schimmer et al., 2010). Based on ongoing research, constantly increasing evidence indicated that abortion waves on dairy goat farms were the most plausible source of infection for humans, primarily affecting people living close to such a dairy goat farm. Based on this information, drastic measures have been implemented, including the large-scale culling of pregnant goats on infected farms (Van der Hoek et al., 2010b).

After Q fever was first diagnosed in the Netherlands in 1956, a rise in seroprevalence was demonstrated with high prevalences not only in high-risk groups but also in controls (Richardus et al., 1984, 1987; Houwers and Richardus, 1987). By unknown causes, human seroprevalences in the Netherlands went down, and before the start of the Q fever epidemic, a seroprevalence survey among 5,654 individuals showed an adjusted overall seroprevalence of 2.4% (Schimmer et al., 2012b), however, differences in seroprevalence should always be interpreted carefully as different serological assays performed in the same study population may lead to significant differences in seroprevalence estimates (Blaauw et al., 2012). The low seroprevalence in the community at the start of the Q fever epidemic combined with a massive shedding of *C. burnetii* on dairy goat farms in a densely populated area, more than likely was the cause of the human Q fever outbreak in the Netherlands between 2007 and 2010.

Before 2007, data about the effectiveness of different antibiotics for the treatment of acute Q fever were scarce. In a Dutch study, different antibiotic treatments were compared with hospitalization risk for patients with acute Q fever, and in case of an initial therapy with doxycycline (200 mg/day), a significant lower risk for hospitalization was shown, subscribing to current guidelines that doxycycline is recommended as antibiotic of first choice (Dijkstra et al., 2012).

In several studies, living close to an infected farm was an independent risk factor for acquiring Q fever (Van der Hoek et al., 2010b, 2011b, 2011c, 2012a; Dijkstra et al., 2012). However, in the surroundings of some dairy goat farms with abortions caused by *C. burnetii*, no human cases were reported. Therefore, the role of local environmental conditions which may influence the transmission of *C. burnetii*

from infected farms, were investigated. Vegetation and soil moisture seemed relevant factors possibly by reducing the amount of dust. Areas without transmission had a higher vegetation density and a relatively high groundwater level (Van der Hoek et al., 2011b).

Since 2010, there has been a sharp decline in the number of notified human cases. In the affected area in the south of the country, up to 15% of the population has been infected, and a rise in cases of chronic Q fever is expected in coming years (Maurin and Raoult, 1999; Nabuurs-Franssen et al., 2008, 2009). This urges the need for reliable diagnostic tools, and a recent analysis of individual serum antibody responses in 344 Q fever patients indicates that not only high IgG phase 1 may be predictive for chronic Q fever, but also that high IgG phase 2 may aid in detecting such putative chronic cases (Teunis et al., 2013). Questions remain about the follow-up of acute Q fever cases, screening of groups at risk for chronic Q fever, screening of donors of blood and tissue, and human vaccination (Van der Hoek et al., 2012a, 2012c).

People at risk

Q fever was first described as a febrile illness in slaughterhouse personnel (Derrick, 1937), and has for a long time afterwards been thought to be mainly an occupational hazard for people working with farm animals, although community outbreaks had been described (Derrick, 1937; Tselentis et al., 1995; Armengaud et al., 1997; Lyytikäinen et al., 1998; Nabuurs-Franssen et al., 2008).

Seroprevalences of more than 75% in high-risk groups have been described in the Netherlands in the eighties (Richardus et al., 1984, 1987; Houwers and Richardus, 1987). In 2006, a cross sectional study confirmed Q fever to be at risk for veterinary students in the Netherlands. A seroprevalence of 18.7% was found and the main risk factors were study direction farm animals, advanced year of study, having had a zoonosis during the study, having ever lived on a farm, and a clear dose-response relation for *C. burnetii* seropositivity and the number of years lived on a farm (De Rooij et al., 2012). In 2009, a survey among 189 Dutch livestock veterinary practitioners, including final year veterinary students, revealed a seroprevalence of 65.1%. Independent risk factors associated with seropositivity were number of hours with animal contact per week, number of years graduated as a veterinarian, being a practicing veterinarian, and occupational contact with swine (Van den Brom et al., 2013c). In a study among 268 Dutch goat farmers and their household members living or working on these farms, a high risk of acquiring human Q fever was concluded from high prevalences of *C. burnetii* antibodies in farmers, spouses and children (Schimmer et al., 2012a). At the end of 2009, the Dutch government decided to cull all pregnant dairy sheep and dairy goats on *C. burnetii* bulk tank milk PCR positive farms. Among 517 persons assisting during culling, 17.5% seroconverted, despite use of personal protective equipment (Whelan et al., 2011). Based on a clear relation between seropositivity and contact with farm animals,

these studies confirmed veterinarians, farmers and their household members, and culling workers to be people at risk for Q fever, and vaccination of these people is suggested in these recent publications.

Detection methods for human Q fever outbreaks

After the peak in human cases in 2009, the relation between notification and *C. burnetii* infections was investigated. This resulted in a ratio of one notification to 12.6 incident infections of *C. burnetii* (Van der Hoek et al., 2012d). During this outbreak, community and physicians became more aware of Q fever, and this resulted in an increase of diagnostic tests performed, and consequently, in an increase of detected smaller outbreaks. Sources were identified using additional research techniques and extensive tracing, and geographic information systems (GIS) were used to identify the most likely source, a single dairy goat farm, of an urban Q fever outbreak in 2008 (Schimmer et al., 2010). GIS was also used to link massive numbers of undetected infections to a single dairy goat farm (Hackert et al., 2012). In a long-term psychiatric institution, an outbreak of forty-five human clinical cases could be related to a flock of *C. burnetii* positive sheep with newborn lambs (Koene et al., 2011). Multivariable logistic regression analysis confirmed the association between visiting a non-dairy sheep farm on so called lamb-viewing days and Q fever in 146 human cases (Whelan et al., 2012). Real-time syndromic surveillance showed to be useful in detecting hidden Q fever outbreaks (Van den Wijngaard et al., 2011). Finally, smooth incident maps of human notifications provided valuable information about the Q fever epidemic and showed Q fever hotspots around infected dairy goat farms (van der Hoek et al., 2012b).

Human Q fever outbreaks with a suspected relation to dairy goat farms have been reported between 2007 and 2009. Since 2005, Q fever outbreaks on dairy sheep and dairy goat farms have been reported. Retrospectively, it was explored whether there was evidence for human Q fever outbreaks between 2005 and 2007. Space-time scan statistics revealed eight suspected Q fever outbreaks based on clusters of low-respiratory infections, hepatitis, and/or endocarditis in hospitalizations during this period. It was concluded that real-time syndromic surveillance can be used to detect hidden Q fever outbreaks (Van den Wijngaard et al., 2011).

Measures taken

Before the start of the human Q fever outbreak in 2007, there were not many formal contacts between the human and veterinary domain in the Netherlands. The questions raised after the first year of this epidemic have stimulated the development and interpretation of the so called one health concept which strives to expand interdisciplinary collaborations and communications in all aspects of health care for humans, animals and the environment, aiming at advancing health

care mainly by expanding the scientific knowledge base. This collaboration has stimulated the development and implementation of a large multidisciplinary research portfolio, to be able to take adequate control measures. Before June 2008, abortion outbreaks in small ruminants were reported on a voluntary basis to the Animal Health Service and farmers were offered the possibility to submit aborted lambs and placentas for post mortem examination. Suspected cases were confirmed by immunohistochemistry (Wouda and Dercksen, 2007). In June 2008, Q fever in small ruminants kept for milk production became a notifiable disease in the Netherlands for farmers and their veterinary practitioners. The notification criterion for farms with more than 100 breeding animals was an abortion wave, defined as an abortion percentage higher than 5% of all pregnant small ruminants. For smaller holdings, a criterion of three or more abortions in a 30-day period was used (Van den Brom and Vellema, 2009).

