Q fever in pregnancy and fetal health: Epidemiological studies

PhD dissertation

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Stine Yde Nielsen
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THE THESIS IS BASED ON THE FOLLOWING MANUSCRIPTS

Paper I

Q fever during pregnancy and maternofetal consequences: a case series from Denmark (in review)

Paper II

Prevalence of *Coxiella burnetii* in women exposed to livestock, Denmark (in review)

Paper III

No excess risk of adverse pregnancy outcomes among women with serological markers of previous infection with *Coxiella burnetii*: evidence from the Danish National Birth Cohort (BMC Infectious Diseases 2013, 13:87 (17 February 2013)

Paper IV

Presence of Antibodies Against *Coxiella burnetii* and Risk of Spontaneous Abortion: A Nested Case-Control Study (Research Article | published 21 Feb 2012 | PLoS ONE10.1371/journal.pone.0031909)

Paper V

Risk of adverse pregnancy outcome in women exposed to livestock: a study within the Danish National Birth Cohort (in review)

LIST OF ABBREVIATIONS

DNBC	The Danish National Birth cohort
LCV	Large-cell variant
SCV	Small-cell variant
LPS	Lipopolysaccharide
QFS	Post Q fever Fatigue Syndrome
IUGR	Intrauterine Growth Retardation
ELISA	Enzyme-linked immunosorbent assay
IFA	Immunofluorescence antibody test
PCR	Polymerase Chain Reaction
SGA	Small for Gestational Age
LMP	Last menstrual period
OD	Optical Density
MLVA	Multiple-Locus Variable number tandem repeat Analysis
MST	Multispacer Sequence Typing

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Introduction

Humans are exposed to and at times affected by an impressive diversity of pathogens. Most of the newly emerging or re-emerging infections causing concern are of zoonotic origin. Global surveillance systems are being developed as public awareness regarding the human health risks of zoonotic infections is rising. The extent may be significant because 75% of pathogens causing emerging infectious diseases are caused by zoonoses, supporting interdisciplinary initiatives such as the One Health concept which is currently gaining momentum. The drivers for emergence interact in complex pathways, and several causal factors have been recognised. A number of the zoonotic pathogens have been recognised for their ability to cause reproductive problems in especially farm animals and in populations at particularly high risk that often include individuals with close contact with livestock [1-6]. Some of these are known, others are suspected to constitute a risk to pregnant women and their fetuses.

Epidemiological and experimental evidence of maternal infection as a significant risk factor for adverse pregnancy outcomes is accumulating [7,8]; untreated infection may cause miscarriage, stillbirth and preterm birth by several mechanisms, including direct fetal infection, placental damage and severe maternal illness. In developed countries, as many as 25% of all fetal deaths are associated with, and likely caused by, an intrauterine infection, and chorioamnionitis has been reported to be present in 85% of preterm births before 28 gestational weeks [9-11]. The aetiologic components of fetal death are often unresolved, and women with positive cultures derived from the fetal membranes or histologic choriamnionitis rarely exhibit clinical signs of infection [8,12]; thus, the impact of zoonotic infections on the course of pregnancy and the newborn are, in all likelihood, somewhat underrated clinically as well as in research . One infection of recent concern, in particular for pregnant women, is Q fever caused by *Coxiella burnetii*.

Q fever is most likely endemic worldwide, but unbiased estimates from relevant populations are scarce because most reports on the incidence and prevalence are from regions with outbreaks or with special medical or scientific interest in the infection [13]. Q fever in pregnancy is suspected to be a potential cause of fetal morbidity and mortality, but the pathogenesis is poorly understood, and the magnitude and range of risks of adverse pregnancy outcome among infected women remain largely unknown.

Until recently, Q fever was considered a rare and imported infection in Denmark.

Existing data and bio bank samples from the Danish National Birth Cohort (DNBC) allowed us to explore associations between animal exposure, seropositivity and pregnancy outcome. When the DNBC cohort was initiated, specific interview questions on exposure to animals during pregnancy were included in the questionnaires. Along with the bio bank, these interview data provide a unique and high quality resource to study some of the unresolved aspects of the impact of zoonotic infections on pregnancy.

This thesis sets out to elucidate the risk of adverse pregnancy outcome among women exposed to livestock (Study V), in particular among women with serologically verified exposure to *C. burnetii* (studies I, III and IV) and to provide further insight into the prevalence of *C. burnetii* infection among pregnant women exposed to livestock (Study II).

BACKGROUND

Q fever does not exist in Scandinavia…

(Medicinsk Kompendium, 17th issue, 2009 [14]).

The history of Q fever

In 1935, a previously undescribed organism was identified almost simultaneously on two different continents. After an outbreak of febrile illness among abattoir workers in Brisbane, Queensland, Australia, Derrick and his colleagues investigated the clinical disease, isolated the causative organism by inoculating blood and urine from infected patients into guinea pigs which became febrile, and named it Q (for query) fever. Some infectious material was sent to Derrick's colleague, Burnet, who continued the quest. Meanwhile, across the Pacific in Montana, USA, Cox and his colleagues were studying the ecology of Rocky Mountain Spotted Fever. A connection between the two groups was established when a laboratory-acquired Q fever infection was discovered in the Rocky Mountain Laboratory in 1938. In honour of Cox and Burnet, who identified it to be a new genus in the Rickettsiaceae, it was named *Coxiella burnetii*; it was later moved to be member of the family Coxiellaceae in the order Legionellales [13,15,16].
The first case of Q fever in pregnancy was described by Bertaud in 1953 [17].

The agent

Coxiella burnetii is a fascinating example of an intracellular parasitism – this small, gram-negative bacterium has uniquely evolved to thrive in the most inhospitable of cellular compartments – the parasitophorous vacuole. The organism has a unique intracellular lifestyle with two distinct morphological forms, a large-cell variant (LCV) and a small-cell variant (SCV). The SCV is thought to be an extracellular survival form showing a high degree of resistance to environmental stressors such as desiccation and heat. When a SCV invades the host, it devel-

ops into the LCV, which is metabolically and divisionally active [15,18].

C. burnetii is further characterised by antigenic phase variation, which is mainly caused by mutational variation in the lipopolysaccharide (LPS). Phase I is highly infectious and can be isolated from infected humans or animals, whereas phase II is less infectious and can only be obtained following passages in embryonated eggs or tissue culture [15,16,19]. The two forms are microscopically indistinguishable, but their impact on serological diagnosis is significant (further described under Laboratory Diagnosis). When infection occurs via the respiratory route, alveolar macrophages in the lungs are supposedly the primary cells to be infected during acute Q fever. *C. burnetii* enters phagosomes, then fuse to phagolysosomes, which fuse progressively to form a large, acidic vacuole [13].

Reservoirs and Transmission

C. burnetii is prevalent throughout the world and infects a large number of animal species including ticks, cats, dogs, rats, birds and kangaroos, but domestic ruminants – cattle, sheep and goats – are considered the main reservoir for transmission to humans. The animals are often asymptomatic, but retained placenta, endometritis and infertility along with several outbreaks of abortions have been reported in the animals, and in some of these animals placental inflammation has been demonstrated [20,21]. The organism is found in the uterus and the mammary glands, and placentas from infected animals contain up to 10^9 bacteria per gram of tissue, and vast amounts may be shed into the environment from birth products [22].

Apart from birth products, infected animals shed the bacteria in urine, milk and faeces, and the predominant mode of infection of humans is via the respiratory tract after inhalation of airborne dust or droplets containing the bacteria [3,13,23]. Human-to-human transmission is rare, but Q fever may be transmitted by blood transfusion [24], sperm [25], maternal-fetal transmission across the placenta [26,27] and transmission from amniotic fluids and placental tissue [28].

Q fever has been shown to travel large distances by wind during natural outbreaks [29,30]; this, combined with the organism's ability to survive for long periods in the environment and because the infectious dose for man is very low (with fewer than 10 organisms sufficient to seed an infection[31]), has made it a potential weapon for use in bioterrorism [16].

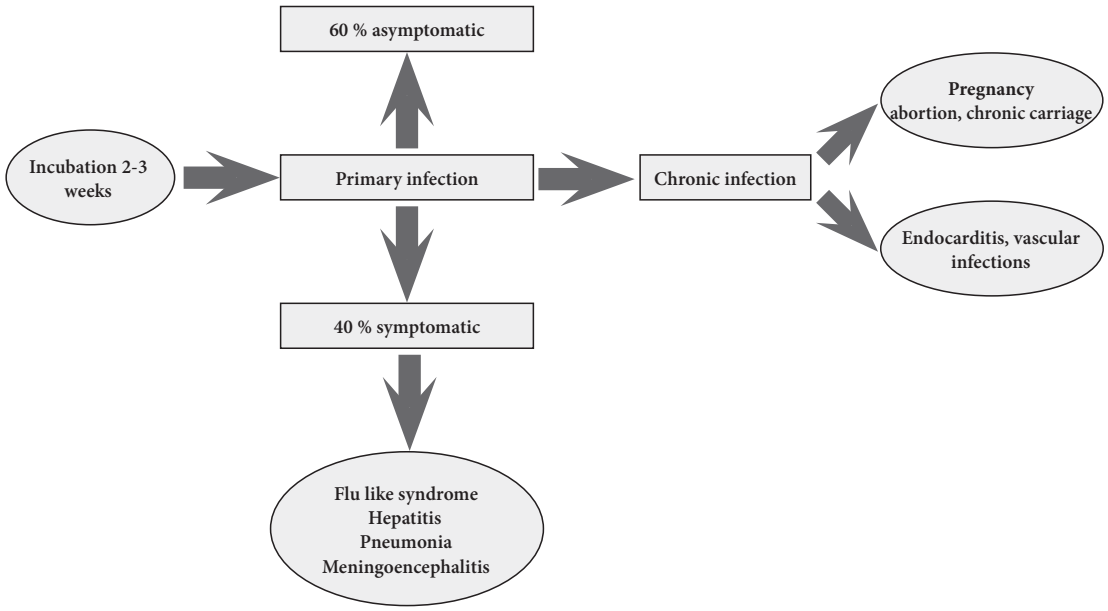
Outbreaks of Q fever have been described as being caused by continuous environmental transmission of *C. burnetii* in large communities (e.g. France, the Netherlands), as single source outbreaks in confined settings (e.g., from ewes at animal markets), or due to occupational

transmission (e.g., in abattoirs, stockyards, rendering plants, laboratories or medical and veterinarian centres where sheep or goats are present). Foodborne outbreaks have also been recognised, e.g., among raw milk drinkers [32-38]. It is conceivable that strain variation, type of host animal, and environmental factors are important for the risk of outbreaks; however, this is poorly understood. Outbreaks have not been recognised in the Scandinavian countries, and most cases of Q fever are probably sporadic (i.e., not related to outbreaks) among individuals directly or indirectly exposed to livestock.

The disease

Humans are the only species known to regularly develop illness as a result of *C. burnetii* infection, but subclinical infection, or a mild flu-like course, is the commonest outcome after exposure. Still, although occurring less frequently, Q fever may also manifest as a severe acute febrile illness with potential complications including hepatitis and pneumonia. Immunologic manifestations including meningitis and meningoencephalitis may also occur, and pericarditis and myocarditis have also been reported. Acute Q fever develops after an incubation period of 15-25 days, depending on the dose. Immunocompromised patients, pregnant women and patients with pre-existing cardiac valve or vascular defects are at increased risk of developing chronic Q fever [24,39-42] (Figure 1).

Figure 1: Different courses of infection with *C.burnetii*



Angelakis, E., Raoult,D. Q fever. Veterinary Microbiology Vol.140 issues 374, 210;pp 297-309

In the Netherlands, which recently experienced the world's largest Q fever outbreak, 20% of the more than 4000 human cases of acute infection were hospitalised [43,44].

In the global population, about 5% of acutely infected patients experience a more severe course of the infection. Endocarditis is the most serious manifestation of chronic Q fever characterised by a poor cellular immune response and valve vegetations with viable coxiella, but chronic infection may also occur as a continuing or recrudescent granulomatous infection of bone, liver and the placenta or the fetus. Post Q fever fatigue syndrome (QFS) may be seen after infection as a clinical expression of a long-lasting fatigue complex involving many organs and with an impaired cellular immune response, a low antibody response and no viable coxiellas [3,15,39,45].

Laboratory Diagnosis

When diagnosing Q fever, a variety of serological methods are available, including complement fixation, enzyme-linked immunosorbent assay (ELISA) and indirect immunofluorescence assay (IFA). ELISA has previously been showed to be suitable for large-scale screening, but IFA is regarded as the gold standard because it is capable of determining both phase I and phase II antibodies simultaneously by the use of two different antigens in a single sample [45,46].

In acute Q fever, primarily antibodies against phase II are elevated, and their titre is higher than is the antibody titre against phase I. As with most other infections, IgM antibodies appear first. Hence, acute infection will show as an increase in the antibody response between paired samples: One taken at onset of symptoms (serum may be negative) and a sample taken 12-25 days after onset of illness [39,47] .

During the last days of the incubation period, C .burnetii will be present in the blood, and PCR (Polymerase chain reaction) detection of coxiella DNA is a valuable diagnostic tool in the early stage of infection during which antibodies cannot be detected [48]. When infected, phase II IgG and IgM antibodies are always elevated, and, although declining, they may remain positive for years. In a chronic infection, positive antibodies against IgG phase I antigens indicate a possible persisting infection [47,49]. PCR may also be useful for the diagnosis of chronic Q fever, and, the diagnosis of chronic Q fever requires more than elevated antibodies, e.g. symptoms and supplementary paraclinical tests like PCR and culture of bone marrow [50].
Due to the high infectivity, the organism should only be handled under bio-safety level 3 containment, and the process of inoculation and isolation of *C. burnetii* is laborious and seldom part of standard diagnostic procedures [45].

Treatment

Most commonly, doxycycline is used to treat acute Q fever, with 100 mg 12 hourly for 14 days being given. Research on newer macrolides and fluorquinilones has been published [51,52], but information on effectiveness is primarily based on in-vitro studies rather than clinical trials [44].

The treatment of chronic Q fever with endocarditis is more challenging, but there is growing evidence suggesting that hydroxychloroquine plus doxycycline should be first line treatment [3,53].

Preventive measures available

In Australia, a vaccine against *C. burnetii* infection has been licensed and in use for abattoir and agricultural workers for many years. In recognition of the importance of protecting individuals at risk of occupational exposure to *C. burnetii*, large Q fever vaccination programs have been serially undertaken in Australia, with vaccination of abattoir workers, farmers, their families and employees in the livestock-rearing industry [54]. Logistical and legal constraints as well as the possibility of adverse reactions in those with previous exposure to the agent are primary reasons why this vaccine has not been used elsewhere [45].

Vaccination of animals using a phase I *Coxiella burnetii*-inactivated vaccine (Coxevac, CEVA) has been found to reduce *C. burnetii* shedding by small ruminants and was widely used in dairy goats and sheep during the recent outbreak in the Netherlands [55-57].

Q fever in Pregnancy

Q fever in pregnancy is suspected to be a potential cause of fetal morbidity and mortality. In animal studies, adverse pregnancy outcome has been reproduced in BALB/c mice, in which infection followed by repeated pregnancies resulted in spontaneous abortion and perinatal death [58], but the precise mechanisms by which the infection compromises pregnancy are largely unknown. One study used a human trophoblast cell line and found that *C. burnetii* infected and replicated within trophoblasts and suggested that normal development of pregnancy may be impaired by the cooperation of trophoblasts and placental immune cells responsive to *C. burnetii* within the placental tissue [59].

Present evidence on the impact on human pregnancy outcome mainly originates from France [26-28,60-62], where a landmark case study of referred infected pregnant patients found that untreated infection was followed by miscarriage, intrauterine growth retardation (IUGR), oligohydramnion, stillbirth or premature delivery. In this study, more than 80% of the 53 women included had obstetric complications, and a chronic profile was reported in more than half of the patients [27].

The French studies conclude that infection in pregnancy is often asymptomatic, and that infected women have a risk of reactivation of a past infection in subsequent pregnancies. Furthermore, infection during the first trimester constitutes a specific risk of miscarriage, and obstetric complications are significantly more frequent in patients who get infected during their first trimester compared to those who get infected later. Carcopino et al. also conclude that there is a link between placentitis and obstetric complications [26,61].

Munster et al. (Netherlands) [63] described placental histopathology and clinical outcome of five cases with asymptomatic *C. burnetii* infection during pregnancy and compared them to symptomatic cases from the literature. Their findings are in line with a study of 153 asymptomatic, seropositive women [64], and the authors concluded that asymptomatic and symptomatic infection during pregnancy are different entities regarding placental pathology and risk of adverse pregnancy outcome and that there may be a linkage between clinical symptoms (fever, fatigue, dyspnoea, etc.) and obstetric complications.

From the Netherlands, two, new large studies evaluated infection in pregnancy and found no increased risk of adverse pregnancy outcome in seropositive pregnancies [65,66].
Dairy goats and sheep were considered to be the source of the Dutch outbreak [67], and one genotype has been found to predominate in these animals. The same genotype was found in a human patient [68], and it has been suggested that the clone harbouring the QpH1 plasmid was responsible for the outbreak [60].

In the latest study from France, pregnancies from 30 women with acute infection in pregnancy were evaluated, and the authors concluded that Q fever is a significant cause of morbidity and may result in miscarriage and that long-term cotrimoxazole treatment prevents complications [61]. The hypothesis that different obstetrical morbidities in different geographical areas are related to strain specificity is also tested in this study. Plasmid types from clinical samples were compared, and four of seven *C. burnetii* strains from infected women with miscarriage harboured the QpDV plasmid; nine human isolates (not placentas) from the Netherlands all harboured the QpH1 plasmid, and the authors consolidate a possible relation between strain specificity and obstetrical complications.

Handling *C. burnetii* infection in pregnancy

Treatment with cotrimoxazole for at least 5 weeks is recommended since doxycycline and hydroxychloroquine are contra-indicated from the 2nd trimester [27,61]. Although the experience from treating pregnant women with cotrimoxazole is limited, it is the best tested treatment. The active ingredient trimethoprim is a folic acid antagonist. Treatment during the 1st trimester entails a small increase in risk of cardiovascular malformations and neural tube defects. This risk can be reduced by simultaneous administration of folic acid.