In the autumn of 2008, a voluntary vaccination campaign in goats was made possible in the high-risk Q fever area in the south of the Netherlands where most of the human cases had occurred. Vaccination was performed with the so far unregistered phase I vaccine containing inactivated *C. burnetii* (Coxevac® , CEVA Santé Animale), and approximately 35,000 goats were vaccinated. The aim of the vaccination was reducing the shedding of *C. burnetii* on dairy goat farms and thus environmental contamination, trying to reduce human exposure (Van den Brom and Vellema, 2009). From 2009 onwards, vaccination became compulsory for dairy sheep and dairy goat farms with more than fifty animals in the south of the country, in the province of Noord-Brabant and parts of the provinces of Gelderland, Utrecht and Limburg, and was compulsory in the whole country from January 2010 onwards for dairy sheep and dairy goat farms, and for small ruminant farms offering recreational activity.

Since February 2009, a stringent hygiene protocol became mandatory for all professional dairy goat and dairy sheep farms, independent of their Q fever status. This protocol included not only some mandatory measures, but also some voluntary measures, aimed at preventing environmental contamination. Farmers were not allowed to take out manure from their stables until at least one month after the lambing season, were obliged to cover manure during storage and transport, and had to plough it under immediately or after composting it for at least three months. Farmers were advised to submit aborted foetuses and placentas for pathological examination and were obliged to render all remaining aborted foetuses and placentas. Administration of all taken measures had to be kept for at least a year. Farmers were also encouraged not to admit pregnant women, children and elderly people into their stables.

Since 2009, compulsory bulk tank milk (BTM) monitoring is carried out on all Dutch dairy sheep and dairy goat farms with more than fifty animals, and PCR-

positive bulk milk has since been used as an additional criterion for veterinary notification of Q fever. A lack of vaccine in 2009 prevented vaccination of all Dutch dairy sheep and dairy goats. This fact combined with a rise in human Q fever patients in 2009 and uncertainty about expectations for human Q fever cases in 2010, did the Dutch government decide to cull all pregnant animals on *C. burnetii* BTM PCR positive dairy sheep and dairy goat farms as a precautionary measure to prevent shedding and subsequent environmental contamination and thus human exposure. A lifetime breeding ban was implemented for all remaining non-pregnant dairy sheep and dairy goats on *C. burnetii* BTM positive farms (Roest et al., 2011a, 2011b).

Conclusion

The human Q fever outbreaks in the Netherlands between 2007 and 2012 could probably occur because two important risk factors were present, namely the susceptibility of the human population indicated by a low seroprevalence of 2.4% before the outbreaks, and shedding of enormous amounts of *C. burnetii* on infected dairy goat farms between 2005 and 2009.

After two years with serious human Q fever outbreaks in 2007 and 2008, the Netherlands have been facing a third outbreak in 2009 (Schimmer et al., 2009). Uncertainty about the risk of new outbreaks in 2010 increased public pressure on the dairy sheep and dairy goat industry, and compulsory measures like hygiene measures, bulk tank milk monitoring and culling of pregnant animals on infected farms were implemented. Since 2010, there has been a sharp decline in the number of notified human cases with 504, 81, and 66 cases notified in 2010, 2011, and 2012, respectively.

Implemented control measures and a rise in the human population with antibodies against *C. burnetii*, most likely resulted in a control of these outbreaks at the end of 2012 (Dijkstra et al., 2012).

Conflicts of interest statement

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no financial support for this work that could have influenced its outcome.

Chapter 12

Summarizing discussion

Introduction (Chapter 1)

The timeline in "Veterinary aspects of a Q fever outbreak in the Netherlands between 2005 and 2012" can be considered from different perspectives. From a veterinary point of view, the first diagnosed abortion waves caused by *C. burnetii* in 2005, indicate the start of the outbreak. Later it was shown that the start of the human outbreak probably also started in 2005. Since 2009, no abortion waves caused by *C. burnetii* have been detected in small ruminants. At the end of 2012, the Dutch National Institute for Public Health and the Environment officially announced that the human Q fever outbreak was over, based on decreasing numbers of human Q fever patients, and a lack in clustering of patients in time and location. Therefore, 2012 was chosen as the year in which the Dutch Q fever outbreak ended.

The aim of this thesis is to describe veterinary aspects of a Q fever outbreak in the Netherlands between 2005 and 2012 to be able to improve control and preventive measures aiming at reducing the shedding of *C. burnetii* and thus environmental contamination, in order to reduce human exposure. As no recent information was available on professionally exposed persons, the research described in this thesis additionally aimed to determine consequences of exposure for livestock veterinarians.

History and situation in 2007 and 2008 (Chapter 2)

In chapter 2, the Q fever situation in the Netherlands in 2007 and 2008 is described. It starts with an overview of the causal agent, the disease and its history, and focuses on the sheep and goat industry in the Netherlands and the recently detected Q fever problems.

Until 2007, Q fever in humans was mainly known as an occupational disease, although community outbreaks, mostly related to a single point contact like a farmers' market (Porten et al., 2006) with shedding non-dairy sheep, had been described (Dupuis et al., 1987; Santoro et al., 2004; Porten et al., 2006; Tissot-Dupont et al., 2007; Gilsdorf et al., 2008; Morovic et al., 2008). Before the 2007 outbreak started, Q fever was a relatively unknown disease in the Netherlands with an average number of notifications in humans per annum of seventeen between 1978 and 2006. In 2007, 168 human cases were confirmed, mainly in the southern part of the country, in an area with a high density of large dairy goat farms. Q fever recurred in 2008, mainly in the same area, and at the end of that year, 1,000 human cases had been registered, making it the largest human outbreak ever recorded.

In 2005, *C. burnetii* was diagnosed for the first time in the Netherlands as a cause of abortion on two dairy goat farms (Rapportage Monitoring Dierziekten Kleine Herkauwers, tweede helft 2005). In 2006, 2007 and 2008, six, seven and seven new

outbreaks on dairy goat farms were confirmed, respectively. The infected dairy goat herds were mainly located in the same area where human cases occurred and they were considered as most plausible source of human infection, although evidence at that time was still inconclusive. In the same period, two outbreaks of abortion caused by *C. burnetii* were confirmed on two dairy sheep farms, one in the southern and one in the northern part of the country, however these two outbreaks did not appear to be related to human cases.

After the first two serious human outbreaks in 2007 and 2008, research started and measures were implemented. First of all, Q fever was made a notifiable disease in small ruminants and an abortion percentage of more than five per cent temporarily became the main notification criterion. Although abortion is the main clinical sign of a *C. burnetii* infection in small ruminants, abortion does not occur in all infected pregnant animals, and with hindsight this possibly means that shedding herds could have been missed by this type of notification, but at that time no validated tools to distinguish herds with and without shedding animals were available.

In October 2008, vaccine (Coxevac®, CEVA Santé Animale) for approximately 40,000 dairy goats became available but at that time the breeding season had already started, while vaccination should be carried out before pregnancy. Vaccination was supposed to prevent abortion and to decrease shedding of *C. burnetii* after infection, but the vaccine was not licensed and almost no published information about its efficacy was available. Also in 2009, when more stringent measures were needed, a lack of vaccine prevented vaccination of all dairy sheep and dairy goats.

In February 2009, implementation of a hygiene protocol became mandatory for all dairy sheep and dairy goat farms aiming at reducing the risk of human infection. Another way to prevent *C. burnetii* infection in humans is to prevent shedding by infected animals, and vaccination is the most appropriate tool to do so. Nevertheless, even vaccination of small ruminants cannot completely prevent each human infection, since other mammals, birds and arthropods can also act as a source of human infection (Babudieri and Moscovici, 1952; Arricau-Bouvery et al., 2005; Berri et al., 2007).

In 2011, a retrospective study demonstrated that *C. burnetii* might have been the cause of hidden Q fever outbreaks with eight clusters of patients with lower respiratory infections or hepatitis in 2005, 2006 and 2007. Real-time syndromic surveillance possibly would have detected these clusters at least two years earlier (van den Wijngaard et al., 2011). This would probably have resulted in an earlier identification of dairy goats and dairy sheep as the most probable source of infection in humans and an earlier implementation of preventive measures on small ruminant farms.

Seroprevalence in small ruminants (Chapter 3)

In the nineteen eighties, seroprevalences in sheep and goats in the Netherlands were low (Houwers and Richardus, 1987). After the 2007 Q fever outbreak in humans, the occurrence of *C. burnetii* infections in small ruminants in the Netherlands was questioned, bearing in mind that the dairy goat industry had changed dramatically after the introduction of the milk quota system in the dairy cattle industry in 1984. In Chapter 3, seroprevalences and associated risk factors are described. A serological survey of 15,186 sheep and goats in 2008 demonstrated that in total 2.4% (95% CI: 2.2–2.7) of the sheep and 7.8% (95% CI: 6.9–8.8) of the goats were positive for antibodies against *C. burnetii*. These seroprevalences were relatively low compared to seroprevalences found in other studies (Martinov et al., 1989b; Hatchette et al., 2002; McQuiston and Childs, 2002; Dolcé et al., 2003; Masala et al., 2004; Psaroulaki et al., 2006; García-Pérez et al., 2009). However, results of studies from other countries are difficult to compare with our results, because of different study designs, and the use of tests with different characteristics.

Dairy sheep and dairy goats had a significantly higher chance of being seropositive than non-dairy sheep and goats. On most dairy goat farms, the animals are housed inside barns throughout the year, and with an average of about nine hundred animals per farm, large numbers of goats are kept on a relatively small area, which may have facilitated transmission. Dairy sheep are also kept in a more intensive way than non-dairy sheep, although they are kept in lower numbers compared to dairy goats, and most of the dairy sheep are kept outside, at least for a couple of months per year (Van den Brom and Vellema, 2009). Non-dairy sheep and goats are usually kept in smaller groups, with less intense between animal contacts than dairy sheep and dairy goats. Nevertheless, susceptible non-dairy sheep and non-dairy goats can become infected with *C. burnetii*, and this can result in shedding of the bacterium after parturition or abortion. Large human Q fever outbreaks have been described as a consequence of such infections (Van den Brom et al., 2014, in press). However, in the Dutch Q fever outbreak, no increased incidence in human Q fever patients was found around infected non-dairy sheep farms (Van der Hoek et al., 2012b).

During pregnancy and in the periparturient period, small ruminants tested significantly more often seropositive than in the early-pregnant or non-pregnant period. In infected small ruminants, massive multiplication of *C. burnetii* can take place during the last weeks of pregnancy. Taking this into account, a breeding ban can be a very effective measure to prevent shedding and thus environmental contamination. However, timely vaccination before the first pregnancy has been demonstrated to reduce shedding significantly (Hogerwerf et al., 2011), and with hindsight, a breeding ban for vaccinated animals might not have been necessary.

Pregnant and periparturient dairy goats in the south-eastern part of the Netherlands had the highest seroprevalence, and this region also had the highest incidence of Q fever in humans. However, seroprevalences in dairy goats found in this study do not prove a causal association with numbers of human Q fever patients. Nevertheless, molecular typing later demonstrated that the main *C. burnetii* genotype found in dairy goats could also be found in human Q fever patients, although numbers of isolated and genotyped *C. burnetii* strains from human Q fever patients were quite small (Roest et al., 2011c; Tilburg et al., 2012a).

Bulk tank milk possibilities and limitations (Chapter 4)

After dairy goats were suspected to be the source of the human Q fever outbreak (Van Steenbergen et al., 2007), the Dutch government decided to implement measures on infected dairy sheep and dairy goat farms. In order to demonstrate an infection with *C. burnetii*, tests for individual animals like ELISA, PCR and immunohistochemistry (Kovacova and Kazar, 2000; Wouda and Dercksen, 2007; Garcia-Perez et al., 2009; Muskens et al., 2011) are available. However, individual sample collection and testing is an expensive and time consuming activity, especially since the average size of Dutch dairy goat farms was around nine hundred in 2008 (Van den Brom and Vellema, 2009), and within herd prevalence may be very low. Bulk tank milk (BTM) testing has proved to be a reliable method to determine disease status in dairy cattle herds for several agents (Veling et al., 2002; Zimmer et al., 2002; Bartels et al., 2005; Kim et al., 2005; Muskens et al., 2011). At the start of the Q fever outbreak, only a study on BTM testing for *C. burnetii* in small ruminants had been published where all BTM samples from 39 Swiss dairy goat farms tested negative in PCR (Fretz et al., 2007), and a short communication estimating the *C. burnetii* prevalence in dairy sheep in Spain (Garcia-Perez et al., 2009).

Chapter 4 describes the *C. burnetii* prevalences in Dutch dairy goat and dairy sheep BTM samples, using a real-time (RT) PCR and ELISA. Results of BTM PCR and ELISA were compared with the serological status of thirteen animals per farm, and correlations with a farm history of abortion caused by *C. burnetii* were determined. For different chosen PCR cut-offs, the highest area under the Receiver Operator Curve of the ELISA was at a PCR cut-off of 100 bacteria/mL.

In 2008, out of 392 farms with more than 200 dairy goats 292 voluntarily submitted a BTM sample, of which 96 (32.9 per cent) were PCR positive and 87 (29.8 per cent) were ELISA positive. The agreement (kappa-value) between results of both diagnostic tests was 0.80. All farms with a history of *C. burnetii* abortions (n = 17) were ELISA positive, 16 out of 17 were also PCR positive. Since the majority of BTM PCR positive farms had not notified abortion waves, is was decided to validate BTM testing to be able not only to detect shedding of *C. burnetii* but also to interpret results.

BTM PCR and ELISA positive farms had significantly higher within-herd seroprevalences than BTM negative farms. After the start of the vaccination campaign, BTM ELISA was no longer an option to distinguish infected from vaccinated farms. Additionally, ELISA positive results do not provide information on shedding of *C. burnetii* (Hogerwerf et al., 2014).

None of the BTM samples from dairy sheep farms (n = 16) were PCR positive, but three of these farms were ELISA positive. BTM samples were submitted mainly in or after August 2008. Since shedding of *C. burnetii* in milk from sheep has been described to occur during a relatively short period after parturition (Rodolakis et al., 2007), sampling shortly after parturition might have led to higher prevalences in BTM samples from dairy sheep farms, as Dutch sheep are seasonal breeders, normally giving birth in the first months of the year.

In the south-eastern part of the country, the area where the human Q fever outbreak started in 2007, a significantly larger proportion of BTM samples was PCR and ELISA positive compared to the rest of the Netherlands, supporting the suspected relation between human cases and infected dairy goat farms.

What we can learn from surveillance (Chapter 5)

Initially, the aim of the BTM surveillance program was to declare dairy goat herds *C. burnetii* free after at least one year of BTM PCR negative results. However, from December 2009 onwards farms were declared infected based on positive results, and additional measures were implemented on those farms like culling of all pregnant dairy goats, and a lifetime breeding ban for the remaining goats. BTM surveillance was assessed retrospectively for its suitability to detect farms with *C. burnetii* shedding animals, and, additionally, it was used to evaluate implemented control measures.