In the 1st trimester, doxycycline is recommended rather than cotrimoxazole. Treatment with cotrimoxazole in 2nd and 3rd trimester is probably fairly uncomplicated. There is, however, a small risk of kernicterus if treatment with sulfamethoxazole is administered immediately prior to giving birth. Experience with other treatment regimens for Q fever in pregnancy is extremely sparse [26,69,70].

Q fever in Denmark

Testing for antibodies in cattle since 2003 has indicated that Q fever is widespread in cattle, dismissing the assumption that the prevalence of *C. burnetii* is low [71,72]; a recent study found a prevalence of 59% antibody positive herds (bulk milk) among 100 randomly selected dairy herds [73].

In a serological analysis of 1613 people tested in 2006-2007, with the majority due to relevant exposure to domestic animals, 177 (11%) were seropositive and 180 had an equivocal result according to the Danish serological cut-off levels. The authors concluded that Q fever should be considered endemic in Denmark [74].

In another recent study, serum samples from a large cohort of farmers, veterinarians, inseminators and hoof trimmers, all having occupational contact with dairy cattle, were tested and 39 of 359 (11%) were found to have antibodies to *C. burnetii*. Veterinarians had the highest seropositivity rate (36%). The study suggests that *C. burnetii* is a recently recognised domestic infection in Denmark and that the risk of infection is associated with occupation [75].

Established risk factors

The risk of infection with *C. burnetii* has been related to particular occupations with close contact to the organism's primary reservoirs, such as domesticated livestock. Examples include veterinary practice and farming [76-79].

In an American study, antibodies against *C. burnetii* were detected in 113 (22.2%) of 508 U.S. veterinarians; those with a mixed small and large animal practice and those with a food animal practice were more likely to be seropositive than were veterinarians with a small animal practice. Furthermore, that study found that ever living on a farm, currently living on a farm, and exposure to ruminants while living on a farm were associated with seropositivity [76].

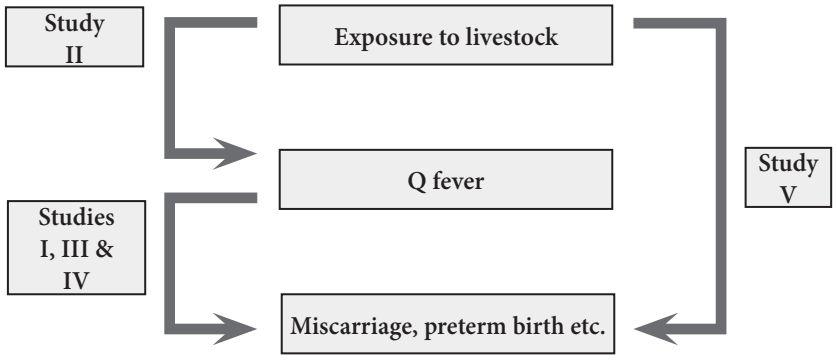
From Australia, it is known that abattoir workers have a higher risk that the background population [45,80].

Summing up, groups at risk in Denmark are veterinarians, especially those working with large animals, farmers, perhaps inseminators and abattoir workers. Since pregnant women are a particular group at risk, focus for further investigation could be pregnant women working within these fields.

AIM OF THE THESIS

This thesis set out to provide further insight into the prevalence of Q fever among Danish pregnant women and the risk of adverse pregnancy outcome among women exposed to farm animals.

Figure 2: Aim of studies



Study I evaluated the course of infection, treatment and pregnancy outcome among Danish pregnant women with elevated antibodies against *C. burnetii* from 2007-2011

Study II intended to quantify risk of infection in pregnant women occupationally and environmentally exposed to *C. burnetii*

Study III set out to examine whether the presence of antibodies to *C. burnetii* during pregnancy or seroconversion was associated with adverse pregnancy outcome

Study IV aimed to assess the potential association between serologic markers of infection with *C. burnetii* and miscarriage

Study V sought to evaluate pregnancy outcome among women with self-reported occupational or domestic contact with farm animals compared to pregnant women without animal contact – using animal contact as a proxy for the risk of zoonotic infection

METHODS

The Danish National Birth Cohort

Between 1996 and 2002, a little more than 100,000 pregnant women were recruited to the DNBC. The women were interviewed during the first half of pregnancy about (among many other items) their occupational exposures. The pregnancies were followed to birth or alternative outcomes of pregnancy, and the children born were (and are continuingly) followed-up with regard to their health through questionnaires and linkages to the Danish Patient Register.

The interviews covered specific questions regarding occupational animal species exposure; women working on farms were asked: "have you worked with animal farming production, meaning live animals?" and "which animals do you work with?" Women with other occupational animal exposure than farming were asked: "which animals do you work with" and: "how are you involved in working with live animals" (veterinarians, veterinary nurses, etc.) and for abattoir workers: "are you directly involved in handling animals at the abattoir?" Hence, the women could be occupationally exposed to living as well as dead animals.

These questions enabled us to define occupational exposure to animals as women who had, during or three months before pregnancy, worked with animals either in an abattoir, on a farm, or in veterinary practice (dairy cattle, meat cattle, pigs, poultry, horses, sheep and goats). Likewise, the women who answered yes to living on a farm with livestock farming were asked: "which species of animals"? As domestic animal exposure of interest we defined cattle, horses, sheep, pigs, poultry, deer, and goat.

The women participating in the DNBC also delivered three blood samples throughout pregnancy: one from gestational week 6-8, one from week 24-26 and a sample from the umbilical cord.

Detection of antibodies against *C. burnetii*

The diagnosis of Q fever relies upon serology. *C. burnetii* expresses two antigens, phase II and phase I. When infected, antibodies against phase II are initially elevated, and their titre is higher than antibodies against phase I with IgM antibodies appearing first. In chronic forms of the disease, antibodies against phase I are elevated [38,40,81]; the antibodies remain positive for many years. A large study from Australia and the U.K. found that phase II IgG antibodies persisted after five and 12 years, respectively [82].

In order to determine antibodies against *C. burnetii*, we used a two-step approach. First all samples were screened in a commercial enzyme-linked immunosorbent assay (ELISA). A variety of serological methods are available; the Panbio ELISA kit has previously been showed to be superior to other and suitable for large-scale screening [83,84]. Positive ELISA samples were confirmed with an immunofluorescence antibody test (IFA).

The commercial ELISA kit was purchased from Panbio (Queensland, Australia) (cat. no. E-QFB01G and E-QFB01M) and used according to the manufacturer's instructions, with minor modifications. Due to the small sample size, the initial total volume was smaller, but the same dilution factors were used.

Samples which were positive for either IgG or IgM antibodies in the ELISA were confirmed with an IFA test from Focus Diagnostics (ca. no. IF0200G and IF0200M). The tests were performed according to the instructions provided by the manufacturer, with the following minor modifications: due to low volume of sample material, the diluted samples 1:10 from the ELISA were used to further dilute the samples as described by the manufacturer. The effect of the dilution in the Panbio buffer was tested prior to the use on patient samples and did not show any influence on the results. Also, the IFA cut-off value suggested by the manufacturer was not used; since the prevalence of the infection varies between geographic areas, the cut-off value suggested by the manufacturer is not necessarily suited for any given area.

A local cut-off value adjusted to the Danish population has been defined, based on 158 anonymous, healthy blood donors from three city areas of Denmark in which people are assumed not to have Q fever [85] (Table 1).

Table 1: Cut-off values in immunofluorescence antibody test (IFA) as applied in Denmark.

	Negative	Equivocal	Positive
IgM phase I	<64	64	>=128
IgM phase II	<64	64-128	>=256
IgG phase I	<128	128-256	>=512
IgG phase II	<128	128-512	>=1024

The equivocal zone was defined in order to address people with an a priori elevated risk of Q fever (such as veterinarians, farmers, etc.), proposing that these high risk groups with an equivocal titre should be considered to be probably positive and managed as such.

Study IV was the first study conducted, cases and non-cases were not selected with any regard to animal exposure, and the Danish cut-off value was used.

When the ELISA positive samples in studies II and III were reanalysed using IFA, a modified version of the Danish cut-off value was used. A sample was considered IFA positive when IgG phase I and II as well as IgM phases I and II were 1:128 or above.

In study I, all analyses were done using IFA, and a sample was considered IFA positive when IgG phases I and II as well as IgM phases I and II were 1:128 or above.

All serological analyses were performed in a certified laboratory at Statens Serum Institut, Denmark. Laboratory personnel were blinded for exposure status, and samples were always analysed in the same batch of commercial kits.

Overview of studies in the thesis

Table 2: overview of study design, sample sizes, data sources, periods of sampling, key dependent and independent variables and data analyses

	Study I	Study II	Study III	Study IV	Study V
Topic	Treatment and pregnancy outcome	Seroprevalence among pregnant women exposed to livestock	Seropositivity and pregnancy outcome	Seropositivity and miscarriage	Exposure to livestock and adverse pregnancy outcome
Design	Case-series	Cohort study	Cohort study	Nested case control	Cohort study
Sample (no.)	19 pregnancies	856 pregnancies	856 pregnancies	218 cases 482 non-cases	82,128 pregnancies
Period of sampling	2007-2011	1996-2002	1996-2002	1996-2002	1996-2002
Independent variables (exposure)		Occupational exposure to livestock, domestic exposure to livestock	Serologic markers of infection with *C. burnetii*	Serologic markers of infection with *C. burnetii*	Occupational and/or domestic exposure to livestock
Dependant variables (outcome)		Seropositivity	Miscarriage, preterm birth, Small for Gestational Age (SGA), stillbirth	Miscarriage (fetal loss before 154 days of gestation)	Miscarriage, preterm birth, SGA, perinatal death
Data analysis	Descriptive	Relative Difference (RD) Relative Risk (RR)	Logistic and linear regression, non-parametric (Wilcoxon) test	Logistic regression	Time-to-event, logistic regression

Study design and populations in the substudies

Study I Case series

Statens Seruminstitut is the only place in Denmark where Q fever serology is performed. For this study, we linked files from departments of obstetrics and infectious diseases from the Regional Hospitals to civil registration numbers from women between 18-45 years who were tested positive for antibodies to *C. burnetii* from 2007-2011. We identified 19 pregnancies in 12 women which fulfilled the following inclusion criteria:

Positive serology with titres available throughout pregnancy allowing for evaluation of *C. burnetii* infection in paired samples.

Studies II, III & IV

These studies were based on pregnancies in the DNBC. In order to address seroprevalence among women with contact to livestock and pregnancy outcome among *C. burnetii* seropositive women, a number of different study populations were sampled: pregnant women with self-reported occupational exposure to livestock, women with self-reported domestic exposure to cattle or sheep and women with no animal exposure (pets not included). Subsequently, the serologic results from these three groups were split into *C. burnetii* seropositive and seronegative, and pregnancy outcome was evaluated comparing these two groups. For the women with animal exposure, seroconversion throughout pregnancy was also assessed.

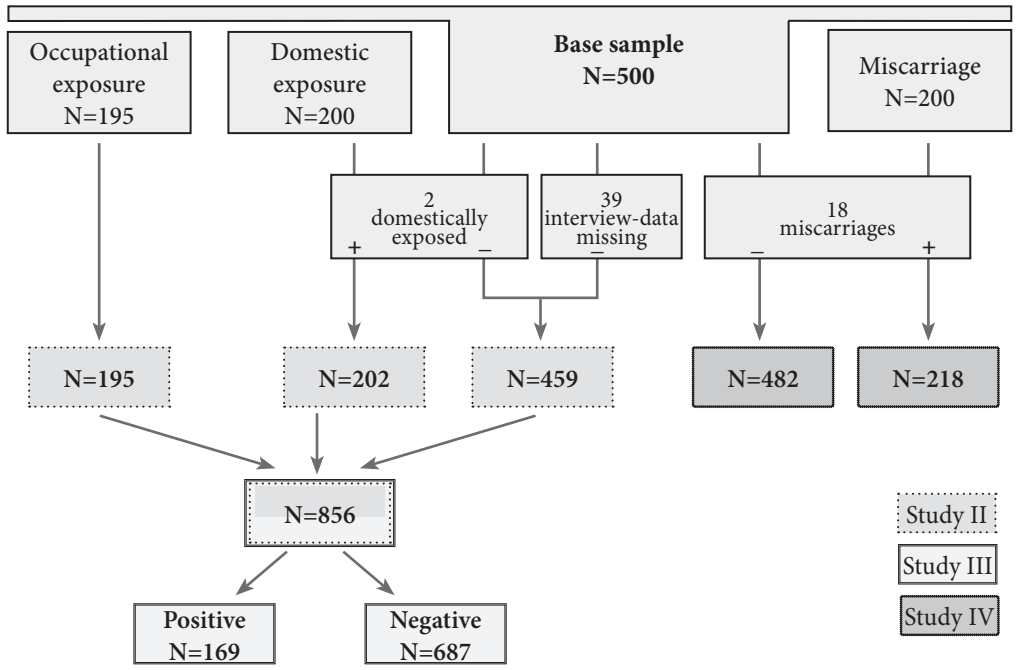
The potential association between serologic markers of infection and miscarriage was also evaluated in a nested case-cohort design, using a case group of women with miscarriage compared to a random sample from the background population.

The group of women exposed through their occupation (n=195) were veterinarians (n=118) and women who worked on a farm with at least 40 dairy cattle (n=77). Domestic exposure was defined as women who reported living with a farmer on a farm with cattle (n=180), sheep (n=22) or both (n=13), but without occupational exposure to these animals.

As a reference group, a sample of 500 pregnancies from the DNBC was randomly selected among the 92,500 participants with an existing blood sample from early pregnancy in the bio bank. This group was used in studies II, III and IV. For studies II and III, the reference group was restricted to women who had participated in the interview in early pregnancy, leaving a reference group of 459 pregnancies.

Study IV was designed as a nested case control study, with 200 pregnancies randomly selected from the 4740 participants who experienced a miscarriage before 154 gestational days (22 gestational weeks) and for whom serum samples from early pregnancy were stored in the bio bank. Eighteen of the pregnancies in the reference group had miscarriage as outcome and were consequently reclassified as cases, leaving 218 cases and 482 non-cases eligible for analysis in this study (Figure 3).

Figure 3: Overview of selection of women's blood samples for Study II, III & IV



Blood samples were analysed for antibodies against *C. burnetii*, and pregnancy outcome was defined as:

Miscarriage: Fetal loss before 154 days (22 gestational weeks) after the first day of the last menstrual period (LMP), with gestational age estimated from the participants' self- reported LMP.

Preterm delivery: Delivery (live births and stillbirths) between gestational weeks 22+0 days and 36 weeks +6 days.

Small for Gestational Age (SGA): for children born from gestational week 37+0 and onwards, SGA was defined as a birth weight corresponding to the lowest 10th percentile in grams and

below for each gestational week. Children with a birth weight above the 10th percentile were used as reference group.

The relationships between serological status, birth weight and gestational age were also evaluated.

***C. burnetii* seroconversion** during pregnancy during pregnancy could be monitored for women with contact to livestock; the strategy was to initially analyse the last existing blood sample. If this sample was tested positive in ELISA, the first blood sample was analysed using ELISA. Confirmatory IFA analyses were performed for the pregnancies in which a change was seen in ELISA from negative in the beginning of pregnancy to positive in the mid-pregnancy or umbilical cord sample, or a doubling in the adjusted ELISA OD (Optical Density) value throughout pregnancy.

Statistical analysis

The strength of the association between exposure to livestock and positive IFA serology was expressed as a risk difference as well as a relative risk for occupational and domestic exposure compared to the reference group according to the prevalence of antibodies against *C. burnetii* in pregnancy. In supplementary analyses, IFA was entered as a continuous variable, and group differences tested with non-parametric (Wilcoxon) test.

Associations between positive *C. burnetii* serology (IFA) and miscarriage, preterm birth and Small for Gestational Age (SGA) were analysed by logistic regression. The association between gestational age at birth and positive IFA serology was tested using a non-parametric (Wilcoxon) test. The association between positive serology (IFA) and birth weight for children born at term was examined by fitting multiple linear regression models.

Study V

Study design, statistical analyses

Four exposure groups were identified from self-reported information in the interviews: occupational as well as domestic exposure to livestock (n=221), occupational but no domestic exposure to farm animals (n=208), domestic but no occupational exposure (n=5,248), and a reference group of women with no occupational or domestic animal contact (n=76,451).

Using these groups, it was investigated whether contact to farm animals was associated with an increased risk of adverse pregnancy outcome, using animal contact as a proxy for the risk of zoonotic infection. The different types of animals were dairy cattle, meat cattle, pigs, poultry, horses, sheep, deer and goats.

Outcome measures were miscarriage, very preterm birth (before gestational week 32), preterm birth (before 37 gestational weeks), Small for Gestational Age (SGA), and perinatal death.

The risk of miscarriage and preterm birth according to animal exposure was estimated as hazard ratios using Cox regression models, with gestational age as the underlying time variable; the association between exposure to livestock and SGA as well as perinatal death was also estimated by logistic regression models.

All analyses were carried out using STATA statistical software, version 11.

SUMMARY OF RESULTS

This section will summarise the main findings of the individual studies. Additional results and more detailed presentations are available in the appended papers.

Study I

We evaluated 19 pregnancies from 12 women and found that 10 out of 19 pregnancies were uncomplicated and resulted in a healthy pregnancy outcome, but nine had obstetric complications; one woman had three miscarriages and an ectopic pregnancy, one had a preterm birth, one had a single fetal demise with a surviving co-twin, and one baby was growth retarded at term. Oligohydramnion and IUGR were found in two pregnancies; of these, one had a healthy outcome, in the other the fetus died a few hours post-partum.

Among these 19 women, three were acutely infected, with symptoms, and at least one was likely to have seroconverted without symptoms close to the beginning of her first pregnancy. Three other women had a serologic profile compatible with probable chronic infection at the end of pregnancy.