Between October 2009 and April 2014, 1,660 (5.6%) out of 29,875 BTM samples from 401 dairy goat farms tested positive for *C. burnetii*. In total, 156 dairy goat farms tested positive for *C. burnetii* at least once in the mandatory BTM surveillance program. The percentage of positive samples dropped from 20.5% in 2009 to 0.3% in 2014. In a multivariable model, significantly higher odds of becoming PCR positive in the BTM surveillance program were found in the months February until November compared to January. The highest odds on BTM PCR positivity were found in July, August and September. A possible explanation for the observed difference in risk in time could be the fact that Dutch dairy goats are seasonal breeders. The higher odds to become *C. burnetii* BTM PCR positive during the breeding season could indicate the possibility of shedding of *C. burnetii* during estrus, as also has been described for *Chlamydia abortus*, another intracellular abortifacient agent in small ruminants (Livingstone et al., 2009; Papp et al., 1994).

Higher odds of becoming BTM PCR positive were also found on farms of which all pregnant dairy goats had been culled. This can be explained by presence of chronically infected goats on officially declared *C. burnetii* BTM PCR positive farms. Finally, the risk for *C. burnetii* BTM PCR positivity significantly decreased after multiple vaccinations.

In the Netherlands, Q fever is a notifiable disease in small ruminants since 2008, and initially an abortion percentage of more than five per cent was the main notification criterion. Nowadays, only farms with an abortion rate deviating from their normal rate have to be notified to the Dutch Food and Consumer Product Safety Authority (NVWA). Using this criterion shedding of *C. burnetii* could be missed, especially in those cases where an infection does not result in abortion or abortions are not noticed. Consequently, non-dairy sheep and goat farmers should be stimulated to pay extra attention to infections that could lead to abortion, and to submit abortion and stillbirth materials, both fetuses and placentas, for post-mortem examination also in cases where only a few animals have aborted. This is even more crucial because vaccination of non-dairy sheep and goats is not mandatory and an active *C. burnetii* surveillance program for these groups of animals does not exist at present.

For dairy sheep and dairy goats, BTM surveillance is a sensible method to detect shedding of *C. burnetii* on a farm. Unfortunately, no methods are available to identify animals in recently infected naive herds that are at high risk for shedding after parturition, and therefore test and cull of high risk pregnant *C. burnetii* infected dairy goats is not possible (Hogerwerf et al., 2014). Tests on excretion fluids can only detect shedding after parturition and contamination of samples can easily result in false positive results (Roest et al., 2012).

Detection of individual shedders (Chapter 6)

In chapter 6, we describe results of individual testing on a BTM PCR positive dairy goat farm where all goats had been vaccinated since 2008 with an inactivated phase one vaccine. All pregnant goats on this farm were culled in 2010, after which BTM PCR became negative. One year later, however, this farm became BTM PCR positive again and from all lactating animals (n = 350), individual milk samples were tested. Five goats born on the farm between 2002 and 2006, appeared *C. burnetii* PCR positive. At post-mortem examination, out of 33 different samples per animal, only milk and mammary tissue were PCR positive. Immunohistochemical examination of several parts of mammary gland and regional lymph node tissues was negative. The replication site of *C. burnetii* as source for the PCR positive milk samples in these five goats remained unknown. After culling of these five positive animals, BTM PCR remained negative until the end of the observation period, April 2014. These results indicate that vaccination does not completely prevent intermittent shedding of *C. burnetii* in previously infected goats.

Although mandatory vaccination has been carried out on all dairy goat farms at least since 2010, four years later, still a small group of farms was officially declared *C. burnetii* infected, and intermittently shedding goats are the most likely explanation. Individually shedding dairy goats should be traced and removed, since their role in maintenance of infection on farms cannot be excluded. Further research is needed to investigate under which circumstances and where multiplication of *C. burnetii* takes place in intermittently shedding animals, especially in non-pregnant animals. Multiplication of *C. burnetii* in trophoblasts of the foetal part of the placenta, found as primary target cells in the study of Roest (2013), is not possible in the latter animals.

Manure and transmission (Chapter 7)

In several outbreaks, manure was identified as the most probable source of a Q fever outbreak (Georgiev et al., 2013). In chapter 7, the role of *C. burnetii* contaminated manure in the transmission of *C. burnetii* to humans, the impact of manure storage in dunghills, and decimal reduction time of the Nine Mile RSA 493 reference strain of *C. burnetii* in different matrices under experimental circumstances, are described.

The findings indicate no association between the incidence of human Q fever and the dispersal of goat manure originating from farms with confirmed abortion storms in 2008 and 2009. This lack of association is supported by our temperature measurements in dunghills on two farms with *C. burnetii* shedding dairy goats. Although we detected *C. burnetii* DNA in goat manure by PCR, we were unable to culture the bacterium from these manure samples. This could have been caused by very low numbers of bacteria present in the samples taken or could result from the absence of viable *coxiella*-bacteria in these manure samples. Even if viable bacteria had been present in manure at the moment of its removal from the stable, it is likely that the composting process in a dunghill would have resulted in killing of the majority of *C. burnetii*.

In contrast, Hermans et al (2014) reported an association between land-applied goat manure and human Q fever cases in the Dutch Q fever outbreak. However, in that study no correction was included for the presence of infected farms in the region where manure was distributed and, moreover, misclassification of infected farms seems likely given the data source they used. Because of these biases, based on the findings reported in this thesis it is still considered unlikely that land-applied goat manure played an important role in the Dutch Q fever outbreak.

Vaccination is essential (Chapter 8)

Vaccination with phase one vaccine Coxevac® (CEVA, Santé Animale) aims to prevent abortion and to reduce shedding of *C. burnetii*. Vaccination should be carried out before pregnancy in order to be effective, and its efficacy had in 2008 only been demonstrated under experimental conditions (Arricau-Bouvery et al., 2005). Since March 2008, it had been tried to import vaccine into the Netherlands, but only in October, when many goats were already pregnant, the non-licensed vaccine became available, and during a voluntary vaccination campaign approximately 40,000 dairy goats within a radius of 45 km around Uden, corresponding with the high human Q fever incidence area, were vaccinated. In 2009, vaccination became compulsory in the southern part of the Netherlands, but because of a lack of vaccine a more extended vaccination campaign was not possible at that time. In the same year, the number of human Q fever cases increased dramatically, and uncertainty what to expect in 2010 made the government decide to cull all pregnant dairy goats and dairy sheep in BTM PCR positive herds.

In chapter 8, we confirmed the effect of vaccination on bacterial shedding. On the day of culling, samples of uterine fluid, vaginal mucus, and milk were obtained from 957 pregnant animals in 13 herds. Prevalence and bacterial load were reduced in vaccinated animals compared with unvaccinated animals. These effects were most pronounced in animals during their first pregnancy. Results indicate that vaccination may reduce bacterial load in the environment and human exposure to *C. burnetii*. In earlier studies, comparable results had been described after experimental infections.

Since 2010, compulsory vaccination is applicable to all dairy goat and dairy sheep farms, farms with a public function and for animals that are participating in shows. Abortion caused by *C. burnetii* has not been diagnosed in small ruminants since 2009 (Van Engelen et al., 2014); it is noteworthy that in previous years (2006-2009), *C. burnetii* was the main abortifacient agent in goats (Van den Brom et al., 2012b). The above mentioned reduction suggests that vaccination with a phase one vaccine is an effective measure to prevent abortion caused by *C. burnetii* in small ruminants.

Infected animals that had been vaccinated before their first pregnancy did not shed large amounts of *C. burnetii* at parturition. Consequently, from a veterinary aspect pre-emptive culling of this group of animals does not seem necessary in future outbreaks.

The small ruminant industry has expressed a wish for an exit strategy for the vaccination programme because of the associated costs and perceived adverse reactions like a (sometimes dramatic) drop in milk production after repeated

vaccinations. Although only low numbers of adverse reactions have officially been reported, and a study in 2009 only showed limited adverse reactions with a reduction in milk yield that did not differ from the reduction measured after a period of warm weather, frequently repeated vaccinations might result in more severe adverse reactions. However, before considering an exit strategy a proper risk assessment should be performed taking into account all pro's and cons of vaccination. Such an assessment should not only take into account veterinary aspects and economic aspects like the probability and costs associated with a new outbreak, but also the perceived risk in the society.

When shedding of *C. burnetii* by infected, non-vaccinated small ruminants occurs, there are no proper measures to prevent environmental contamination other than culling of animals on infected farms. Sheep and goat farmers who are not obliged to vaccinate their animals should consider vaccination, and farmers who do not wish to vaccinate should be aware of the risks and consequences when shedding in their herd or flock occurs.

Veterinarians at risk (Chapter 9)

Q fever has long been considered primarily an occupational zoonotic disease for abattoir workers, sheep shearers, livestock farmers, and especially veterinarians, because of their contact with potentially infected animals (Marrie and Fraser, 1985; Richardus et al., 1987; Valencia et al., 2000; Abe et al., 2001; Monno et al., 2009; Whitney et al., 2009; Chang et al., 2010). In 1984, 84% out of 222 livestock veterinarians in the Netherlands were serologically positive for antibodies against *C. burnetii* (Richardus et al., 1984). As no recent information was available regarding the consequences of the Dutch Q fever outbreak for occupationally exposed persons, in chapter 9, seroprevalence in Dutch livestock veterinarians and possible risk factors were determined.

Blood samples from 189 veterinarians, including veterinary students in their final year, were collected, and screened for IgG antibodies against phase I and phase II antigens of *C. burnetii* using an indirect immunofluorescent assay, and for IgM antibodies using an ELISA. Antibodies were demonstrated in 123 (65.1%) out of 189 veterinarians. Participants also filled in a questionnaire, and number of hours with animal contact per week, number of years graduated as veterinarian, rural or sub urban living area, being a practicing veterinarian, and occupational contact with swine were associated with seropositivity.

Several other studies have been conducted in the Netherlands in which seroprevalences and associated risk factors among occupationally exposed people were determined (Whelan et al., 2011; De Rooij et al., 2012; Schimmer et al., 2012a; Whelan et al., 2012; Van den Brom et al., 2013c; De Lange et al., 2014). Generally,

high seroprevalences were found, and in most cases intensive contact with livestock was an important risk factor. To protect occupationally exposed people their vaccination has been suggested in many studies. Although vaccination of people belonging to risk groups is common in Australia (Marmion, 2007; Gidding et al., 2009), vaccination of humans in the Netherlands evoked much debate, not only because the Australian vaccine was not licensed in the Netherlands but also because vaccination may be associated with adverse effects. Eventually, between January and May 2011, 1,366 patients with specific vascular disorders were vaccinated, after intensive pre-vaccination screening (Bults et al., 2012; Isken et al., 2013).

Veterinarians seem to have been at risk for decades in the Netherlands, and probably still are because of high prevalences in Dutch livestock (Muskens et al., 2011; Van den Brom et al., 2013a). However, not many veterinarians reported clinical illness possibly related to *C. burnetii* infections, and it is difficult to predict whether or not vaccination would have been of any benefit. Livestock veterinarians are at risk for acquiring Q fever, and it is of importance that they and their physicians are aware of this risk.

Remaining questions

Nowadays, there are still many questions that need to be answered in order to be able to fully understand *C. burnetii* infections in small ruminants and the risk of subsequent human exposure. Some of the main questions arising from this thesis are:

- are current surveillance programs for *C. burnetii* sufficient to detect shedding small ruminants?
- where does *C. burnetii* survive and multiply in so called chronically infected animals?
- under what conditions can *C. burnetii* shedding start in non-pregnant animals, while vaccination is mandatory?
- how often does shedding of *C. burnetii* occur in non-dairy unvaccinated small ruminants?
- why are seroprevalences in occupationally exposed high, while clinical Q fever is rarely reported?
- what was the quantitative effect of each of the implemented measures, which finally stopped the human Q fever outbreak?

Final remarks arising from this thesis and future perspectives

In the future, individual Q fever infections in humans will most likely occur on a regular base, and in most cases probably no source will be identified. Ruminants are the main reservoir for *C. burnetii*, but other mammals, birds and arthropods may also act as sources of incidental occurrence of human Q fever, and especially,

occupationally exposed are at risk. Infection of single persons or small groups are difficult to prevent, since hygiene measures are often not sufficient to prevent infection (Whelan et al., 2012). Therefore, physicians should be aware of Q fever (and other zoonotic diseases), especially in case of illness in occupationally exposed.

Worldwide, large human Q fever outbreaks are mainly related to shedding sheep and goats and often originate from a single farm or on a single event, e.g. a farmers' market (Porten et al., 2006). In order to prevent human Q fever outbreaks caused by shedding small ruminants, several measures were implemented in the Netherlands, e.g. compulsory notification in small ruminants, mandatory vaccination, bulk tank milk surveillance, manure measures, a stringent hygiene protocol, a breeding ban, and culling of pregnant animals. Additionally, formal contacts between the veterinary and human health field have improved resulting in communication and collaboration on a regular basis.

Of the implemented measures, mandatory vaccination with a phase one vaccine (Coxevac®, CEVA Santé Animale) has played an important role in the reduction of shedding, especially when administered before first pregnancy (Hogerwerf et al., 2011). Since mandatory vaccination is applicable, the number of *C. burnetii* BTM PCR positive dairy goat farms has decreased in time. However, it is difficult to quantify the effect of each of the other control measures that were implemented more or less in the same period.

On dairy sheep and dairy goat farms, bulk tank milk surveillance is an effective method to detect shedding of *C. burnetii*. To be able to detect infected and shedding non-dairy small ruminants, notification of abortion is important, but probably not sufficient to detect all cases, because the notification threshold might not be reached. In those cases, voluntary submission of abortion and stillbirth materials should be encouraged, especially since decreasing numbers of submissions from small ruminants are of concern in the Netherlands (Van den Brom et al., 2012b). Submission of such materials is not only of importance for detection of *C. burnetii* infections, but also for several of the other infectious causes of abortion in small ruminants which also have zoonotic potential (van Engelen et al., 2014). Therefore, everyone involved should realize that post-mortem examination not only is of importance for the economy of individual farmers, but also can be of benefit for the health of farmer, family members, visitors and, in some cases, even others. In the future, additional surveillance methods should be developed, such as air or dust sampling on small ruminants farms, which can also be used on non-dairy small ruminant farms.

In a situation where the current surveillance efforts fail to detect shedding of *C. burnetii* by small ruminants, real time syndromic surveillance in humans serves as the final method to prevent new large community outbreaks from happening. This

will detect clusters of human Q fever patients in an early stage (Van den Wijngaard et al., 2011), and when combined with possibilities of modern geographic information systems can be useful in identifying the most probable source of infection (Schimmer et al., 2010), enabling quick implementation of measures to prevent further human exposure. Nevertheless, it is undesirable that humans serve as a sentinel for Q fever outbreaks in ruminants, so should this happen it should be a trigger to further improve surveillance in small ruminants.

Culling of pregnant animals as a measure to prevent human Q fever is under discussion, since environmental contamination has already been established at the time infection has been confirmed (Stuen and Longbottom, 2011). Nevertheless, when massive shedding of *C. burnetii* in small ruminants is detected among non-vaccinated small ruminants, culling of pregnant animals from that herd should seriously be considered in order to prevent further massive shedding, causing environmental contamination and subsequent human exposure. Additionally, culling of non-pregnant adult animals that have given birth previously should be considered, since shedding mainly starts at parturition, and can last for months (Roest et al., 2012). Another consideration to cull non-pregnant adult animals in infected herds is the possibility of chronic infection in infected animals not vaccinated before their first pregnancy. They possibly could shed *C. burnetii* in successive lambings (Berri et al., 2002). The role of chronically infected animals in a vaccinated herd or flock is unknown, as well as their risk for human health.