Reactivation of an earlier infection in a subsequent pregnancy was seen in two patients who had a post-partum titre fall followed by a fourfold or more increase in titres in their next pregnancy. And although her titres were negative in a previous pregnancy 2 years earlier, one more patient had a serologic profile indicating reactivation.

None of the 10 placentas tested in our study were PCR positive; in the cases of fetal death, no placental or embryo material was tested for *C. burnetii*. In seven pregnancies, treatment with cotrimoxazole was initiated; six of them had healthy, term babies and no mention of severe side effects were found in the women's medical records; one woman who was treated had obstetric complications. In comparison, among the 12 pregnancies with no treatment, eight experienced obstetric complications.

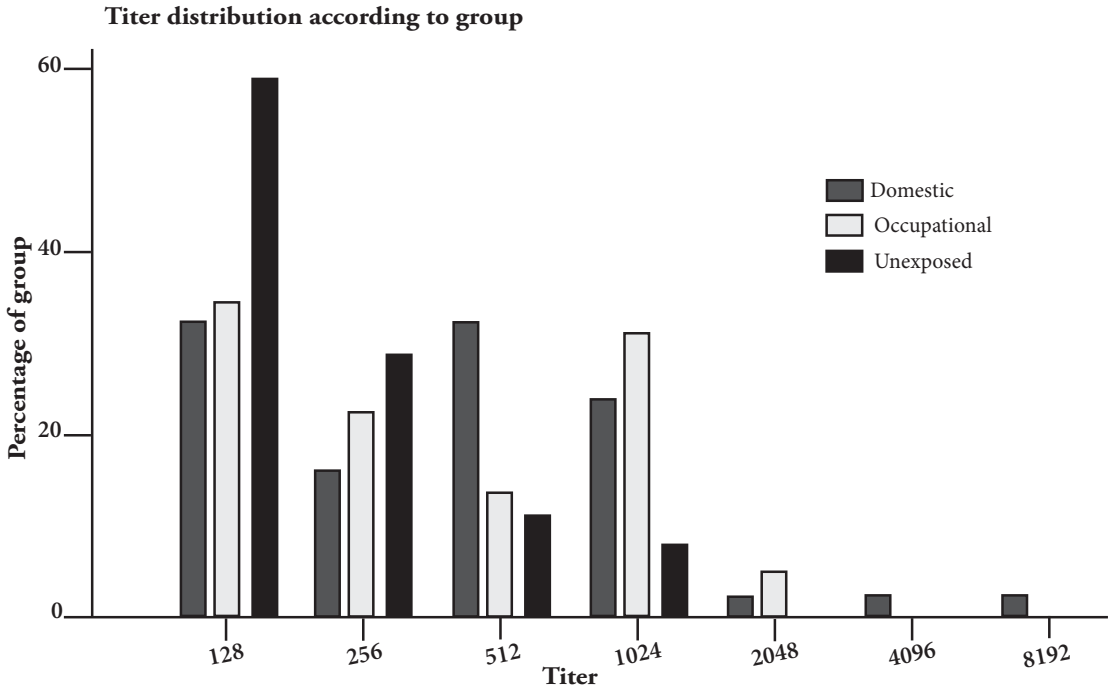
Study II

In the cohort of 856 pregnancies, the proportion of *C. burnetii* seropositive women was substantially and statistically significantly higher in women with occupational exposure to livestock (47.2%) as well as those with domestic exposure to livestock (32.2%) in comparison with unexposed women (4.8%). The risk difference for seropositivity between the occupationally exposed and unexposed women was 42 per 100 (95%CI: 0.35-0.5); the occupationally exposed had a 9.8 times higher risk of being seropositive than did the unexposed women (relative risk: 9.8; 95%CI: 6.4-15.2). The risk difference between the domestically exposed and unexposed women was 27 per 100 (95%CI: 0.2-0.3); the domestically exposed had a 6.7 (95%CI: 4.3-10.6) times higher risk of being seropositive than did the unexposed women.

Analyses based on specific animal contact according to IFA status showed that 23 (74.2%) of veterinarians working with cattle were seropositive, and that the risk of being IFA positive was 2.7 times higher in veterinarians who worked with cattle than it was in those who did not (RR: 2.7; 95%CI: 1.8-4.0), whereas among the domestically exposed women who were exposed to cattle, 64 (33.2%) were IFA positive.

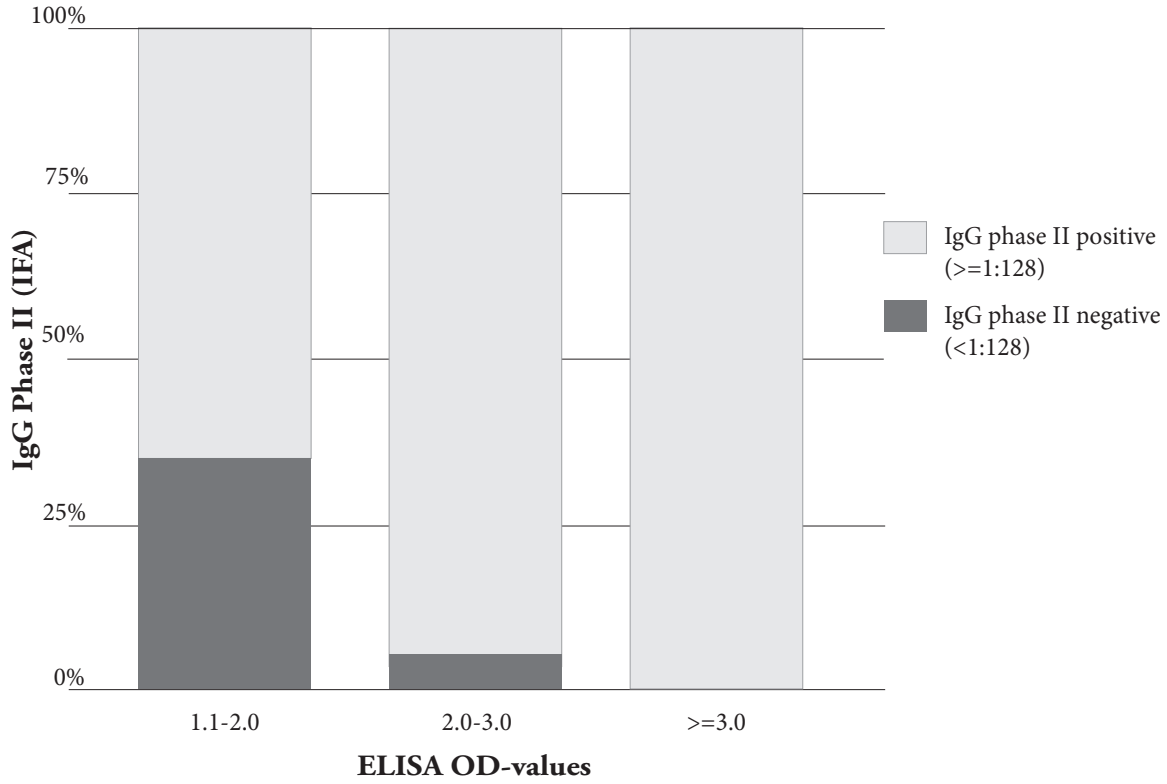
Figure 4 shows the distribution of positive IgG phase II titres in the three groups and illustrates how unexposed women are mainly "low-positive", whereas the "high-positive" titres are primarily from the two groups of exposed women.

Figure 4: Distribution of IgG phase II titres in the three exposure groups (Study II)



In order to illustrate coherence between OD values in ELISA and IFA results and to support the choice of strategy in the analysis, the relationship between IgG phase II positive ELISA and IFA results is illustrated in Figure 5. The IFA positive results were those with high levels of adjusted OD values (optical density values measuring antibody concentrations) in ELISA.

Figure 5: Immunofluorescence antibody (IFA) IgG phase II according to enzyme-linked immunosorbent assay (ELISA) optical density (OD) values



It was concluded that pregnant women with occupational or domestic exposure to cattle and/or sheep had a high prevalence of antibodies to *C. burnetii* compared to unexposed pregnant women and that contact with livestock is a risk factor for *C. burnetii* in Denmark.

Study III & IV

Among the 856 women, antibodies against *C. burnetii* (IFA) were detected in 169, while 687 women were IFA negative. In Study III, no association was found between positive serology and risk of miscarriage (adjusted OR: 1.5; 95%CI: 0.3-7.6) or preterm birth (adjusted OR: 0.4; 95%CI: 0.1-1.1).

Infants born to seropositive mothers had a 0.9 day older gestational age than infants born to seronegative mothers, and this difference was not significant (p = 0.06, Wilcoxon non-parametric test).

When evaluating the birth weight for all newborns, there was a significant weight difference (168 g; 95%CI: 70-267 g), with the IFA positive babies being heavier; results were similar when restricting analyses to term babies (37 completed gestational weeks or more): (134 g; 95%CI: 47-221 g).

No association was found between SGA and seropositivity (IFA) (OR: 0.4; 95%CI: 0.8-1.0). One IFA negative woman had an induced abortion after gestational week 12 due to fetal disease. One preterm birth was a stillbirth in gestational week 23; two women had stillbirths in gestational week 35; all were IFA negative.

A total of 14 women met the criteria for seroconversion during pregnancy in ELISA. These were confirmatory tested in the IFA; 10 of them seroconverted during pregnancy. All had occupational or domestic contact to livestock. All gave birth to live babies at term; however, two newborns were SGA (Table 4, Study III).

None of the seroconverters reported episodes of fever during pregnancy at the interview done at the beginning of the 3rd trimester.

Based on these findings, it was concluded in Study III that seropositivity was not associated with adverse pregnancy outcomes in this study as no elevated risk of miscarriage, preterm birth, or low birth weight among pregnancies positive for *C. burnetii* was found in comparison with seronegative Danish pregnant women.

When evaluating the association between antibodies to *C. burnetii* and miscarriage independently among 218 pregnancies ending in miscarriage and 482 non-cases (pregnancies without miscarriages until 22 gestational weeks) (Study IV), one (0.46%) case was IFA positive and three (0.62%) non-cases were IFA positive. No relation was found between serologic markers of Q fever and miscarriage (OR: 1.19; 95%CI: 0.12-11.70). In this study (IV) the Danish cut-off value was used, with the equivocal zone classified as negative; therefore adjusted odds ratios were also calculated using the ELISA results, and findings were similar (OR:0.94; 95%CI:0.12-11.7), i.e. that there was no major association between *C. burnetii* infection and miscarriage in humans.

Study V

Among the 82,128 women included in Study V, a total of 5830 (6.9 %) reported occupational or domestic contact with livestock in their pregnancy or 3 months prior to becoming pregnant.

Neither occupational nor domestic animal exposure was found to be associated with miscarriage, preterm birth, SGA or perinatal death (Tables II & III, Study V).

Interview data was obtained after miscarriage for a considerable number of miscarriages in the cohort. However, in the analysis restricted to women who were interviewed while still pregnant the estimates obtained were essentially the same.

Stratified analysis by different types of animal contact (sheep, cattle, pigs, poultry and other) did not change this, and analysis restricted to women who were employed or had been unemployed for a maximum period of 6 months prior to becoming pregnant did not change any of the outcome measures significantly.

DISCUSSION

The present research was designed in the light of a paucity of previous investigations on the impact of Q fever in pregnant women and the fetus. The picture painted at the time of design was one of complete lack of clinical experience in handling the infection in pregnancy in Denmark, challenged by a sudden and serious concern arising from findings in France.

Our approach was to use existing biological specimens and interview data as well as to follow the pregnancies in which an infection was a high risk, suspected or confirmed, beginning from 2007-2008 when Statens Serum Institut had to initiate a "Q fever hotline" due to a very steep increase in demand for serological analyses and interpretation. Furthermore, we wanted to address zoonotic pathogens and their possible impact during pregnancy on a larger scale. The population-based design, the almost complete follow-up, and the availability of blood samples throughout pregnancy made the DNBC unique, valuable and obvious for the purposes of our research, and we regard the size of our studies grounded in this cohort a major strength.

In Study I, we identified a total of 12 seropositive women who underwent 19 pregnancies; obstetric complications were recorded in nine of the 19 pregnancies included.

In Studies II, III & IV we found a high prevalence of antibodies to *C. burnetii* among pregnant women exposed to livestock compared to the prevalence in randomly selected unexposed pregnant women. In none of the population-based studies using pregnancies and blood samples from the DNBC did we find any increased risk of adverse pregnancy outcome among seropositive women.

Moreover, we found no association between exposure to Danish livestock and adverse pregnancy outcome (Study V). We found it reasonable to assume that most of the women with domestic or occupational contact with livestock were exposed to zoonotic pathogens, primarily campylobacter and salmonella, but also toxoplasma, listeria, *Yersinia enterocolitica*, VTEC or *C. burnetii* exposure [74,86-91]. But analyses in separate categories for occupational and domestic exposure, restrictions to women in the labour market, and stratification on specific animals failed to change any results.

Cut-off value

Denmark experienced a rising interest in Q fever from 2007-2008. The increased attention was primarily due to diagnostic awareness and probably testing "in excess" rather than to the true emergence of a new infection [74]. Following the increased attention, Villumsen et al. established a national cut-off value [85]. The authors chose a very restrictive cut-off value when defining a local baseline. This was based on the assumption that Q fever only occurred sporadically in Denmark and on the aim to obtain a high specificity as well as a high predictive value of a positive result.

When diagnosing Q fever, some countries have defined their own cut-off values, while others use the cut-off value recommended by the manufacturer. This use of different cut-offs or criteria for the interpretation of serological results hampers the generalisability and inference of results reported in studies from different countries, and this has prompted many discussions over the past years. A recent study used a cohort of Q fever patients to compare serological and PCR results. Although the same IFA method was used, there was large discrepancies in the IFA results between three reference laboratories, and the authors proposed the establishment of an international standard of Q fever serological investigation [92].

In order to bypass the inevitable choices included when defining a cut-off value, we performed supplementary analyses based on quantitative measures. In Study IV, the quantitative comparison of ELISA OD values between cases and non-cases independent of cut-off values further supported our conclusion; in Study II, analyses with IFA (IgG phase II) as a continuous variable rather than dichotomised into negative and positive showed that the titres were significantly higher in the two exposure groups than in the unexposed.

Results from recent studies reveal that, particularly in the rural populations of Denmark, Q fever is more widespread than earlier assumed [74,75]. One may now argue that the Danish cut-off value is too conservative. Furthermore, the purpose of obtaining a high specificity is not ideal in all settings and clinical situations since infected patients may be falsely classified as seronegative without further testing.

As mentioned earlier, Villumsen et al. defined an "equivocal zone" (Table 1), proposing that patient with relevant symptoms and an a priori elevated risk that had equivocal titres should be considered probably positive. The predictive value of a serological indicator would be higher among such patients than among asymptomatic individuals with low risk of exposure. Studies I, II and III were conducted later than Study IV and included groups with high risk of exposure. Consequently, we decided to use a modified version of the Danish cut-off value (a sample was considered IFA positive when any of the phases were 128 or above). In Studies II and III, analyses were repeated with the equivocal titres classified as negative. The latter

conservative interpretation of the serological values would theoretically lead to a lower positive prevalence and higher predictive value. However, the trend was the same for all results, and no associations between seropositivity and adverse outcome of pregnancy were revealed.

We acknowledge that even the adjusted Danish cut-off value is high compared with the cut-off value used in some other studies. However, application of a lower cut-off value could have falsely classified additional women as seropositive and led to misclassification and thus a higher risk of overlooking a potential association between true seropositivity and adverse outcome of pregnancy.

Methodological considerations

C.burnetii seroconversion versus acute Q fever

Interestingly, seroconversion was not very frequent as most of the seropositive women (studies II and III) had markers of previous infections and only 10 met the criteria for IFA seroconversion. This number is low in terms of quantifying the risk of adverse pregnancy outcome among seroconverters.

However, a large part of the anxiety as regards Q fever and pregnancy concerned the risk of reactivation of infection among women with signs of past infection. It was exactly this question that our studies had the power to address, whereas the inferences as regards outcomes in women with acute infection or clear-cut chronic infection are much more limited.

Moreover, a high seroprevalence of *C. burnetii* accompanied by a low frequency of clinical symptoms in farmers and veterinarians has been found in Denmark as well as abroad [74,76]. The vast majority of the seropositive women in our studies were exposed to animals, and because the number of unexposed seropositive women was small, we cannot draw conclusions regarding adverse pregnancy outcome in this group or clarify whether the dynamics of infection differ in unexposed women compared to women more heavily exposed to *C. burnetii*.

Validity of the data on early pregnancy loss (Study III, IV & V)

The median gestational age at recruitment to the DNBC was 10 weeks (25 and 75 percentiles: 7 weeks; 13 weeks). One third of all implanted pregnancies fail to survive beyond midpregnancy, with the vast majority occurring in the 1st trimester [93,94]; although gestational age at recruitment was less than 8 weeks for 40% of the cases (miscarriages) in Study IV, very early fetal

loss, which constitutes the largest proportion of miscarriages, is not included in Studies II–V. These very early miscarriages are insufficiently registered and a general challenge when studying pregnancy outcome, as the high mortality in early pregnancy becomes an element when interpreting almost every pregnancy outcome that follows [94]. Consequently, we cannot exclude a harmful effect of *C. burnetii* infection in very early pregnancy.

Sample size and Statistical issues

Studies II–V were based on prospectively collected data from what is still the largest cohort of pregnant women in the world, but the number of participants in Studies II, III and IV was limited by accessibility to blood samples. We included all veterinarians in the DNBC. In order to obtain sufficient statistical power, we also sampled a similar sample size of women with domestic exposure to livestock and a larger reference population from the DNBC.