Although the human Q fever outbreak may have been declared as ended in 2012, for several groups of people this is not the case. Many human Q fever patients still suffer from complaints related to infection, and they are sometimes daily confronted with the consequences of this disease. For the Dutch dairy goat and dairy sheep industry, mandatory vaccination and BTM surveillance are still applicable, and for many individual farmers and others involved, Q fever is a very difficult and painful subject. Also from a political point of view, Q fever remains an actual topic. In 2010, evaluation of the Q fever outbreak has been performed by commission Van Dijk, and this commission concluded that both involved ministries had acted too hesitant on various occasions. Q fever patients accused dairy goat farmers in 2013 and the Dutch government in 2014 for their role in the Q fever outbreak. Finally, several research projects continue, and quite some questions are still waiting to be answered.

Before the start of the Q fever outbreak, there were not many formal contacts between the human and veterinary domain in the Netherlands. The questions raised after the first year of this outbreak have stimulated the development and interpretation of the so called one health concept which strives to expand interdisciplinary collaborations and communications in all aspects of health care for humans, animals and the environment, aiming at advancing health care mainly

by expanding the scientific knowledge base. This collaboration has stimulated the development and implementation of a large multidisciplinary research portfolio. It also stimulated a structure in which signals from both fields are shared on a monthly base, in an accessible way, and in between, important signals are exchanged immediately. It can be concluded that the human Q fever outbreak encouraged the so called one health thought in the Netherlands.

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Nederlandse samenvatting

De Q-koorts uitbraak in Nederland, die in 2007 begon en de beide volgende jaren in omvang toenam, heeft ons veel geleerd over deze aandoening door het multidisciplinaire onderzoek dat heeft plaatsgevonden. Dit proefschrift beschrijft diergeneeskundige aspecten van die uitbraak, beginnend met de eerste abortusuitbraken op melkgeitenbedrijven in 2005 en eindigend in 2012 met de aankondiging van het RIVM dat met de afname van het aantal patiënten de Q-koorts uitbraak voorbij is. Het doel van de onderzoeken in dit proefschrift is om met toegenomen kennis over Q-koorts, de controle en preventieve maatregelen te verbeteren, de uitscheiding van *Coxiella burnetii* en dus omgevingscontaminatie te verminderen en zodoende blootstelling van mensen te verlagen. Omdat er geen recente informatie was over de risico's voor beroepsmatig blootgestelde personen, is tevens de blootstelling van dierenartsen, werkzaam in de sector landbouwhuisdieren, onderzocht.

Hoofdstuk 2 beschrijft de Q-koortssituatie in Nederland in 2007 en 2008 en geeft achtergrondinformatie over de schapen- en geitensector, de verwekker van de ziekte, de ziekte zelf en over de historie van de uitbraak. Tot 2007 stond Q-koorts bij mensen met name bekend als een beroepsziekte, hoewel ook enkele uitbraken van Q-koorts zijn beschreven die meestal gerelateerd waren aan contact met vleesschapen die massaal *C. burnetii* uitscheidden.

Vóór 2007 was Q-koorts een relatief onbekende ziekte in Nederland met gemiddeld 17 patiënten per jaar tussen 1978 en 2006. In 2007 kregen 168 mensen Q-koorts. De meeste patiënten woonden in Zuid-Nederland, een gebied met veel grote melkgeitenbedrijven. In 2008 zorgde Q-koorts opnieuw voor veel problemen en kregen 1.000 mensen deze ziekte. Daarmee was deze uitbraak, voor zover beschreven, toen al de grootste ooit.

In 2005 werd *C. burnetii* in Nederland voor het eerst vastgesteld als oorzaak van abortus op twee melkgeitenbedrijven. In 2006, 2007 en 2008 werd deze diagnose op respectievelijk nog eens zes, zeven en zeven melkgeitenbedrijven gesteld. Deze bedrijven waren vrijwel allemaal gelegen in dezelfde regio waar zich de humane Q-koortsuitbraak voordeed. Geiten werden gezien als de meest waarschijnlijke bron van de humane infecties, hoewel het bewijs op dat moment niet sluitend was. In dezelfde periode werd abortus door *C. burnetii* op twee melkschapenbedrijven vastgesteld, maar aan deze twee uitbraken leken geen (clusters) humane patiënten te zijn gerelateerd.

Na de eerste twee Q-koortsuitbraken in 2007 en 2008 startte aanvullend onderzoek en implementatie van maatregelen volgde. Vanaf juli 2008 gold een meldplicht

voor Q-koorts bij kleine herkauwers met als meldcriterium een abortuspercentage van boven de vijf procent. Hoewel bij kleine herkauwers abortus het belangrijkste klinische symptoom is van een *C. burnetii* infectie, treedt abortus niet op bij alle geïnfecteerde drachtige dieren. Het is dus mogelijk dat op basis van het meldcriterium uitscheiding door kleine herkauwers is te missen.

In oktober 2008 kwam het vaccin Coxevac® (CEVA Santé Animale) beschikbaar voor ongeveer 40.000 melkgeiten. Vaccinatie zou abortus moeten voorkomen en uitscheiding van *C. burnetii* na infectie moeten verminderen, maar het vaccin was op dat moment niet geregistreerd en er was slechts beperkt gepubliceerde informatie over het vaccin beschikbaar.

In februari 2009 werd een hygiëneprotocol voor alle melkschapen- en melkgeitenbedrijven verplicht gesteld met als doel het risico op humane infectie te verkleinen. Een andere manier om humane *C. burnetii* infecties te voorkomen is het tegengaan van uitscheiding door geïnfecteerde dieren. Vaccinatie leek de meest aangewezen manier om dat te bewerkstelligen. Desalniettemin zal vaccinatie van kleine herkauwers niet alle humane infecties kunnen voorkomen, aangezien ook andere zoogdieren, vogels en geleedpotigen een bron van humane infectie kunnen zijn.

In de jaren tachtig van de vorige eeuw was de seroprevalentie van *C. burnetii* infecties onder kleine herkauwers in Nederland laag. **Hoofdstuk 3** beschrijft seroprevalenties en bijbehorende risicofactoren in 2008. In een serologisch onderzoek van 15.186 schapen en geiten had 2,4% (95% betrouwbaarheidsinterval (BHI): 2,2–2,7) van de schapen en 7,8% (95% BHI: 6,9–8,8) van de geiten afweerstoffen tegen *C. burnetii*. De gevonden prevalenties waren relatief laag vergeleken met studies in andere landen, hoewel de resultaten moeilijk vergelijkbaar zijn vanwege verschillen in studieopzet en het gebruik van testen met verschillende eigenschappen.

Melkschapen en melkgeiten hadden een significant hogere kans om serologisch positief te worden getest dan niet-melkgevende schapen en geiten. Gedurende de dracht en rond de aflamperiode testten kleine herkauwers significant vaker positief vergeleken met niet drachtige cq. vroeg drachtige dieren. Drachtige melkgeiten en melkgeiten rond de aflamperiode in het zuidoosten van Nederland hadden de hoogste seroprevalentie. In deze regio was ook de incidentie van Q-koorts patiënten het hoogst. De gevonden seroprevalenties bij geiten tonen geen causaal verband aan met het aantal humane patiënten.

Hoofdstuk 4 beschrijft de *C. burnetii* prevalentie in tankmelkmonsters afkomstig van melkgeiten- en melkschapenbedrijven, op basis van PCR en ELISA. De resultaten van de tankmelk PCR en ELISA zijn vergeleken met de serologische status per bedrijf en met een eventuele bedrijfshistorie van abortus ten gevolge van

C. burnetii. Voor verschillende PCR afkapwaarden bleek het oppervlak onder de "Receiver Operator Curve" van de ELISA het grootst wanneer de afkapwaarde van de PCR 100 bacteriën/ml bedroeg.

In 2008 stuurden 292 van de 392 bedrijven met meer dan 200 melkgeiten vrijwillig een tankmelkmonster in. In totaal bleken 96 (32,9 procent) van de tankmelkmonsters PCR positief en 87 (29,8 procent) ELISA positief. De overeenkomst (kappa-waarde) tussen de resultaten van beide testen was 0,80. Alle deelnemende geitenbedrijven met een historie van abortus door *C. burnetii* (n = 17) waren ELISA positief, 16 van de 17 waren ook PCR positief. Het overgrote deel van de tankmelk positieve bedrijven had geen abortusstorm gemeld. Vervolgens is validatie van tankmelkonderzoek uitgevoerd, niet alleen om uitscheiding van *C. burnetii* vast te stellen, maar ook om de resultaten te kunnen interpreteren.

Coxiella burnetii tankmelk PCR en ELISA positieve bedrijven hadden significant hogere binnenbedrijfsprevalenties dan tankmelk negatieve bedrijven. Na het begin van de vaccinatiecampagne was het niet meer mogelijk om onderscheid te maken tussen geïnfecteerde en gevaccineerde bedrijven. Daarnaast zegt een ELISA resultaat niets over uitscheiding van de bacterie.

Geen enkel tankmelkmonster afkomstig van een melkschapenbedrijf was *C. burnetii* PCR positief, maar drie melkschapenbedrijven waren wel ELISA positief.

In het zuidoosten van Nederland, het gebied waar in 2007 de humane Q-koorts uitbraak begon, was een significant groter deel van de tankmelkmonsters PCR en ELISA positief in vergelijking met de rest van Nederland. Deze bevinding was een ondersteuning voor het vermoeden van een relatie tussen humane patiënten en uitscheidende melkgeitenbedrijven.

In eerste instantie was het doel van een monitoringsprogramma op basis van tankmelkmonsters om koppels melkgevende dieren die een jaar lang *C. burnetii* PCR negatief waren, vrij te verklaren van *C. burnetii*. Echter, eind 2009 nam de druk op de politiek toe na opnieuw een toename in het aantal Q-koorts patiënten en de onzekerheid over wat er in 2010 zou volgen. Er werd besloten om bedrijven besmet te verklaren op basis van een *C. burnetii* PCR positieve tankmelkuitslag. Er volgden aanvullende maatregelen als ruiming van drachtige dieren en een levenslang fokverbod voor de overgebleven geiten. In **hoofdstuk 5** is de tankmelkmonitoring retrospectief geanalyseerd door te kijken of deze methode geschikt is om bedrijven met *C. burnetii* uitscheidende dieren op te sporen en om aanvullend de geïmplementeerde controlemaatregelen te evalueren. Tussen oktober 2009 en april 2014 testten 1.660 (5,6%) van 29.875 tankmelkmonsters afkomstig van 401 melkgeitenbedrijven positief op aanwezigheid van *C. burnetii* DNA. In totaal testten 156 melkgeitenbedrijven ten minste één keer positief op de

aanwezigheid van *C. burnetii* in de verplichte tankmelkmonitoring. Het percentage positieve monsters daalde van 20,5% in 2009 tot 0,3% in 2014. In een multivariabel model bleek de kans op een *C. burnetii* PCR positieve tankmelkuitslag van februari tot en met november significant groter dan in januari. De kans op een *C. burnetii* PCR positieve tankmelkuitslag was het grootst in juli en augustus. Bedrijven waar drachtige dieren waren geruimd hadden ook een significant grotere kans op een *C. burnetii* PCR positieve uitslag. Tenslotte verminderde de kans op *C. burnetii* positiviteit naarmate het aantal vaccinaties op een bedrijf toenam.

Hoofdstuk 6 beschrijft de resultaten van individueel geteste melkmonsters op een *C. burnetii* PCR tankmelk positief melkgeitenbedrijf waar alle geiten sinds 2008 zijn gevaccineerd met een geïnactiveerd fase één vaccin. Alle drachtige geiten op dit bedrijf zijn geruimd in 2010, waarna de tankmelkmonsters *C. burnetii* PCR negatief werden. Een jaar later was dit bedrijf opnieuw *C. burnetii* PCR positief in tankmelkmonsters, waarna van alle 350 aanwezige lacterende geiten individuele melkmonsters zijn onderzocht. Vijf geiten die tussen 2002 en 2006 waren geboren op dit bedrijf bleken *C. burnetii* PCR positief. Tijdens pathologisch onderzoek waren van 33 verschillende weefselmonsters per dier alleen melk en uierweefsel PCR positief. Immunohistochemische kleuring van verschillende delen van de uier en de regionale lymfeknoop waren negatief. De locatie van replicatie van *C. burnetii* als bron voor de PCR positieve melkmonsters in deze vijf geiten kon niet worden achterhaald. Sinds de vijf uitscheidende dieren het bedrijf hebben verlaten, is op het bedrijf de *C. burnetii* PCR in elk geval tot het einde van de observatieperiode in 2014 negatief gebleven in de landelijk verplichte tankmelkmonitoring. Op basis van deze resultaten kan worden gesteld dat vaccinatie intermitterende uitscheiding door eerder geïnfecteerde geiten niet geheel kan voorkomen.

In verschillende uitbraken is mest aangewezen als meest waarschijnlijke oorzaak van een Q-koorts uitbraak. In **hoofdstuk 7** wordt de rol van met *C. burnetii* gecontamineerde mest in de transmissie naar mensen in de Nederlandse uitbraak beschreven, alsook de impact van mestopslag in mesthopen en de hitteresistentie van de Nine Mile RSA 493 referentiestam van *C. burnetii* in verschillende substanties. De resultaten laten geen correlatie zien tussen de incidentie van Q-koorts patiënten en de verspreiding van geitenmest afkomstig van bedrijven met een abortusstorm ten gevolge van *C. burnetii* in 2008 of 2009. Het ontbreken van een correlatie wordt ondersteund door de temperatuurmetingen in een mesthoop gerelateerd aan de hitteresistentie van *C. burnetii*. Hoewel *C. burnetii* DNA in geitenmest werd aangetoond, is het niet gelukt de bacterie te kweken uit monsters van geitenmest afkomstig uit de potstal en de mesthoop. De aanwezigheid van kleine aantallen bacteriën in de monsters kan hier de reden van zijn, of het feit dat er geen levende bacteriën in de geitenmestmonsters aanwezig waren. Zelfs wanneer er levende bacteriën in de geitenmest aanwezig zijn is het aannemelijk dat door compostering het overgrote deel van de aanwezige bacteriën zou zijn gedood.