Power calculations were based on the literature, and, on existing Danish data [95]; it was assumed that the prevalence among exposed women would be 10% and 2% of that in the background population. With a sample size of 200 exposed and 200 unexposed, an odds ratio of 5 could be detected by a power of 88% and a two-sided significance level of 0.05. The incidence of miscarriage and preterm birth in the Danish National Birth Cohort is about 5% for both. By sampling 200 exposed (seropositive) and 500 unexposed (seronegative), an odds ratio of 3 could be detected by a power of 80% at a two-sided significance level 0.05.
When addressing pregnancy outcome, Studies II, III and IV turned out to be underpowered due to the much lower than expected frequency of adverse pregnancy outcome. Thus, we experienced limited statistical power at some point in most of the analyses.

Selection bias

Selection bias is present if the association between exposure and outcome differs between study participants and those theoretically eligible for the study, including those who were invited but did not participate [96,97].

Only about 30% of the women eligible were enrolled in the DNBC. Recruitment to the DNBC occurred at the first antenatal visit, and selection took place at various levels, first at the general practitioner, where about only 50% of the general practitioners informed the pregnant women about the study, and secondly at an individual level.

We consider it unlikely that women exposed to animals should be more liable to not participate than other women and besides, this would only be a problem if associated to the outcome, i.e. if women with animal exposure had a higher proportion of adverse pregnancy outcome. Nohr et al. have quantified the impact of the initial selection into the DNBC and found that

the participants were more often of normal weight, nonsmokers or previous smokers, and more likely to have IVF (In vitro fertilisation) pregnancies. They also had a lower rate of preterm births and infants with SGA. However, when compared to a more complete dataset, the estimated effect on the risk estimates was small and selection bias a minor problem [98,99]. Data also showed that women with fetal death and those with early miscarriage were more prone to be non-participants [100]. However, some of the women who chose to not participate in interviews delivered a blood sample in early pregnancy. This was disclosed in Study IV, where aborters and non-aborters were chosen at random and irrespective of participation in interviews; for 28% of women with miscarriage no interview data were available, whereas this was the case in 5% of the non-aborters (Table I, Study IV). The study population was biased towards a "healthy pregnant population" due to selection of women with fewer miscarriages; as *C. burnetii* infection in the 1st trimester may constitute a specific risk of miscarriage [27], there is the possibility that an increased risk in early pregnancy may in Studies III, IV and V be reflected by a "protective" effect in later pregnancy if studying pregnancies that have successfully survived through the most vulnerable period.

Information bias

Information bias occur as a consequence of measurement error, i.e. if the exposure or the outcome is subject to misclassification, this will bias the results. Misclassification will be differential if the exposure is associated with the outcome; if not, it is non-differential. Differential misclassification may lead to unpredictable information bias, while non-differential misclassification of dichotomous variables most often will bias the results towards the null [96,101].

We used self-reported contact with livestock as a proxy for zoonotic infections. During the years of data collection to the DNBC (1996-2002), women would presumably not link an adverse pregnancy outcome to animal exposure. However, some differential misclassification cannot be ruled out among women who were interviewed after a fetal loss because they may have a tendency to report all exposures more accurately, resulting in recall bias.

Ascertainment of pregnancy outcome in the DNBC was based on national registers. Due to uncertainty about the exact time of conception, determination of gestational age at birth has inherent limitations, and some degree of measurement error will persist. The use of obstetric ultrasound increased during the years of data collection for the DNBC, with a trend towards ultrasound becoming more common than the LMP date in determining gestational age [102]. Some degree of non-differential misclassification must be expected in any analyses using gestational age, and our results could be biased if data on gestational age from the last years of data collection were based on ultrasound to a greater extent than during the first years, and adjustment for calendar year could therefore have been justified.
It is, however, unlikely that determination and reporting of gestational age should be differen-

tial by infection status. We used the self-reported LMP in analyses of miscarriage and registry data for all other outcomes.

Confounding

Confounding is a systematic error that leads us to confuse the effect of an exposure with the effect of another variable, the confounder. In contrast to information and selection bias, confounding can be accounted for not only in the study design (by randomisation, restriction or matching) but also in the analyses of data (e.g. by standardisation, restriction or matching or in multivariate regression models). A confounder must be associated with the exposure as well as the outcome and should not be a part of the causal pathway (e.g. an intermediate step) [96,101,103].

Confounding in our studies could arise from differences in health behaviour as a result of the reproductive experience of the women [104]. If women exposed to livestock had knowledge of a suspected increased risk of adverse pregnancy outcome within their profession, then those with a history of negative outcome might be more prone to participate than women with a previous healthy pregnancy outcome. Also, those with a history fetal loss or other adverse outcomes may have a higher baseline probability of adverse pregnancy outcome. Conversely they might alter their behaviour in subsequent pregnancies as a result of their awareness of this, having a tendency to avoid animal exposure which could introduce behaviour modification bias. To capture uncontrolled confounding by any behavioural modification related to knowledge about reproductive history, success or the opposite, analyses were replicated with restriction to a subgroup of primigravid women with a short time to pregnancy interval (<6 months); the results were, however, essentially unchanged (Study V). Also, in Study III, stratified analysis on contact with livestock and pregnancy outcome (miscarriage abortion and preterm birth), irrespective of titre status, showed no significant difference between the groups.

Furthermore, there could be characteristics entailing different behaviours in women living or working on farms that could alter their pregnancy outcome. Heavy physical work with occupational lifting throughout pregnancy and less focus on healthy lifestyle in pregnancy should have been taken into account if we had found an association between animal contact and adverse pregnancy outcome. Other confounding factors could be socioeconomic status or strenuous leisure time physical exercise [105]. However, for women who work or live on a farm with physical activity incorporated in daily routines, a possible effect of leisure time exercise can be difficult to quantify. If socioeconomic status was an essential risk factor for any of the outcomes included in this cohort, it would have resulted in different estimates in subanalyses on women with a connection to the work force (Study V). As illustrated in Table 3 the women in the DNBC with animal exposure were essentially similar to unexposed women,

and although it does not eliminate additional confounding, it justifies our a priori decision to adjust all events for three important risk factors for adverse reproductive outcome.

Table 3:Characteristics of 82,128 women from the DNBC (Study V)

	Animal exposure (n=5,677)	No animal exposure (n=76,451)
Alchohol (weekly consumption)		
0 drinks	3,308 (58.3%)	46,786 (61.8%)
<1 drinks	914 (16.1%)	11,819 (15.5%)
1-3 drinks	17,767 (23.4%)	1,446 (25.5%)
Coffee (daily consumption)		
0 cups	62 (1%)	957 (1.25%)
< 2 cups	3,675 (64.7%)	54,328 (71%)
2-<4 cups	935 (16.5%)	10,961 (14.3%)
4+ cups	1,004 (17.7%)	10,174 (13.3%)
Smoking (daily)		
Non-smokers	4,827 (85%)	64,222 (84%)
1-<10 cigarettes	407 (7.2%)	6,173 (8.1%)
10+ cigarettes	441 (7.8%)	6,016 (7.9%)
Pre-pregnancy BMI		
<18.5	180 (3.2%)	3,507 (4.6%)
18.5-<25	3,496 (61.6%)	51,334 (67.2 %)
25-<30	1,322 (23.3%)	14,305 (18.7%)
30+	581 (10.2%)	6,004 (7.9%)
Exercise (hours per week)		
No exercise	3,722 (65.6%)	47,330 (61.9%)
Exercise, but less than 3.5 hours	1,521 (26.8%)	23,585 (30.9%)
3.5 hours or more	423 (7.5%)	5,362 (7%)
Parity		
0	2,185 (38.5%)	39,265 (51.4%)
1	1,820 (32%)	25,934 (34%)
2	1,237 (21.8%)	9,239 (12.1%)
>2	432 (7.6%)	1,963 (2.6%)
Spontaneous abortions		
0	4,579 (80.7%)	63,289 (82.8%)
1	780 (13.7%)	9,973 (13%)
2	217 (3.8%)	2,211 (2.9%)
3+	95 (1.7%)	910 (1.2%)
Time to pregnancy		
none	864 (15.2%)	11,685 (15.3%)
<=12 months	3,974 (70%)	54,486 (71.3%)
>12 months	817 (14.4%)	10,036 (13.1%)

Age is an important factor determining miscarriage risk, and smoking is a well-known risk factor for preterm birth. Adjustment for smoking in the analyses of miscarriages was justified by the inconsistency of previous findings related to smoking and miscarriage.

When studying reproductive epidemiology, adjustment for previous pregnancy outcome is controversial because one adverse pregnancy outcome may strongly predict the occurrence of another [94,106]. Women may change behaviour as a result of a previous poor pregnancy outcome, and analysis adjusting for previous pregnancy outcome without having data on the exposures for previous pregnancies may be biased by a dependency between current exposure and past pregnancy history [94,107].

It is unlikely that women would associate a poor pregnancy outcome with animal exposure during the years of data collection to the DNBC, and the above-mentioned stratified analyses in primigravid women in Study V justifies adjustment for earlier pregnancies since the results were essentially the same as in the primary analyses. However, linking pregnancy outcome to exposure to livestock may have occurred among the case women in Study I due to the increasing focus on Q fever among veterinarians in recent years. This should be taken into account if we were to conduct a study now, as a previous adverse pregnancy outcome could potentially be an intermediate factor in the analyses of adverse pregnancy outcome for women exposed to livestock.

Main findings in the light of other studies

Our findings of a high prevalence of antibodies to *C. burnetii* among pregnant women with occupational or domestic exposure to livestock are in line with two recent Danish studies on the presence of antibodies to *C. burnetii* among people working with livestock; they found the highest prevalence of antibodies among veterinarians (36% and 39%,respectively) [74,75].
In general, a higher seroprevalence has been found in studies evaluating seroprevalence in groups handling livestock, especially veterinarians, than in studies of seroprevalence in the background population [76,78,108-110] (Table 4). In one Dutch study of veterinarian students, 18.7% were seropositive [111]; in another, 65% of 189 veterinarians were seropositive; the number of hours with animal contact per week, the number of years since the participants had graduated, living in a rural area, and working as practicing livestock veterinarian were risk factors in that study [79]. In a third study, seroconversion was 17.5% among Dutch culling workers who were seronegative before culling, demonstrating the extreme infectivity of *Coxiella burnetii* [112].
Few large population-based studies have examined the seroprevalence in the background population. A Dutch study analysed sera from a large population-based study conducted in

2006-2007, before the outbreak, and found a seroprevalence of 1.5% [113] . An Irish study tested 2,394 randomly selected blood samples from a population-based survey of cardiovascular risk factors performed in 1986 and 1987 and found a seroprevalence of 12.8%, with 48.8% of farmers being seropositive [114]. An American study tested sera from 4437 participants from the National Health and Nutrition Examination Survey, 2003–2004, and found 3.1% to be seropositive [115]. The comparability of the seroprevalence studies are limited by the use of different cut-off values (Table 4).

The evidence of Q fever's adverse impact on pregnancy outcome mainly originates from French case studies of referred infected pregnant patients and pregnancies with Q fever diagnosed retrospectively after an adverse pregnancy outcome [26,27,60].

The overall outcome of our research failed to corroborate the findings from the French studies, but is much in line with recent studies from the Netherlands. Here two large studies on the risk of Q fever in pregnancy have been conducted. One study included serum samples from early pregnancy (from the 12th gestational week) of 1174 pregnant women living in the high-risk area. They found no association between positive Q fever serology and preterm delivery (delivery before 37 gestational weeks), low birth weight (<2,500 g), low birth weight for gestational age (<10th percentile), and fetal or neonatal mortality, congenital malformations or a 5-minute Apgar score less than 7 [66]. However, early miscarriages were not included. The other study was a large randomised trial of 1229 pregnant women. In the intervention group, antibiotic treatment was given in the case of acute or chronic infection; in the control group, sera was frozen and analysed after delivery. This study found 15% of the women to be seropositive in the intervention group as well as the control group, no significant difference in obstetric complications was found, and routine screening in pregnancy of women living in high-risk areas was not recommended [65]. As in our studies III and IV, the vast majority of positive samples in the two Dutch studies indicated previous infection.

Table 4: Overview of seroprevalence studies

	Numbers included	Numbers seropositive	Test used	Cut-off values	Sera collected	Published
Studies of seroprevalence in people with animal exposure						
Bacci S et al.	1613	177 (11%)	IFA	Danish cut-off values: IgG phase I: Titre >=1:512 IgG phase II: Titre>=1:1024 IgM phase I: Titre >=1:128 IgM phase II: Titre >= 1:256	2006-2007	2009
Bosnjak E et al.	359	39 (11%)	IFA	Danish cut-off values	2008	2009
Whitney EA et al.	508	113 (22%)	ELISA (PanBio) Samples equivocal or positive (>9 "PanBio units") tested with IFA	IgG phase I: Titre >=1:16 IgG phase II: Titre >=1:16	2006	2009
Marrie TJ	110	24 (21.8%)	Complement Fixation test; IFA	Fourfold rise in CF or IFA	1982-1983	1985
Abe T et al.	267	36 (13.5%) IgG positive	IFA	Titre >=1:64 for all phases	1997-2000	2001
Casolin A	829	89 (10.7%)	IFA and complement-fixing antibody	Titre >=1:10 for all phases; complement-fixing antibody >= 1:2.5	1996-1997	1999
Whelan J et al.	246	43 (17%)	ELISA (Virion) Titres>30IU/mL were confirmed by IFA	Titre >=1:32 for all phases	2009-2010	2011
Van den Brom et al.	189	123 (65.1%)	ELISA (Focus) and IFA	IgG phase I: Titre >=1:32 IgG phase II: Titre >=1:32	2009	2012
De Rooij MMT et al.	674	126 (18.7%)	IFA	Titre >=1:32 for all phases	2006	2012
Studies of population-based seroprevalence						
McCaughey C et al.	2394	306 (12.8%)	ELISA	Adjusted OD value: >11	1986-1987	2008
Anderson A et al.	4437	180 (3.1%)	ELISA (Panbio) equivocal/positive samples confirmed by IFA	IgG phase I: Titre >=1:16 IgG phase II: Titre >=1:16	2003-2004	2009
Schimmer B et al	5654	85 (1.5%)	ELISA (Virion/ Serion) >20 U/ml were confirmed by IFA	IgG phase I: Titre >=1:32 IgG phase II: Titre >=1:32	2006-2007	2012

Munster et al. (Netherlands) described placental histopathology and clinical outcome of five women with asymptomatic *C. burnetii* infection, and found that the four available placental biopsies were PCR negative [64].

In the French landmark study of 53 cases [27], placentitis was found in eight of 23 placentas tested; in the latest French study with 30 cases, no placentitis or isolation of *C. burnetii* was found in the 14 available biopsies [60].

In a recent German study by Boden et al., none of the seven placentas investigated were culture or PCR positive PCR, and none of 11 women who were seropositive had a chronic serologic profile [116].

None of the 10 placentas tested in our study (Study I) were PCR positive.

Regarding handling Q fever in pregnancy, there is no consensus about screening method or treatment [117]. The French recommendation of treatment of seropositive women with cotrimoxazole throughout pregnancy is widely practised, but has recently been questioned [116,118]. Boden et al. found no obvious association between *C. burnetii* infection and negative pregnancy outcome among 11 women infected in pregnancy; nine women were treated with antibiotics but only three received the recommended long term cotrimoxazole, and the authors conclude that the recommendation of long-term cotrimoxazole treatment for every pregnant woman with laboratory confirmed Q fever is questionable.

To our knowledge, no strain was isolated from placentas during the Dutch outbreak. In the latest French study, eight of nine human isolates from six different cities in the Netherlands were identified as genotype MST33, and they all harboured the QpH1 plasmid [60]. One dominant MLVA (Multiple-Locus Variable number tandem repeat Analysis) genotype was identified among goats and sheep throughout the infected area [68,119], and MST (Multispacer Sequence Typing) found the presence of genotype MST33 in clinical samples from goats and sheep [120].

Although *C. burnetii* infection is widespread in cattle in the Netherlands, the human outbreak, with more than 4000 humans infected, was not linked to cattle but most likely resulted from *C. burnetii* with the genotype MST33 in the goat population [120-122].

The plasmid type is associated with the genetic content [123,124]; among the human isolates investigated, Angelakis et al. found that many of the French strains carried the QpDV plasmid which has been associated with acute Q fever. The authors found that strains isolated from placentas of infected women with miscarriage primarily harboured the QpDV plasmid and suggest that strains with this plasmid more often lead to miscarriage [60].

In comparison to France and the Netherlands, there are few sheep and goats in Denmark; the source of infection here is primarily cattle, and as far as we know Denmark has never experienced a clinically verified Q fever outbreak. Likewise, epidemic abortions in cattle have never been reported, and the number of *C. burnetii* shed overall in dairy milk is in low [21,125].

Nielsen et al. have found that the level of antibodies in bulk tank milk from Danish dairy cattle may be associated with perinatal mortality in calves, but their results were not consistent [126]. Hansen et al. have examined cotyledons from Danish dairy cattle and found that placental infection was more likely in herds with intermediate or high antibody levels in bulk tank milk, and that *C. burnetii* infection was rarely associated with inflammation. The authors conclude that the pathogen will be excreted during calving even in herds that are bulk tank milk negative, and that lack of inflammation may indicate that the pathogen is in a latent state and thus less virulent [127]. This could partially explain why Q fever in cattle is usually not clinically apparent and therefore does not show up as a risk factor in our studies.

CONCLUSION

Our findings of high levels of antibodies against *C. burnetii* in Danish pregnant women exposed to livestock show that *C. burnetii* is not a newly emerged pathogen in Denmark and that Q fever is endemic here, as it probably is in most other countries.

In our DNBC studies, we found no association between exposure to Danish production animals and adverse outcome of pregnancy, and our studies failed to show a higher risk of miscarriage, preterm birth or SGA weight among pregnant women positive for *C. burnetii* than among seronegative Danish pregnant women.

We hypothesised that being seropositive in pregnancy would be associated with adverse pregnancy outcome, potentially mediated by reactivation of a latent infection.
In Study I, reactivation in a subsequent pregnancy of a previous infection was seen in two patients, and another also had a serologic profile indicating reactivation.

The seropositive women in Study III did not have a history of a higher proportion of previous miscarriages compared to the seronegative women, indicating that recurrent miscarriage caused by reactivation of *C. burnetii* was not an issue in this population. The few women with seroconversion indicating acute as well as chronic Q fever suggests that seropositivity is primarily a sign of past exposures rather than a cause of great concern.

The 10 women who experienced seroconversion in Study III, the asymptomatic seroconverters, and those reporting symptoms of acute Q fever in Study I are too few to quantify pregnancy outcome in women with acute and, in particular, symptomatic infections. The risk of miscarriage/stillbirth was zero for the 10 seroconverters. In an attempt to estimate the risk of negative outcome in this material, we used an exact method, the Clopper-Pearson interval. This gave a confidence interval of 0 to 0.3085, meaning that, in the DNBC material, a risk above 31% for an adverse pregnancy outcome can be excluded. This contrasts the findings in the case-series with adverse outcome among 47% of the pregnancies, and expresses the difference in using population based studies and clinical cases which always entails bias.

Addressing chronic infection and how to handle these postpartum is also limited as only three women in Study I had a serologic profile compatible with probable chronic infection at the end of pregnancy.

The fact that placental material was unavailable or tested negative in all 9 out of 19 pregnancies with obstetric complications (Study I) limits a definite conclusion regarding Q fever as the cause of adverse pregnancy outcome for these women. Still, in contrast to Studies II–IV, this study reveals that Q fever in pregnancy may be problematic in Denmark, although not to the same extent as in France.

With this thesis, we have sought to achieve applicable results and, importantly, to conduct studies large enough to challenge the extremely precautious clinical guidelines written when Denmark was struck by concern related to Q fever in pregnancy 2007-2008.

We trust that we have contributed to further delineation of how to handle Q fever in pregnancy. Our results substantiate how contact to livestock is a risk factor for *C. burnetii*, and we conclude that Q fever should be considered a possible differential diagnosis in people with close contact with livestock, especially veterinarians and women domestically exposed to cattle. We have used the evidence from present studies in the revision of guidelines, deeming it safe for women exposed to livestock to continue to work during pregnancy as long as they are monitored with serology, along with a de-emphasis of the precautions regarding labour for infected women.

After years of studying this infection and following numerous discussions with international experts in the field, I am now strongly convinced that the Danish cut-off value is too restrictive from a clinical as well as from an epidemiological point of view. My suggestion is to change the equivocal zone to positive, as we have in Studies I, II and III. Along with clinical guidelines underlining that infection should always be evaluated in paired samples and never in just one blood sample, a valuable increase in the number of correct diagnoses of Q fever infection would be obtained in Denmark.

PERSPECTIVES

An exposure or a disease qualifies as potentially hazardous when statistical associations are found in epidemiological studies. However, with possible publication bias in mind, it is more difficult to interpret a number of studies with no association and therefore to agree on the absence of risk.

The complexity in segregating harmless seroconversion from infection that may jeopardise maternofetal health has been thoroughly illustrated in most of the studies on Q fever and pregnancy.

Against the background of the existing studies on pregnancy and Q fever, we now know that pregnancy outcome may be healthy for a majority of seropositive women without use of cotrimoxazole. On the other hand, based on our findings, we cannot dismiss the incrimination of a hazardous effect on pregnancy, and further insight is warranted regarding when this infection poses a threat to the mother and the fetus.

Optimisation of risk assessment calls for further research on risk groups, preventive measures, routes of transmission, strain specificity, trimester of infection, diagnosis and indications for treatment.

Defining groups at risk has been partially accomplished. The predominant role of livestock, particularly during parturition, in the transmission of human Q fever is irrefutable, but livestock management practices may change.

The human vaccine against Q fever (Q-VAX® Q Fever Vaccine, CSL Biotherapies) has been available since 1989 [80]. A recent study has confirmed the effectiveness of the National Q fever vaccination programme in substantially reducing the burden of Q fever (notifications and hospitalisations) among occupationally exposed groups in Australia [128]. The vaccine was made from the Henzerling strain, is rather old, and legal constraints along with the risk of adverse reactions in people previously exposed makes it highly unlikely that it will ever be widely licensed in Europe.

A new generation of human vaccine is required to confer protection against Q fever infection without the necessary screening for prior immunity before vaccination. The likelihood that Q-

vax will not be approved for licensing outside Australia has led to a search for the development of safe and effective new vaccines, including projects aiming to produce a vaccine through the combined cloning of portions of the O antigen of the LPS molecule heterogeneously with immunogenic proteins [129]. The animal vaccine (Coxevac, CEVA) has been used in France and the Netherlands; it has been licensed for use in Denmark and is currently used sparsely in Danish cattle [55,56].

Complete removal of risk groups from exposure to livestock appears inordinate, but preventive measures in Denmark could be vaccination of risk groups, if and when a new vaccine effective against the strains present is developed.

Coxiella burnetii with its resilient nature remains a query microorganism, and it is not well understood why its outbreak potential seems to be different in different settings. The recent findings of Hansen et al. [127] indicate that the organism found in Danish cattle is in a latent state and is therefore less virulent. This could be a partial explanation why outbreaks of clinical Q fever in humans have not been related to cattle, and the disease may be less severe than described from e.g. France, consolidating our results.

The organism has an unusual stability, and environmental factors may contribute to the spread of infection by, for example, airborne dissemination in a dry, dusty and windy rural environment. Doses of infectious material may be higher in an outbreak setting than in a more silent endemic transmission scenario (as seen in Denmark). Furthermore, numbers of shed bacteria and types of strain may differ from one host animal or one geographic setting to another. It is intriguing that some of the drivers for outbreak potential may also be related to the heterogeneity found in different clinical outcomes, which may arise from differences in virulence as well as host reservoirs.

The increasing availability of complete genome sequences has increased our understanding of the genetic diversity among different strains, but still, the intricacies of its genomic information leave many questions.

Expansion of typing methods that can discriminate strains is pivotal when trying to improve our understanding of Q fever and has been assessed during situations like the outbreak in the Netherlands [130,131].

There are still pregnancies at risk which are not adequately approached and lack of knowledge on whether infection in very early pregnancy poses the biggest threat or not. Moreover, addressing the indication for treatment of all seropositive pregnant women with potentially teratogeneous antibiotics cannot wait for future studies but is a present, clinical challenge.

Munster et al. conclude that the existing evidence is insufficient to recommend routine screening for *C. burnetii* infection during pregnancy in high-risk areas in the Netherlands, based on their review using the Wilson and Junger criteria [132]. This cannot be applied to the setting in Denmark with no outbreaks, but the suggestion in some studies that Q fever should be added to the aetiological agents responsible for intrauterine infections associated with morbidity and mortality during pregnancy, grouped under the term TORCH for *Toxoplasma*, "others" (including *Listeria*, hepatitis B, and HIV), rubella, cytomegalovirus, and herpes, could be relevant in a Danish, clinical setting [13].

For now, pregnant women with a recognised, relevant exposure must be followed with serology, and indications for treatment must be evaluated individually for pregnant women with rising/positive titres.

Further insight on this query organism and its clinical impact is of paramount importance, also outside the scope of pregnancy¸ and determining correlation between strain and disease based on genotyping of Danish isolates is one of the next challenges; this will commence shortly. But genotyping is only meaningful when it is linked to epidemiologic and clinical data, and studies addressing the dynamic interaction between reservoirs, routes of transmission, vectors and population diversity are requisite.

ENGLISH SUMMARY

Q fever in pregnancy and fetal health: Epidemiological studies

Background

Q fever is a zoonotic infection which may be of particular concern to pregnant women since infection in pregnancy is suspected to be a potential cause of fetal morbidity and mortality. Unfortunately, an estimate of the risk of adverse pregnancy outcome in pregnant Danish women is difficult to give because of the limited insight into the complex pathogenesis of *C. burnetii*.

Aim

The overall aim of this thesis was to provide further insight into the prevalence of Q fever among Danish pregnant women and the risk of adverse pregnancy outcome among women exposed to livestock.

We sought to quantify the risk of infection in pregnant women occupationally and environmentally exposed to *C. burnetii*, and to improve our understanding of the association between the presence of antibodies against *C. burnetii* during pregnancy, seroconversion and adverse pregnancy outcome. Moreover, we evaluated pregnancy outcome in a large cohort of women with the use of self-reported exposure to farm animals.

Methods

The thesis combined interview data and blood samples from The Danish National Birth Cohort (DNBC) and files from women infected with Q fever in Denmark between 2002 and 2011. In a case-series, we evaluated 19 pregnancies in 12 women. The DNBC collected interview data and blood samples from 100,418 pregnant women (1996–2002) and using a number of sampled study populations, we investigated seroprevalence among pregnant women exposed

to livestock compared to unexposed (n=856), pregnancy outcome among women with serologically verified exposure to *C. burnetii* (n=856), prevalence of *C. burnetii* among women with miscarriage (n=700) and pregnancy outcome among groups of pregnant women with various exposures to farm animals (n=82,128).

Blood Samples were screened for antibodies against *C. burnetii* in a commercial enzyme-linked immunosorbent assay (ELISA). Positive samples were confirmed with an immunofluorescence (IFA) test.

Findings

Study I evaluated 19 pregnancies in 12 women and found that 9 had obstetric complications. Study II concluded that pregnant women with occupational or domestic exposure to cattle and/or sheep have a high prevalence of antibodies to *C. burnetii* compared to unexposed pregnant women.

Study III found that *C. burnetii* seropositivity was not associated with adverse pregnancy outcome since we found no elevated risk of miscarriage, preterm birth, or low birth weight among pregnant women positive for *C. burnetii* compared to seronegative, pregnant women.

Study IV assessed the risk of Q fever and miscarriage and found no relation between *C. burnetii* positivity and miscarriage.

Study V found neither occupational nor domestic animal exposure to be associated with miscarriage, preterm birth, growth restriction or perinatal death. Stratified analysis by different types of animal contact did not change any of the outcome measures significantly.

Conclusion

Overall, we found that contact to livestock is a risk factor for *C. burnetii* and that Q fever is endemic and not a newly emerged pathogen in Denmark. We found few women with seroconversion indicating acute Q fever as well as chronic Q fever, but in 19 pregnant women with evidence of seroconversion, almost half had obstetric complications. However, our DNBC studies failed to show a higher risk of miscarriage, preterm birth or Small for Gestational Age among pregnancies positive for *C. burnetii* than in seronegative Danish pregnant women.

DANSK RESUMÉ

Q-feber I graviditeten og fosterhelbred: Epidemiologiske studier

Baggrund

Q-feber er en zoonose af særlig interesse for gravide kvinder, eftersom infektion i graviditeten er mistænkt for at være en potentiel årsag til føtal morbiditet og mortalitet. Desværre er risikoen for negative graviditetsudfald for danske, gravide kvinder vanskelig at estimere på grund af den begrænsede viden om *C. burnetii*'s komplekse patogenese.

Formål

Denne afhandling sigtede mod at øge viden om forekomst af Q-feber blandt danske gravide og risikoen for negative graviditetsudfald blandt kvinder med forskellig eksponering for husdyr. Formålet var at kvantificere risikoen for infektion blandt gravide kvinder med erhvervs-eller miljømæssig eksponering for *C. burnetii*, samt at forbedre forståelsen af sammenhængen mellem forekomsten af antistoffer mod *C. burnetii* under graviditeten, serokonvertering og graviditets-og fødselskomplikationer. Derudover evaluerede vi graviditetsudfald i en stor kohorte af kvinder med forskellige former for selvrapporteret eksponering for husdyr.

Metoder

Vi anvendte interview data og blodprøver fra Bedre Sundhed for Mor og Barn (BSMB) samt journaler fra Q-febersmittede kvinder i Danmark mellem 2007 og 2011. I en case-serie evaluerede vi 19 graviditeter fra 12 kvinder. BSMB indeholder interviewdata og blodprøver fra 100.418 gravide kvinder (1996-2002). Ved hjælp af en række samplede studiepopulationer undersøgte vi seroprævalensen blandt gravide kvinder eksponeret for husdyr sammenlignet med ueksponerede (n = 856), graviditetsudfald blandt kvinder med serologisk verificeret eksponering for *C. burnetii* (n = 856), forekomsten af *C. burnetii* blandt kvinder med spontan abort (n = 700) og graviditetsudfald blandt grupper af gravide kvinder med forskellige slags

dyreeksponering (n = 82.128).
Blodprøverne blev screenet for antistoffer mod *C. burnetii* i enzyme-linked immunosorbent assay (ELISA). Positive prøver blev genanalyseret med en immunofluorescens (IFA) test.

Fund

Studie I evaluerede 19 graviditeter fra 12 kvinder hvoraf at 9 havde obstetriske komplikationer.

Studie II konkluderede, at gravide kvinder med erhvervs- eller miljømæssig udsættelse for kvæg og/eller får havde en høj forekomst af antistoffer mod *C. burnetii* sammenlignet med ueksponerede gravide kvinder.

Studie III fandt, at antistoffer mod *C. burnetii* ikke var associeret med negative graviditetsudfald i form af spontan abort, for tidlig fødsel eller vækstretardering.
Studie IV vurderede risikoen for Q-feber og spontan abort og fandt ingen sammenhæng mellem antistoffer mod *C. burnetii* og spontan abort.

Studie V konkluderede, at hverken erhvervs- eller miljømæssig eksponering for husdyr var forbundet med spontan abort, for tidlig fødsel, væksthæmning eller perinatal død. Stratificerede analyser af forskellige typer dyrekontakt ændrede ikke resultaterne.

Konklusion

Samlet set fandt vi, at kontakt til husdyr er en risikofaktor for *C. burnetii* i Danmark, og at Q-feber er endemisk forekommende og ikke et nyopstået patogen herhjemme. Vi fandt få kvinder med serokonvertering som indikation på akut såvel som kronisk Q-feber, men I en case-serie med 19 graviditeter havde næsten halvdelen obstetriske komplikationer. Vores BSMB undersøgelser fandt dog ingen forøget risiko for spontan abort, for tidlig fødsel eller væksthæmning blandt *C. burnetii* positive graviditeter sammenlignet med seronegative danske gravide kvinder.

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Paper I

Q fever during pregnancy and maternofetal consequences: a case series from Denmark

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Article Summary Line: Adverse pregnancy outcome was found in nine of 19 (47%) C. burnetii positive women in Denmark - a country with a high seroprevalence but low emergence of clinical Q fever.

Key words: Q fever, *Coxiella burnetii*, infection, pregnancy, spontaneous abortion, miscarriage, preterm birth

Running title: Q fever in pregnancy and maternofetal consequences

ABSTRACT

Past case series have reported a very high risk of obstetric complications among women infected with Q fever, whereas recent studies have failed to find an increased risk of adverse pregnancy outcome in seropositive women.
From 2007-2011, we found 19 pregnancies with a positive or equivocal test for antibodies to *C. burnetii* (IgM phase I & II titres >=64; IgG phase I & II titres >= 128).
Ten of the 19 pregnancies were uncomplicated with a healthy outcome, whereas nine (47%) had obstetric complications (miscarriage, preterm delivery, small for gestational age, oligohydramnion, fetal growth restriction and perinatal death).
The number of adverse pregnancy outcomes in our study is comparable with previous retrospective findings; however, the number of cases with miscarriage and intrauterine fetal death was lower. Specific knowledge is needed to guide general practitioners and obstetricians in their handling of pregnant women at risk or with symptoms of Q fever.

INTRODUCTION

In recent years, Q fever, a zoonotic infection caused by *Coxiella burnetii*, has been the focus of increasing interest in several European countries, including Denmark (1-4). In ruminants, infection with *C. burnetii* is associated with high numbers of bacteria in the placenta, and the infection is known to cause abortions, retained placenta, endometritis and infertility (5, 6). Humans are infected predominantly by inhalation of contaminated aerosols, and individuals with contact to livestock are at risk of exposure to *C. burnetii* (7). Up to 90% of pregnant women with antibodies suggesting recent infection with *C. burnetii* remain asymptomatic (8). However, studies from France have associated symptomatic and asymptomatic *C. burnetii* infection during pregnancy with obstetric complications, including miscarriage, preterm delivery and fetal death (9-11). By contrast, recent studies from northern Europe have not found an association between *C. burnetii* and adverse pregnancy outcome (12-15).A recent Danish study on the seroprevalence of *C. burnetii* in cattle found that bulk tank milk samples tested positive at 59 of 100 randomly selected farms (16). Among Danish veterinarians, the prevalence of antibodies to *C. burnetii* ranged from 36% to 39% (1, 2). This shows that in Denmark Q fever is prevalent both in the animal reservoir and in those who are occupationally exposed or living in rural areas with livestock contact. A review of signs and symptoms among seropositive individuals found that infections were asymptomatic or associated with mild illness (1). However, the risk and implications of infection in Danish pregnant women have not been exhaustively described (12, 15).Therefore, we reviewed national data (from Aarhus University Hospital, Aalborg University Hospital, Hospital of Southwest Jutland, Viborg Regional Hospital,

Regional Hospital West Jutland, and Hilleroed Hospital) to identify a series of Danish women with elevated antibodies to *C. burnetii* in pregnancy in order to evaluate their course of infection, treatment with cotrimoxazole (trimethoprim-sulfamethoxazole) and pregnancy outcome. The collection of this case series was in particular motivated by the controversies between the findings from the French case series and the population-based studies from the Netherlands and Denmark mentioned above.

MATERIALS AND METHODS

In Denmark, Q fever serology is only performed at the Statens Serum Institut (SSI) by indirect immunofluorescence assay (IFA, Focus Diagnostics, Cypress, CA, USA), according to manufactures instructions.
Every resident in Denmark is provided with a permanent and unique civil registration number that enables individual-level linkage between different national registries. By linking data from health records at obstetric and infectious disease departments to civil registration numbers from women between 18 and 45 years who had positive or equivocal tests at the SSI for antibodies to *C. burnetii* from 2007 to 2011, we identified pregnant women who could be included in the study according to the following criteria:
Positive serology, with titres available throughout pregnancy that allowed evaluation of *C. burnetii* infection in paired samples.