Vaccinatie met het fase één vaccin Coxevac® (CEVA, Santé Animale) beoogt abortus te voorkomen en de uitscheiding van *C. burnetii* te verminderen. Om dit effect te bereiken is vaccinatie vóór de dracht vereist. Al in het voorjaar van 2008 is geprobeerd vaccin naar Nederland te halen. Pas in oktober echter, toen een deel van de geiten al drachtig was, konden tijdens een vrijwillige vaccinatiecampagne ongeveer 40.000 geiten in een straal van 45 kilometer rondom Uden worden gevaccineerd met het toen niet geregistreerde vaccin Coxevac®. In 2009 kreeg de vaccinatiecampagne een verplicht karakter in het zuidelijke deel van Nederland, het gebied van de humane Q-koortsuitbraak. Een uitgebreidere vaccinatiecampagne was opnieuw niet mogelijk vanwege een gebrek aan vaccin. Omdat in 2009 het aantal humane patiënten drastisch toenam besloot de overheid om drachtige geiten op tankmelk PCR positieve bedrijven te ruimen, in de hoop nieuwe ziektegevallen in 2010 te beperken. **Hoofdstuk 8** beschrijft het effect van vaccinatie op bacteriële uitscheiding. Hiertoe zijn bij 957 drachtige dieren afkomstig uit 13 koppels monsters genomen van baarmoederinhoud, vaginaal slijm en melk op de dag van ruiming. De prevalentie en hoeveelheid DNA van *C. burnetii* bleken in gevaccineerde dieren lager te zijn dan in ongevaccineerde dieren. Deze effecten waren het meest uitgesproken bij dieren die voor het eerst drachtig waren. De resultaten indiceren dat vaccinatie de omgevingscontaminatie en daarmee blootstelling van de mens aan *C. burnetii* vermindert. Eerdere studies onder experimentele omstandigheden gaven vergelijkbare resultaten.

Q-koorts stond lange tijd primair bekend als een beroepsziekte bij slachthuispersoneel, schapenscheerders, veehouders en met name bij dierenartsen, vanwege hun contact met mogelijk besmette dieren. In 1984 had 84% van 222 dierenartsen werkzaam in de sector landbouwhuisdieren afweerstoffen tegen Q-koorts. Tijdens de humane Q-koorts uitbraak was er geen recente informatie beschikbaar over de gevolgen voor beroepsmatig blootgestelde personen. **Hoofdstuk 9** beschrijft de seroprevalentie onder dierenartsen en mogelijke risicofactoren. Van 189 dierenartsen, inclusief studenten diergeneeskunde in het laatste jaar van hun studie, is bloed afgenomen. Afweerstoffen werden aangetoond bij 123 (65,1%) van de 189 dierenartsen. Tevens hebben deelnemende dierenartsen een vragenlijst ingevuld. Risicofactoren die gerelateerd bleken aan seropositiviteit waren het aantal uren diercontact per week, aantal jaren afgestudeerd als dierenarts, wonend op het platteland of aan de rand van de stad, werkzaam als praktiserend dierenarts en beroepsmatig contact met varkens. Slechts bij een klein deel van de deelnemende dierenartsen is Q-koorts ooit aangetoond als oorzaak van ziekteverschijnselen.

Hoofdstuk 10 geeft een opiniërend overzicht van *C. burnetii* infecties bij kleine herkauwers. **Hoofdstuk 11** beschrijft de humane Q-koorts uitbraak tussen 2007 en 2012 in Nederland.

Hoofdstuk 12 bevat een samenvattende discussie die de bevindingen van dit proefschrift bespreekt en relateert aan internationale literatuur. Het gebied met de meeste Q-koorts patiënten kwam overeen met het gebied met de hoogste seroprevalenties in drachtige melkgeiten. Tankmelkonderzoek op melkgeitenbedrijven bevestigde dit beeld. Deze bevindingen waren een indicatie voor de relatie tussen de ziekte bij mensen en geiten en zodoende een bevestiging van een reeds bestaand vermoeden, maar vormden geen bewijs voor een causaal verband. Tankmelkonderzoek werd geïnitieerd om bedrijven vrij te kunnen verklaren van *C. burnetii*, maar de uitkomsten daarvan werden vervolgens gebruikt om onderscheid te kunnen maken tussen bedrijven met en zonder uitscheidende dieren. Het bleek mogelijk om op een bedrijf met een *C. burnetii* PCR positieve tankmelk de individueel uitscheidende dieren door middel van pooling van melkmonsters op te sporen. Na afvoer van de uitscheidende geiten bleven tankmelkmonsters van dit bedrijf PCR negatief. Op het land verspreide geitenmest lijkt een ondergeschikte rol in de humane Q-koorts uitbraak te hebben gespeeld. Vaccinatie van geiten is een zeer effectieve maatregel gebleken om uitscheiding van *C. burnetii* tegen te gaan. De seroprevalentie onder dierenartsen werkzaam in de sector landbouwhuisdieren blijkt hoog, ondanks dat er maar zelden ziekteverschijnselen ten gevolge van Q-koorts worden vastgesteld.

Veel onderzoek naar Q-koorts startte na de humane uitbraak in Nederland in 2007. Desondanks blijven vele vragen nog onbeantwoord. Zo is het de vraag of, behalve in de melkschapen- en melkgeitenhouderij, de huidige monitoringssystematiek voldoende is om *C. burnetii* uitscheidende dieren op te sporen. Ook is niet bekend waar de bacterie in chronisch geïnfecteerde dieren kan overleven en zich kan vermeerderen. Onder welke omstandigheden kunnen niet-drachtige dieren ondanks een vaccinatieplicht, de bacterie gaan uitscheiden? Hoe vaak komt uitscheiding van *C. burnetii* voor bij niet-gevaccineerde dieren? Ook is het de vraag hoe het kan dat de seroprevalentie onder beroepsmatig blootgestelde personen hoog is, terwijl deze groep mensen maar zelden ziekte ten gevolge van een infectie met *C. burnetii* meldt. Tot slot is niet bekend wat het kwantitatieve effect is geweest van elk van de geïmplementeerde maatregelen.

Vóór de start van de Q-koorts uitbraak waren er niet veel formele contacten tussen de humane en veterinaire gezondheidszorg in Nederland. Samenwerking aan openstaande vraagstukken tijdens de uitbraak heeft er toe geleid dat er nu een vaste overlegstructuur bestaat, waarin de humane en veterinaire gezondheidszorg op laagdrempelige wijze en op regelmatige basis informatie delen. Daarnaast vindt uitwisseling van belangrijke signalen direct plaats. De humane Q-koorts uitbraak heeft zodoende gezorgd voor stimulering van de one health gedachte in Nederland.

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Curriculum vitae

René van den Brom was born on February 25th 1977 in Amsterdam, the Netherlands, and grew up in Abcoude, where he visited primary school 'Nellestein'. In 1989, he started high school at 'Hervormd Lyceum Zuid' in Amsterdam, where he passed his VWO-exam. In 1997, he started his study Veterinary Medicine at Utrecht University and obtained his degree in veterinary medicine (DVM) in 2004 with merit. During his study he was for one year (1999-2000) president of the Veterinary Students Association (DSK), and for two years (2001-2002) member of the Faculty board. In 2002, he received the 'Intervet award', reached out annually at the Faculty of Veterinary Medicine to a student who excellently contributed to integration among veterinary students.

In 2004, René started as a livestock veterinarian in veterinary practice 'Het Westelijk Weidegebied' in Harmelen, which started working together with the veterinary practice of the Faculty of Veterinary Medicine, Utrecht, in 2009.

In 2008, René started working part-time at GD Animal Health, Deventer, as veterinarian in small ruminant health. Due to the human Q fever outbreak with a suspected relation to dairy goats, a large portfolio of (veterinary) research started with the aim to obtain additional information on *Coxiella burnetii* infections in small ruminants, in which René participated from the beginning. Since 2010, René works full-time at GD Animal Health, and from that moment on he started writing scientific papers. His external PhD-program started after publication of his first research paper. In 2013, René qualified as specialist in small ruminant health management, and currently is diplomate of the European College of Small Ruminant Health Management (DipECSRHM).

Nowadays, René is still employed at GD Animal Health, where he is working as a veterinary specialist with an extra interest in surveillance of small ruminant health and infectious causes of abortion in small ruminants.

René lives with his wife Astrid and their children Stijn and Mirthe in Haarzuilens, and they spend their time outside working hours swimming, gardening, running, and taking care of their small flock of sheep.

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