Detection of antibodies against *C. burnetii*

C. burnetii expresses two antigens, phase I and phase II. When infected, phase II IgG and IgM antibodies are elevated, and they may remain positive for months to years. In acute Q fever, primarily antibodies against phase II are raised, and titres are higher than antibodies against phase I. As with most other infections, IgM antibodies appear first. In chronic forms of the disease, antibodies against phase I are elevated.
A local cutoff value adjusted to the Danish population has been defined (17), including negative, equivocal and positive titres. In this study patients with equivocal and positive titres were included.A sample was considered IFA positive when IgM phase I or phase II titres were 64 or above; for IgG when any of the phases were 1:128 or above.
A 4-fold increase in titres between two paired samples was defined as diagnostic of a recent or an acute infection.

PCR analysis

DNA from urine samples were subjected to a Chelex® 100-based DNA extraction method as previously described (18). DNA from placental and bone marrow samples was extracted using the DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions, and DNA from the cream layer of fresh breast milk samples was extracted using a previously described protocol that included washing with PBS and subsequent extraction using the DNeasy Blood & Tissue Kit (19). PCR was conducted with primers targeting the multi-copy gene IS1111 as previously described by Koch et al. (20).

RESULTS

We identified a total of 12 women with equivocal and positive titres who underwent 19 pregnancies in the 5-year period. All women were farmers or veterinarians and resided in rural areas of Denmark. Obstetric complications were recorded in nine (47%) of the 19 pregnancies included in the study.

Two patients (#1 and #10) reported dry cough and short episodes of fever and both had antibody titres consistent with acute infection during the 1st trimester. They were both treated with cotrimoxazole from gestational week 15 or 10, respectively. Patient #1 was treated throughout pregnancy and patient #10 until gestational week 39. Patent #1 had a PCR positive urine sample in gestational week 10, but PCR on a bone marrow biopsy from gestational week 15 and PCR on amniotic fluid and placenta were all negative. For patient #10, no *C. burnetii* DNA was detected by PCR on placental tissue and breast milk. In her second pregnancy, serology for *C. burnetii* was negative, PCR on placenta was not performed and she had a healthy outcome.

Patient #2 reported weeks of dry cough without fever during the weeks just before the first pregnancy that ended with miscarriage; she had three miscarriages and an extrauterine pregnancy within a period of 2 years, and from her titres it is reasonable to assume that she was acutely infected weeks prior to the first miscarriage. Her titres reached a maximum after the 2nd miscarriage. Unfortunately, no embryo material was tested from any of her miscarriages.

Three of the women (#3, 5 and 12) had a serologic profile compatible with probable chronic infection (according to the new Dutch guidelines (21)), with IgG phase I titre of 1024 at the end of pregnancy; two of the women were treated during pregnancy. None of the three had any symptoms, were diagnosed with endocarditis, or received long-term post-partum treatment.

Patient #4 seroconverted before her second pregnancy in which she experienced a single fetal demise around gestational week 8 and a surviving co-twin. She had a decrease in IgG phase I during pregnancy and had a healthy, term baby.

Patient #5 was treated for acute Q fever prior to her second pregnancy. Her titres were stable in the second pregnancy, and she gave birth to a term but slightly small for gestational age infant. After a short interpregnancy interval, she had a significant increase in IgG phase II titres in her third pregnancy, and due to fetal growth restriction and oligohydramnion from gestational week 28, she had a caesarean section in gestational week 38. The placentas in the two pregnancies were not investigated. Patient #8 had rising titres during her first pregnancy, and due to bleeding and contractions she had an acute caesarean section in week 27 and gave birth to a severely growth retarded and malformed infant who only lived a few hours. The fetus and placenta were not tested for *C. burnetii*, but tests for toxoplasmosis, cytomegalovirus and parvovirus B19 were negative, as were genetic testing for neuromuscular diseases. There was a slight decrease in titres postpartum, but in her second pregnancy, her titres increased significantly, and treatment with cotrimoxazole was initiated around gestational week 22. In gestational week 30 she spontaneously went into labour and gave birth to a healthy baby. Treatment was terminated immediately postpartum and her antibodies decreased, indicating that she was not chronically infected.

The remaining pregnancies (#6, #7, #9, #11) had an uncomplicated course with a healthy pregnancy outcome apart from patient #7 who had an acute caesarean due to rupture of the uterus (she also had had a caesarean section in her first pregnancy).

In seven pregnancies, treatment with cotrimoxazole was initiated; six of them had healthy, term babies, and no mention of severe side effects was found in the women's medical records. One of the women with obstetric complications received treatment with cotrimoxazole (patient #8 in her second pregnancy). By comparison, among the 12 pregnancies with no treatment, eight experienced obstetric complications. The effect of treatment with cotrimoxazole on complications was tested using a Fisher's exact test (p-value: 0.057).

PCR was performed on placentas from 10 pregnancies and in four of these, breast milk was also tested; none were positive. Seven of the 10 women who had their placentas investigated had received treatment with cotrimoxazole during pregnancy.

DISCUSSION

In 10 of the 19 pregnancies during which equivocal or positive tests for *C. burnetii* antibodies were found, the pregnancy was uncomplicated and resulted in a healthy pregnancy outcome, whereas nine (47%) were associated with obstetric complications. One woman had three miscarriages and an extra-uterine pregnancy, one experienced preterm delivery, one had a single fetal demise with a surviving co-twin, and one delivered a small for gestational age baby. Oligohydramnion and fetal growth restriction were found in two pregnancies; one had a healthy outcome, in the other the fetus died a few hours post-partum. The total numbers of adverse

pregnancy outcomes in our study is comparable with previous retrospective findings; however, the number of cases with miscarriage and intrauterine fetal death was much lower.

The evidence for an adverse pregnancy outcome in humans in relation to Q fever and pregnancy mainly originates from French case studies of referred, infected (acute, chronic and seroconversion) pregnant patients and pregnancies with Q fever diagnosed retrospectively after an adverse outcome (9-11). Carcopino et al. reported clinical symptoms in 32 of 53 cases (60.4%), a chronic serology profile in more than half of their patients, and concluded that Q fever in pregnancy may cause severe complications (10). Among the 53 women infected during pregnancy, Carcopino et al. found four cases of intrauterine fetal death, all with placentitis, whereas placentitis was not found in 15 patients whose newborns were alive at birth. Placental analysis was performed in 23 patients, and *C. burnetii* was identified in nine, and the authors propose a link between placentitis and obstetric complications (10). In a recent Dutch study, Munster et al. described the placental histopathology and clinical outcome of five cases with asymptomatic *C. burnetii* infection during pregnancy and compared them with symptomatic cases from the literature. They conclude that asymptomatic and symptomatic infection during pregnancy may be different entities regarding placental pathology and risk of adverse pregnancy outcome and that there may be a link between clinical symptoms (fever, fatigue, dyspnoea, etc.) and obstetric complications (22).

None of the placentas in our study were studied by histopathology; 10 of the placentas were tested with PCR, none were positive. Two of these 10 women experienced symptoms and seven received treatment. A possible explanation for the lack of findings related to the placenta in seven of the cases could be the treatment or focal placental infection. However, results similar to those of Munster et al. have been shown in a larger cohort of 153 asymptomatic seropositive women (23), suggesting a low rate of placental infection in asymptomatic women and that obstetric complications in symptomatic cases may be explained by massive placental necrosis following either a higher bacterial load in the placenta, systemic infection, or both.

Untreated Q fever in one pregnancy may be reactivated in a subsequent pregnancy (24). We found that two patients (#5 and #8) had a post-partum decline in antibody titres followed by a fourfold or more increase during their next pregnancy. Furthermore, one patient (#12) had a serologic profile indicating reactivation although her titres had been negative in a previous pregnancy 2 years earlier.

A recent French study investigated 30 pregnant women with serological indication of acute infection in pregnancy (11); 17 of the 30 women were asymptomatic, five were tested due to previous negative pregnancy outcome, one because of animal exposure and 13 had clinical symptoms. Among the 17 asymptomatic patients, only two had an uncomplicated pregnancy, but no placentitis or isolation of *C. burnetii* was found in 14 available biopsies. In our study three patients (#1, #2 and #10) reported symptoms of acute Q fever, and at least one, patient #8, was likely to have seroconverted without symptoms close to the beginning of her first pregnancy. One of these had three recurrent miscarriages; the others had a healthy pregnancy outcome. This illustrates the complexity regarding separation of harmless seroconversion from

infection threatening maternal and fetal health.
The women with symptoms in our study were not the ones with a serologic profile compatible with a probable chronic infection at the end of pregnancy (#3, #5 and #12); symptomatic chronic infection may have been prevented in some of the women who received treatment throughout pregnancy.
The indication for serologic testing is a crucial point; in our study the indication for testing was exposure to livestock in the majority of women; two (#1 and #8) were tested in a subsequent pregnancy because of an adverse pregnancy outcome; none were tested because of symptoms. In contrast, the women in the French case series were primarily tested because of pathologic conditions during their current pregnancy, clinical symptoms (fever, hepatitis), or retrospectively because of an adverse pregnancy outcome. Among the 53 pregnancies evaluated by Carcopino et al., 16 pregnancies did not receive treatment with cotrimoxazole. These were all diagnosed after delivery, which is an important selection bias to be taken into account when interpreting their results.
In all likelihood, the most important bias when interpreting the association between *C. burnetii* and pregnancy outcome is conditional upon the indications for the investigation.
The very high complication rate in both ours and others retrospective studies is in contrast with several large epidemiological studies.
The recent, unprecedented Q fever outbreak in the Netherlands prompted two large studies in pregnancy women; a large population based study of 1174 serum samples (from the 12th week of pregnancy) found no association between antibodies to *C. burnetii* and adverse pregnancy outcome among women living in the area with the highest Q fever incidence. A large randomised trial testing 1229 pregnant women living in high-risk areas during the outbreak in the Netherlands found 15% of the women to be seropositive in both the intervention group and the control group. Only seven women in the intervention group were acutely infected and treated during pregnancy, and no significant difference in obstetric complications was found. Hence, routine screening in pregnancy of women living in high-risk areas was not recommended (13). A recent, Danish study assessed the association between presence of antibodies to *C. burnetii*, seroconversion and pregnancy outcome and found that seropositivity was not associated with miscarriage, preterm birth or low birth weight (15).
When treating Q fever in pregnancy, cotrimoxazole for at least 5 weeks is recommended, based on the French case studies, since doxycycline and hydroxychloroquine are contra-indicated from the 2nd trimester (10, 24). The active ingredient trimethoprim is a folic acid antagonist. Treatment in the 1st trimester entails a small increase in risk of cardiovascular malformations and neural tube defects. This risk can be reduced by simultaneous administration of folic acid. In Denmark, doxycycline is recommended rather than cotrimoxazole during the first trimester. Treatment with cotrimoxazole in the 2nd and 3rd trimester is relatively uncomplicated. However, a small risk of kernicterus in the newborn after maternal treatment with sulfamethoxazole immediately prior to birth may be considered (25, 26), but to our knowledge, no reports of kernicterus attributable to maternal ingestion of sulfonamides have appeared (27).

Experience with other treatment regimens for Q fever in pregnancy is extremely sparse. The French recommendation that seropositive women be treated with cotrimoxazole throughout pregnancy is widely practised, but the indications are being questioned as more studies fail to corroborate the French results (28). In a recent study, Boden et al. found no obvious association between *C. burnetii* infection and negative pregnancy outcome in 11 women. All nine seroconverted during pregnancy, three presented with symptoms (fever); nine women were treated with antibiotics but only three received the recommended long-term cotrimoxazole. None of seven placentas investigated were positive, and none of 11 women had a chronic serologic profile. The authors conclude that the recommendation of long-term cotrimoxazole treatment for every pregnant woman with laboratory confirmed Q fever is questionable (28). Angelakis et al. (11) showed that QpDV plasmid was present in four of seven *C. burnetii* strains isolated from infected women who had had a miscarriage. The authors suggest that the different obstetric morbidity found in different geographical areas could be related to strain specificity, potentially based on differences in plasmid types.
Among the present cases, no breast milk samples were PCR positive of the four samples tested. *C. burnetii* has been found in human milk (29, 30), but the implications for the breast-fed child are unclear, and due to lack of evidence, breastfeeding has been deemed safe according to the Danish obstetric guidelines on the treatment of seropositive, pregnant women and their newborns.

Complications in almost half of the pregnancies may seem a high rate, but the causal relation of the finding may not be clear. For example, in the cases of fetal death (patients #2 and #8), no placental or embryo material was tested for *C. burnetii*. This, along with the entirely negative findings in the placentas tested by PCR limit any definite conclusion regarding Q fever as the cause of adverse pregnancy outcome among the women included in this study. In any case, the risk of complications is lower than reported from France where most untreated seropositive women experienced a negative outcome. Still, we did find complications in eight (67%) out of 12 pregnancies in which the women were not treated, which supports the effect of treatment. The disagreements of serious adverse outcome between the French, the Dutch, the German and our study could be partly related to strain virulence; further clarification would improve the indications of treatment of Q fever during pregnancy. However, pregnancy outcome may very well be healthy for the majority of seropositive women without cotrimoxazole treatment if they are infected with a strain of low virulence. Outbreaks of Q fever have been described to occur only in small ruminants. In France, goats and sheep have been the source of infection, and in the recent Dutch outbreak it was goats. In Denmark, goat and sheep farms are rare, and despite high clinical awareness during the last 7 years, there has, to our knowledge, never been a microbiologically verified outbreak of Q fever in humans or a case of chronic Q fever that was definitely acquired in Denmark. However, as stated earlier, a very large percentage of Danish dairy cattle shed the bacteria, which suggests that there could be some difference in the virulence between strains, as previously suggested by Angelakis et al. (11). As all the included

women had contact with livestock in Denmark, it is reasonable to assume that these women had been occupationally exposed to endemically occurring *C. burnetii* infections among cattle. Re-exposure to *C. burnetii* is a risk for pregnant women who are exposed to infected animals during pregnancy; Along with this, the fact that all women in this study had contact with cattle enhances the relevance of regular serologic follow-up throughout pregnancy for women who are exposed to domestic animals.

CONCLUSION

We describe 12 women with positive or rising titres against *C. burnetii* in 19 pregnancies from Denmark - a country with high seroprevalence of *C. burnetii* but low prevalence of clinical Q fever. Almost half of the women included in this study had obstetric complications; seven pregnant women received long-term treatment with cotrimoxazole. We found complications in eight out of 12 untreated pregnancies and two cases of fetal death. However, in none of our cases, was a result suggesting a causal relationship between seropositivity and adverse pregnancy outcome identified.
Further knowledge is needed, and specifically general practitioners and obstetricians need guidelines regarding the treatment of pregnant women at risk or with symptoms of Q fever.

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Table 1: Summary of patient characteristics and pregnancy outcome from Danish women with positive *C. burnetii* titres in pregnancy

	Age	Parity	Symptoms	Contact to animals	Treatment in pregnancy	Gestational age at delivery (weeks +days)	Birth weight	Clinical outcome	*C. burnetii* in placenta tissue or breast milk or other	Comments
Patient #1	33	2	Fever and cough first weeks of pregnancy	Yes, cattle, veterinarian	Yes from week 15	38+6	3570g	Healthy	PCR urine positive in pregnancy week 10Bone marrow biopsy pregnancy week 15: negativePCR breast milk, amniotic fluid, and placenta: negative	
Patient #2	40	1	Dry cough for weeks just prior to first included pregnancy	Yes, cattle, veterinarian	No	8 weeks		Miscarriage	Not available	During a period of 2 years: 3 spontaneous abortions and 1 extra-uterine pregnancy
Patient #3	30	1	No	Yes, cattle, veterinarian	Yes, from week 10	39+1	3500g	Healthy	PCR placenta negative	
Patient #4	34	1	No	Yes, cattle, veterinarian	No	39+0	3030g	Single fetal demise around week 8 with a surviving co-twin with a healthy outcome	PCR breast milk and placenta: negative	Q fever in 2006 (not pregnant)
Patient #5										
Pregnancy 1	32	1	No	Yes, cattle, veterinarian	No	40+3	3000g	Dysmature	Not available	Acute Q fever in 2006 (not pregnant), 3 weeks doxycycline
Pregnancy 2	33	2	No		No	38+0	2360g	Caesarean due to IUGR (23 % from week 28) and oligohydramnion		

Patient #6	26	1	Not available	Yes, cattle, farmer	No	39	3720g	Healthy	PCR placenta:negative	
Patient #7	32	1	No	Yes, cattle, veterinarian	Yes, from week 10	41	3210g	Acute Caesarean due to uterine rupture	PCR placenta:negative	2 spontaneous abortions prior to this pregnancy
Patient #8										
Pregnancy 1	24	0	No	Yes, cattle, assisting female farmer	No	27+2	Not available	IUGR, oligohydramnion/ malformations, died few hours post partum	Not available	
Pregnancy 2	26	1	No	Yes, cattle, assisting female farmer	Yes, from week 22	30	1570g	Preterm	PCR placenta:negative	
Patient #9:										
Pregnancy 1	30	0	No	Yes, cattle, veterinarian	From week 20	39+4	3790g	Healthy	PCR placenta and breast milk: negative	
Pregnancy 2	33	1	No	Yes, cattle, veterinarian	No	40+2	4170g	Healthy	Not available	
Patient # 10										
Pregnancy 1	30	0	1 month of dry cough beginning of pregnancy, a short episode of fever	Yes, cattle, veterinarian	Yes, from week 10	39+6	3420g	Healthy	PCR placenta and breast milk: negative	
Pregnancy 2	33	1	No	Yes, cattle, veterinarian	No	41+2	3400g	Healthy	Not available	
Patient #11	30	1	No	Yes, cattle, veterinarian	No	40	4230g	Healthy	PCR placenta negative	
Patient #12	31	1	No	Yes, cattle, veterinarian	Yes, from week 22	39+5	3570 g	Healthy	PCR placenta negative	

Table 2: Specification of *C.burnetii* titres in 19 pregnancies from 12 Danish women

	First sample (pregnancy week)	Titer IgM II	IgMI	IgGII	IgGI	Last sample (pregnancy week)	Titer IgM II	IgMI	IgGII	IgGI	Comments
Patient #1	7	2048	8000	1024	512	38	<64	<64	256	<128	Negative titres 6 months prior to this pregnancy
Patient #2	After miscarriage in pregnancy week 8	256	<64	256	128	After extrauterine pregnancy week 7	512	128	512	512	Maximum titres after second miscarriage: IgG phase II: 4096; IgG phase I: 2048; IgM phase I: 512; IgM phase II: 256
Patient #3	3	<64	<64	<128	512	33	<64	<64	128	1024	1st titres taken 2 months prior to this pregnancy with IgG phaseI: 1024.Maximum titres in pregnancy week 9 with IgG phase I: 4096 and IgG phase II: 256*
Patient #4	10	<64	<64	1024	512	1 day post partum	<64	<64	1024	128	First titres taken 3 months prior to this pregnancy: identical to titres from pregnancy week 10.No further rise of titres in this pregnancy**
Patient #5 Pregnancy 1	9	<64	<64	256	512	38	<64	<64	256	256	First titres taken 6 months prior to this pregnancy: IgM phase II: <64; IgM phase I: <64;IgG phase II: 512; IgG phase I: 1024. No further rise of titres in this pregnancy**
Pregnancy 2	12	<64	<64	128	1024	31	<64	<64	512	1024	No further rise of titres in this pregnancy**
Patient #6	13	<64	<64	256	<128	At birth	<64	<64	128	<128	No titres available before this pregnancy. Maximum titres in pregnancy: IgG phase II: 256*

Patient #7	7-8	128	<64	4096	<128	At birth	64	<64	512	<128	Titres positive 6 months prior to this pregnancy: IgM phase II: 1:128; IgM phase I: <64; IgG phase II: 2048; IgG phase I: <128. No further rise of titres in this pregnancy**
Patient#8											
Pregnancy 1	12	1:128	<64	512	256	26	256	<64	1024	256	Maximum titres in this pregnancy: IgG phase II:2048*
Pregnancy 2	10	<64	<64	256	128	26	<64	<64	256	256	Maximum titres in this pregnancy:IgG phase I:1024; IgG phase II:1024*
Patient #9											
Pregnancy 1	10	<64	<64	256	<128	39	64	<64	128	<128	Maximum titres in this pregnancy: phase II IgG: 1024*
Patient #9 Pregnancy 2	12	<64	<64	256	<128	36	<64	<64	256	<128	No further rise of titres in this pregnancy**
Patient #10											
Pregnancy 1	8	512	<64	4096	<128	36	<64	<164	256	<128	No titres available before this pregnancy. No further rise of titres in this pregnancy**
Pregnancy 2	9	<64	<64	128	<128	14	<64	<64	<128	<128	No further rise of titres in this pregnancy**
Patient #11	7	<64	<64	128	128	26	256	256	512	<128	Titres positive one month prior to this pregnancy: IgM phase II: <64; IgM phase I: <64; IgG phase II: 1024; IgG phase I: <128No further rise of titres in this pregnancy**
Patient #12	10	<64	<64	2048	<128	37	<64	<64	1024	1024	Negative titres in a pregnancy 2 years earlierMaximum titres in this pregnancy: IgG phaseII:2048

*Remaining titres have not risen above/beyond values in beginning/end of pregnancy

** No further rise of titres in this pregnancy indicates that the titres have not risen beyond values in beginning/end of pregnancy

Paper II

Prevalence of *Coxiella burnetii* in women exposed to livestock animals, Denmark

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ABSTRACT

Introduction

Q fever is a zoonotic infection, which may be of particular concern to pregnant women. To our knowledge, Denmark has never experienced a clinically verified Q fever outbreak. We aimed to quantify risk of infection in pregnant women occupationally and environmentally exposed to *C. burnetii*.

Methods

Methods
The Danish National Birth Cohort collected blood samples from 100,418 pregnant women (1996-2002). We sampled 195 women with occupational exposure to livestock (veterinarians and female farmers), 202 women with domestic exposure (dairy cattle and/or sheep) and a random sample of 459 unexposed women. Samples were screened for antibodies against *C. burnetii* in a commercial enzyme-linked immunosorbent assay (ELISA). Positive samples were confirmed with an immunofluorescence (IFA) test (cutoff titre >=1:128).

Results

The proportion of seropositive women was higher in the occupationally exposed population (47.2 % seropositive, RR: 9.8; 95%CI: 6.4-15.2) and in the domestically exposed population (32.2% seropositive, RR: 6.7; 95%CI: 4.3-10.6) when compared to unexposed women (4.8% seropositive).

Conclusion

We found a high prevalence of antibodies to *C. burnetii* among pregnant women with occupational or domestic exposure to cattle and/or sheep compared to unexposed pregnant women. Our findings suggest that contact to livestock is a risk factor for *C. burnetii* infection in Denmark.

INTRODUCTION

Most emerging infectious diseases are of zoonotic origin [1], and populations at particular high risk often include individuals with occupational exposure to live animals such as veterinarians, farmers and those living in close contact with domestic livestock. One disease of recent concern, in particular for pregnant women, is Q fever caused by *Coxiella burnetii*.
In small ruminants, infection with *C. burnetii* is known to cause abortions, retained placenta, endometritis and infertility, and placentas of infected animals contain high numbers of bacteria [2,3].

Q fever is most likely endemic worldwide, but unbiased estimates from relevant populations are scarce because most reports on incidence and prevalence are reported from regions with outbreaks or with particular medical or scientific interest in the infection [4].
In Denmark, Q fever has previously been considered a rare and imported disease, but testing for antibodies in livestock animals since 2003 has indicated that the infection is widespread. A recent study found a prevalence of 59% antibody positive animals from 100 randomly selected dairy herds [5].

Human infection is usually acquired through inhalation of contaminated aerosols from infected animals, which contaminate the environment through excretion of bacteria in large amounts in birth-by-products, especially placenta [6-8].
For healthy humans, Q fever infection often has a mild, flu-like course, but may also cause severe pneumonia. Immunocompromised patients and patients with pre-existing cardiac valve or vascular defects are at risk of a more severe course of the infection [6,8].

Q fever in pregnancy is suspected to be a potential cause of foetal morbidity and mortality. French case studies have suggested risk of spontaneous abortion, intrauterine growth retardation, oligohydramnion, stillbirth and premature delivery in untreated pregnancies [4,9,10]. Recent studies have not found any association between presence of antibodies against *C. burnetii* and adverse pregnancy outcome, but knowledge on the topic is sparse [11-15].

The risk of infection with *C. burnetii* has been related to particular occupations with close contact to the organism's primary reservoirs, such as domesticated livestock animals. Examples include veterinary practice and farming [16,17].

In order to conduct risk assessment, it is important to quantify the risk of infection in exposed populations. The aim of the present study was to investigate the prevalence of elevated antibody titres against *C. burnetii* in occupationally and domestically exposed women compared with unexposed women sampled from a population based study of pregnant women.

MATERIALS AND METHODS

Participants

The Danish National Birth Cohort (DNBC), a nationwide cohort of 100,418 pregnant women and their offspring (12), served as base for sampling of the study population.

Enrolment in the DNBC took place between 1996 and 2002. All Danish pregnant women were invited for the study in connection with the first antenatal visit to the general practitioner. Information on exposures before and during the early part of pregnancy, including occupation and exposure to livestock, was collected by means of a computer assisted telephone interview scheduled to take place in gestational week 12 (interview forms available at the website for the cohort).

Interviews with the women included data on reproductive history, age, smoking status, domestic contact to animals and very detailed questions regarding occupational exposure to different animals.

Women who confirmed to having worked on a farm or with live animals during their pregnancies or 3 months prior to becoming pregnant were further questioned about the type of animals, the size of the herd, occupation, and etcetera. During pregnancy, two blood samples were collected; one around gestational weeks 6 to 12, the second around gestational week 24; samples were stored in a bio-bank.

A detailed description of the cohort can be found elsewhere [18].
We sampled three groups from the DNBC cohort: pregnant women with self-reported occupational exposure to livestock (n=195), women with self-reported domestic exposure to cattle or sheep (n=202) and a randomly sampled reference group (n=459). It was a criterion for all three groups that the women had participated in the interview in early pregnancy and had delivered a blood sample to the bio-bank.

Women exposed through their occupation (n=195) were veterinarians (n=118) or women who worked on a farm with at least 40 dairy cattle (n=77).

Domestically exposed women was defined as women who reported cohabiting with a farmer and living on a farm with cattle (n=180), sheep (n=22) or both (n=13), but without occupational exposure to these animals. Unexposed women were randomly selected (n=459); two of these were domestically exposed to animals and were consequently reclassified as such.
In order to evaluate a possible association between geographic area and seropositivity, the participants were classified using NUTS3 (nomenclature of territorial units for statistics) [19], which subdivides the regions of Denmark into 11 areas.

Detection of antibodies against *C. burnetii*

The diagnosis of Q fever relies upon serology. *C. burnetii* expresses two groups of antigens, phase II and phase I.

In acute Q fever, primarily antibodies against phase II are elevated, and their titre is higher than antibodies against phase I. As with most other infections, IgM antibodies appear first. In chronic forms of the disease, antibodies against phase I are elevated.
When infected, phase II IgG and IgM antibodies are always elevated, and IgG remain positive for many years. A large study from Australia and England found that phase II IgG antibodies persisted after five and 12 years, respectively [20].

In order to determine antibodies against *C. burnetii*, we used a two-step approach. First all samples were screened in a commercial enzyme-linked immunosorbent assay (ELISA). Positive ELISA samples were confirmed with an immunofluorescence antibody test (IFA). When investigating the association between exposure, Q fever titres and pregnancy outcome we consider IFA to be gold standard.

The commercial ELISA kit were purchased from Panbio (Queensland, Australia) (cat. no. E-QFB01G and E-QFB01M) and used according to the manufacturers' instructions with minor modifications. Due to small sample size the initial total volume was smaller but same dilution factors were used.

Samples which were positive for either IgG or IgM antibodies in the ELISA were confirmed with an IFA test from Focus Diagnostics (ca. no. IF0200G and IF0200M). The tests were performed according to the instructions provided by the manufacturer, with the following minor modifications: due to low volume of sample material, the diluted samples 1:10 from the ELISA were used to further dilute the samples as described by the manufacturer. The effect of the dilution in the Panbio buffer was tested prior to the use on patient samples and did not show any influence on the results (results not shown).

Also, the IFA cutoff suggested by the manufacturer was not used; since the prevalence of the infection varies between geographic areas, the cutoff suggested by the manufacturer is not necessarily suited for any given area [21].

A local cutoff adjusted to the Danish population has been defined, including negative, grey zone and positive titres [22] (Table 1). The grey zone was defined in order to address people with an a priori elevated risk of Q fever (such as veterinarians, farmers etc.), proposing that these high risk groups with a grey zone titre should be considered to be probably positive. When the ELISA positive samples in our study were reanalysed using IFA, a modified version

of the Danish cutoff was used. A sample was considered IFA positive when any of the phases were 1:128 or above.

All serological analyses were performed in a certified laboratory at Statens Serum Institut, Denmark. Laboratory personnel were blinded for exposure status and samples were always analysed in the same batch of commercial kits.

Statistical analysis

The strength of the association between exposure and positive IFA serology was expressed as a risk difference as well as a relative risk for occupational and domestic exposure compared to the reference according to the prevalence of antibodies against *C. burnetii* in pregnancy. We included all veterinarians and women in DNBC who reported occupational exposure to cattle in the occupationally exposed group. In order to obtain sufficient statistical power, we sampled from DNBC a similar sample size of domestically exposed women and a larger reference population. All analyses were carried out using STATA statistical software, version 11.

RESULTS

Age and distribution of urban or rural residence can be seen in Table 2. Age was normally distributed in all three groups (Table 2).

The median age among occupationally exposed women was 31 years (interquartile range: 28; 33), compared to 30 years (interquartile range: 27; 33) in domestically exposed women, and 29 years (interquartile range: 26; 32) in the unexposed.

When looking at age and seropositivity, the smallest number of IFA positive women were found in the age group below 25 years (13.5% seropositive), whereas findings from other age groups were similar (age 25 to 34 years and 35+ were similar (22.7% and 18.1% seropositive, respectively). There was no correlation between age and seropositivity.

Figure 1 illustrates the relationship between IgG phase II positive ELISA and IFA results. The IFA positive samples were the ones with high levels of adjusted OD-values (optical density values measuring antibody concentrations) in ELISA.

In the confirmatory IFA analysis, 92 (47.2%; 95%CI: 40.0-54.4) occupationally and 65 (32.2%; 95%CI: 25.8-39.0) domestically exposed were *C. burnetii* antibody positive in IFA compared to 3 (4.8%; 95%CI: 3.0-7.1) in the unexposed group (Table 3).

The proportion of seropositive women was significantly higher in women with occupational exposure to livestock as well as with domestic exposure to livestock when compared to unexposed women. The risk difference between the occupationally exposed and unexposed women was 42 per 100 (95%CI: 35-50); the occupationally exposed had a 9.8 times higher risk of being seropositive compared to the unexposed women (relative risk: 9.8; 95%CI: 6.4-15.2).

The risk difference between the domestically exposed and unexposed women was 27 per 100 (95%CI: 0.21-0.34); the domestically exposed had 6.7 (95%CI: 4.3-10.6) times higher risk of being seropositive compared to the unexposed women (Table 3).

Reporting the IFA results according to the Danish cutoff with grey zone titres classified as negative, the trend was the same. Here the proportion of seropositive women was also significantly higher in women with occupational exposure to livestock (19% seropositive, RR: 29; 95%CI: 9.1-93.0). This was also found in women with domestic exposure to livestock (11.0% seropositive, RR: 16.7; 95%CI: 5.0-55.0) when compared to unexposed women (0.7% seropositive).

Figure 2 shows the distribution of positive IgG phase II titres in the three groups and illustrates how unexposed women are mainly “low-positive” whereas the “high-positive” titres are primarily from the two groups of exposed women.

Previous versus recent infection

In the occupationally exposed women, 79 were IgG phase II positive, 43 were IgG phase I positive, 41 of them were positive in both. Three women’s IgM phase II were positive, one of these were also positive in IgG phase II, another in both IgG phases. None was IgM phase I positive.

In the domestically exposed women, 59 were IgG phase II positive, 30 were IgG phase I positive, 26 of them were positive in both phases. Three were IgM phase II positive, with one of them also being positive in IgM phase I, and two in IgG phase II. One was only IgM phase I positive.

In the unexposed women, 21 were positive in IgG phase II, 6 of these were also IgG phase I

positive. One was positive in IgM phase I as well as IgG phase II and one was IgM phase II positive but negative in all other phases.

Altogether, we mainly found serological evidence of previous infection.

Specific animal contact

Apart from working with live animals, 38 of the 118 veterinarians lived on a farm with animals; none of the veterinarians who lived on a farm had a job without animal contact. Among the 77 female farmers who all worked on farms with at least 40 dairy cattle, 69 of them lived on cattle farms. Four of them also worked with meat cattle and 5 worked with sheep. All 202 women domestically exposed were married to a farmer. 193 of these lived on a farm with cattle, 22 had sheep and 13 women had cattle as well as sheep at the farm. Two women with domestic exposure were also exposed to animals at work.

Analyses based on specific animal contact according to IFA status showed that 23 (74.2%) of veterinarians working with cattle were seropositive, and that the risk of being IFA positive were 2.7 times higher in veterinarians who work with cattle compared to those who did not (RR: 2.7; 95%CI: 1.8-4.0). The positive predictive value of being seropositive given that you are a veterinarian working with cattle was 48.9%.
Among the domestically exposed women who were exposed to cattle, 64 (33.2%) were IFA positive, and the positive predictive value of being seropositive for these women was 98.4%, whereas PPV for domestic exposure to sheep was only 9.2% (Table 4).

Urban versus rural area

Among the 427 women living in rural areas, 128 (30%) were IFA positive compared to 48 (11.5%) seropositive among women living in urban areas. The risk of being IFA positive was 2.6 times higher in women living in rural areas (RR: 2.6; 95%CI: 1.9-3.5).

In the unexposed women, 151 (33%) lived in rural areas. Eleven (7.3 %) of these were seropositive, compared to 11 (3.6 %) seropositive women among the unexposed living in urban areas.

DISCUSSION

We found a high prevalence of antibodies to *C. burnetii* among pregnant women with occupational or domestic exposure to cattle or sheep compared to the prevalence in randomly selected unexposed pregnant women. The highest predictive values for being seropositive were found among pregnant veterinarians and women with domestic exposure to cattle.

To our knowledge, this is the first population-based seroepidemiologic study in which the prevalence of antibody titres against *C. burnetii* in occupationally and domestically exposed women was compared to unexposed pregnant women.

In general, a higher seroprevalence has been found in studies evaluating seroprevalence in groups handling livestock, especially veterinarians, than in studies of seroprevalence in the background population [23-29]. In one Dutch study of veterinarian students, 18.7% were seropositive [30]; in another, 65% of 189 veterinarians were seropositive; the number of hours with animal contact per week, the number of years since the participants had graduated, living in a rural area, and working as practicing livestock veterinarian were risk factors in that study [31]. An American study found antibodies against *C. burnetii* in 113 (22.2%) of 508 U.S. veterinarians; compared with veterinarians with a small animal practice, those with a mixed small and large animal practice and those with a food animal practice were more likely to be seropositive. Furthermore that study found that ever living on a farm, currently living on a farm, and exposure to ruminants while living on a farm were associated with seropositivity [16].

A recent Danish study examined the presence of antibodies to *C. burnetii* among people working with domestic animals and found the highest prevalence of antibodies (36%) among veterinarians [32].

Close contact to birth products when performing caesareans and other kinds of labour assistance is a possible explanation for the higher prevalence of antibodies among veterinarians compared to domestically exposed women found in this study.

Denmark experienced a rising interest in Q fever from 2007-2008, but the increased attention was primarily diagnostic rather than indicating true emergence of a new infection. In the present study, some of the blood samples analysed date back to 1996, and this indicates that *C. burnetii* is not a newly emerged pathogen in Denmark; most likely it has been common among people with contact to cattle for a long time.

In the present study, we applied a cutoff of 1:128 which is higher than in other studies [16,24,33,34]. The Danish cutoff was based on the assumption that Q fever was uncommon

in the general Danish population [22]; however, two other studies have shown that in the rural populations of Denmark, Q fever is more widespread than earlier assumed [32,35].

In the definition of the Danish cutoff, the authors included a grey zone in order to address individuals with an a priori elevated risk of Q fever (Table 1), proposing that high risk groups such as veterinarians, farmers etc. with a grey zone titre should be considered probably positive and managed as such (the predictive value of a positive result is likely to be higher in an exposed population than in the general population).

Moreover, the Danish cutoff was based on the assumption that blood donors from urban areas of Denmark are not exposed to *C. burnetii*, but the prevalence of antibodies among women with no animal exposure in our study (4.8%) is rather high compared to, for instance, the seroprevalence in the general population in the Netherlands before the recent outbreak of about 2.4% [36]. This may indicate that *C. burnetii* is generally widespread in Denmark, but could also be an argument in favour of not lowering the cutoff too much.

Consequently, we have decided to apply 1:128 as cutoff for all phases in this study.
Human outbreaks of Q fever have only been described to occur from small ruminants; in France, goats and sheep have been the source of infection. The Netherlands have recently experienced the world's largest outbreak of Q fever with more than 4000 humans infected [37] and here the source of infection was goats [38].

There are different strains of *C. burnetii*, and, as for other bacteria, these are probably expressed with varied pathogenicity and different manifestations among animals as well as humans. Variation in strains and pathogenicity could be a partial explanation for the variation in incidence of illness reported from different countries. From the Dutch outbreak it has been suggested that one genotype is responsible for the human Q fever epidemic, since the genotypes found in humans and goats were very similar [38]. An alternative explanation could be the possibility that small ruminants shed larger doses of bacteria than cattle usually do.
In comparison to France and the Netherlands, there are few sheep and goats in Denmark; the source of infection here is primarily cattle [39], and as far as we know Denmark has never experienced a clinically verified Q fever outbreak.

Our study has limitations since we have no PCR or culture positive samples to verify 'true positivity'. But we regard the size of this cohort a major strength to this study.

In conclusion, this study found that Danish pregnant women exposed to livestock animals have significantly higher levels of antibodies against *C. burnetii* when compared to unexposed with the highest prevalence of antibodies found among veterinarians who worked with cattle.
Our findings enhance how *C. burnetii* is not a newly emerged pathogen in Denmark and that Q

fever is endemic here as is probably the case in most other countries.

Our results suggest that contact with livestock is a risk factor for *C. burnetii*. Keeping in mind the high prevalence of symptoms and severe pneumonia reported from the recent Dutch outbreak, Q fever should be considered a possible differential diagnosis in people with close contact to domestic animals, especially veterinarians and women domestically exposed to cattle.

Table 1: Cutoff values immunofluorescence antibody test (IFA) as applied in Denmark (16). In the present study, a cutoff of 1:128 was used for all phases

	Negative	Grey zone	Positive
IgM phase I	<64	64	>=128
IgM phase II	<64	64-128	>=256
IgG phase I	<128	128-256	>=512
IgG phase II	<128	128-512	>=1024

Table 2: Distribution of selected characteristics among 856 women from the Danish National Birth Cohort

	Occupationally exposed (N=195)	Domestically exposed (N=202)	Unexposed reference (N=459)
AGE: (N=856)			
<25:	13 (6.7%)	26 (12.9%)	65 (14.2%)
25-<35:	148 (75.9%)	140 (69.3%)	343 (74.7%)
35+:	34 (17.4%)	36 (17.8%)	51 (11.1%)
Living in rural area (n=427)	113 (58.5%)	163 (81.9%)	151 (33.3%)
Living in urban area (n=418)	80 (41.5%)	36 (18.1%)	302 (66.7%)

Table 3: Risk Difference and Relative Risks for occupationally and domestically exposed compared to an unexposed reference group according to prevalence of antibodies against *C. burnetii* in pregnancy using Immunofluorescence (IFA). RD = Risk Difference, RR = Relative Risk.

	Occupationally exposed (N=195)	Domestically exposed (N=202)	Unexposed reference group (N=459)
IFA negative	103 (52.8%)	137 (67.8%)	437(95.2%)
IFA positive	92(47.2%)	65 (32.2%)	22 (4.8%)
RD (95%CI)	0.42 (0.35-0.50)	0.27 (0.21-0.34)	
RR (95%CI)	9.84 (6.37-15.20)	6.71 (4.26-10.57)	

Table 4: Specified characteristics according to Immunofluorescence antibody (IFA) seropositivity among women with animal contact. PPV = Positive predictive value, NPV = Negative predictive value

	IFA positive (n=62)	IFA negative (n=783)	Risk ratio	PPV	NPV
Veterinarians (N=118)					
Specific work related contact to cattle					
Yes (n=31)	23(74.2%)	8 (25.8%)	2.7 (95%CI:1.8-4.0)	48.9%	88.7%
No (87)	24 (27.6%)	63(72.4%)			
Slaughtery					
Yes (n=20)	9 (45%)	11 (55%)	1.2 (95%CI:0.7-2.0)	19.1%	85.5%
No (n=98)	38 (38.8%)	60 (61.2%)			
Veterinarians with domestic animal contact					
Yes (n=38)	23 (60.5%)	15 (39.5%)	2.0 (95%CI:1.3–3.1)	48.9%	69.1%
No (n=80)	24 (30%)	56 (70%)			
Domestic exposure (N=202):					
Cattle					
Yes (n=193)	64 (33.2%)	129 (66.8%)	2.98 (95%CI:0.5-19.1)	98.4%	5.8 %
No (n=9)	1 (11.1)	8 (88.9%)			
Sheep					
Yes (n=22)	6 (27.3%)	16 (72.3%)	0.8 (95%CI:0.4-1.7)	9.2%	88.3%
No (n=180)	59 (32.8%)	121(67.2%)			
Cattle and sheep					
Yes (n=13)	5 (38.5%)	8(61.5%)	1.21 (95%CI: 0.6-2.5)	7.7%	94.2%
No (n=189)	60 (31.8%)	129 (68.2%)			

Figure 1. Immunofluorescence antibody (IFA) IgG phase II according to enzyme-linked immunosorbent assay (ELISA) Optical Density (OD) values.

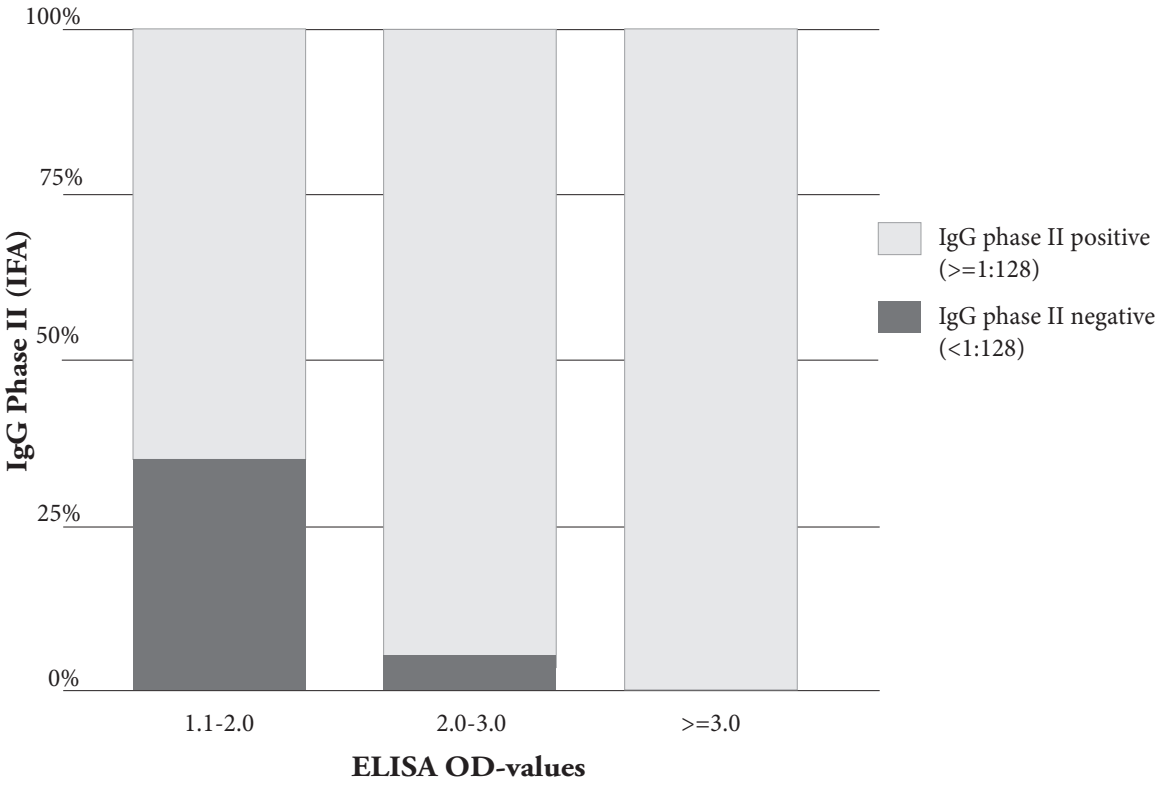
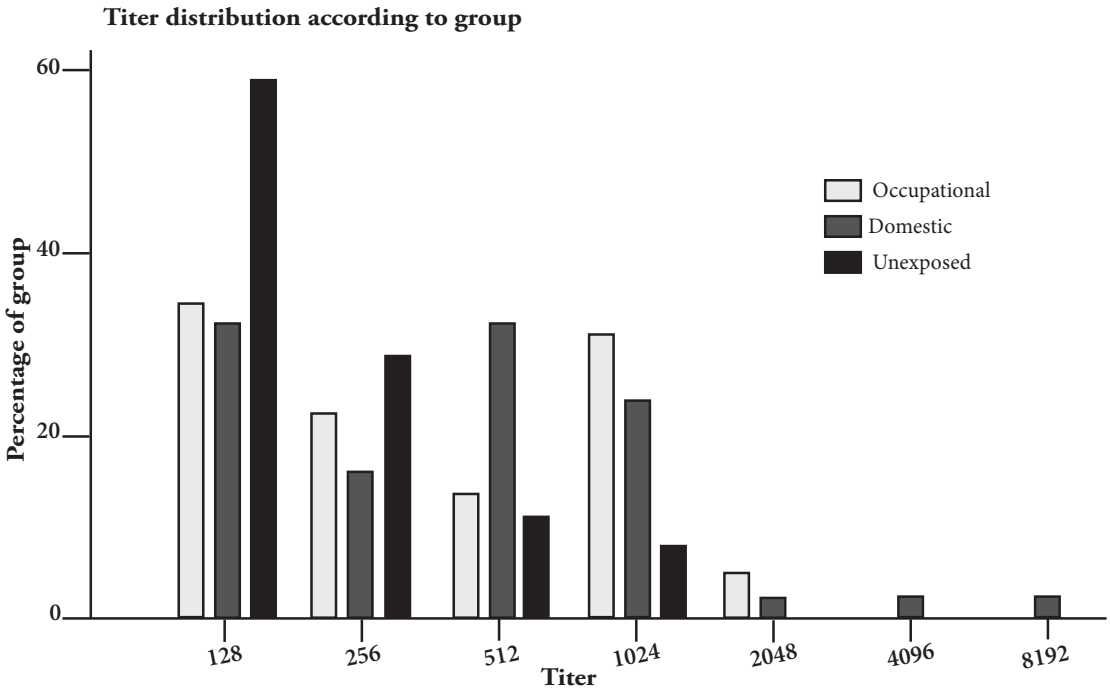


Figure 2. Titre distribution of immunofluorescence (IFA) IgG phase II according to exposure groups.



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RESEARCH ARTICLE Open Access

No excess risk of adverse pregnancy outcomes among women with serological markers of previous infection with *Coxiella burnetii*: evidence from the Danish National Birth Cohort

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Abstract

Background: Q fever caused by *Coxiella burnetii* is transmitted to humans by inhalation of aerosols from animal birth products. Q fever in pregnancy is suspected to be a potential cause of fetal and maternal morbidity and fetal mortality but the pathogenesis is poorly understood, and even in Q fever endemic areas, the magnitude of a potential association is not established.
We aimed to examine if presence of antibodies to *C. burnetii* during pregnancy or seroconversion were associated with adverse pregnancy outcomes.

Methods: The Danish National Birth Cohort collected blood samples and interview data from 100,418 pregnant women (1996–2002). We sampled 397 pregnant women with occupational or domestic exposure to cattle or sheep and a random sample of 459 women with no animal exposure. Outcome measures were spontaneous abortion, preterm birth, birth weight and Small for Gestational Age (SGA).
Blood samples collected in pregnancy were screened for antibodies against *C. burnetii* by enzyme-linked immunosorbent assay (ELISA). Samples positive for IgG or IgM antibodies in the ELISA were confirmed by immunofluorescence antibody test (IFA).

Results: Among the 856 women, 169 (19.7%) women were IFA positive; 147 (87%) of these had occupational or domestic contact with livestock (IFA cutoff > =1:128).
Two abortions were IFA positive vs. 6 IFA negative (OR: 1.5; 95%CI: 0.3-7.6). Three preterm births were IFA positive vs. 38 IFA negative (OR: 0.4; 95% CI: 0.1-1.1). There was a significant difference in birth weight of 168 g (95% CI: 70-267 g) with IFA positive being heavier, and the risk of being SGA was not increased in the newborns of IFA positive women (OR: 0.4; 95%CI: 0.8-1.0).
Most seropositive women were IgG positive indicating previous exposure. Seroconversion during pregnancy was found in 10 women; they all delivered live babies at term, but two were SGA.
(Continued on next page)

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Conclusion: We found no increased risk of adverse pregnancy outcome in women with verified exposure to *C. burnetii*.
To our knowledge, this is the first population-based seroepidemiologic study evaluating pregnancy outcome in women with serologically verified exposure to *C. burnetii* against a comparable reference group of seronegative women.

Keywords: Q fever, *Coxiella burnetii*, Infection, Human, Pregnancy, Spontaneous abortion, Preterm birth

Background

Q fever is a zoonotic infection caused by *Coxiella burnetii,* an intracellular pathogen. In small ruminants Q fever is known to cause abortions, retained placenta, endometritis and infertility. Placentas of infected animals contain high numbers of bacteria [1,2]; the bacteria remain viable for months in the environment.

Human infection is usually acquired through inhalation of contaminated aerosols from infected animals that contaminate the environment in particular through excretion of the bacteria in large amounts in birth-by-products, especially placenta [3-5].

Q fever has previously been considered a rare, imported infection in Denmark, but recent studies have found antibodies against *C. burnetii* in a large percentage of Danish dairy cattle as well as in humans exposed to livestock [6-8].

For otherwise healthy people, Q fever infection is often asymptomatic or has a mild, flu-like course, but may also cause severe pneumonia. Pregnant women, immunocompromised patients and patients with pre-existing cardiac valve- or vascular defects are at risk of a severe course of infection [3,5].

Q fever in pregnancy is suspected to be a potential cause of fetal morbidity and mortality, but the pathogenesis is poorly understood, and even in Q fever endemic areas the magnitude of a potential association is not established.

Present evidence mainly originates from French case studies of referred infected pregnant patients in which untreated infection was followed by spontaneous abortion, intrauterine growth retardation, oligohydramnion, stillbirth or premature delivery [9]. Infection in pregnancy is often asymptomatic but may imply an increased risk of chronic infection and a risk of reactivation of a past infection in subsequent pregnancies has been suggested [9-11].

Two new studies evaluated infection in pregnancy and found no increased risk of adverse pregnancy outcome in seropositive pregnancies [12,13].

Although Q fever is endemic worldwide, the reported prevalence seems to be highest in areas with medical or scientific awareness of the infection and many obstetricians know little about the infection [10]. Since the evidence of pregnancy outcome in women with Q fever infection relies primarily on case reports, unbiased estimates of the risks of adverse pregnancy outcome among infected women remain largely unknown.

Our primary objectives were to evaluate the association between antibodies to *C. burnetii* and pregnancy outcome and to compare pregnancy outcome in women who seroconverted during pregnancy with seronegative pregnant women.

Methods

Participants

The study was based on interview data and blood samples from the Danish National Birth Cohort (DNBC), which is a nationwide cohort of 100,418 pregnant women and their offspring.

Enrolment in the DNBC took place between 1996 and 2002. The women were recruited in connection with the first antenatal visit to the general practitioner. Information on variables reflecting exposures before and during the early part of pregnancy was collected by means of a computer assisted telephone interview scheduled around gestational week 12. A second interview was scheduled in week 30 (interview forms are available at the website for the cohort).

During pregnancy, two blood samples were collected; one between gestational weeks 6 to 12, the second in gestational week 24. A sample was also drawn from the umbilical cord.

The interviews were performed if the women were reached within four phone calls, and if they agreed to participate.

The interviews covered reproductive history, age, smoking status, domestic contact to animals as well as very detailed questions regarding occupational contact to different animals.

A detailed description of the cohort can be found elsewhere [14].

In women who participated in the first interview and who also provided a blood sample (n = 95000) the study population was defined as follows: Occupational contact with livestock (n = 195), domestic contact with cattle or sheep (n = 202) and a randomly selected sample with no contact to livestock (n = 459). Blood samples from

these 856 women were analyzed for antibodies against *C. burnetii.*

Pregnancy outcome was defined as:

Spontaneous abortion: fetal loss before 154 days (22 weeks) after the first day of the last menstrual period with gestational age estimated from the participants' self- reported last menstrual period.
Preterm delivery: delivery (live births and stillbirths) between gestational weeks 22 + 0 days and 36 weeks + 6 days.
Small for gestational Age (SGA): for children born from week 37 + 0 and onwards, SGA was defined as a birth weight corresponding to the 10^{th} percentile in gram and below. Children with a birth weight above the 10^{th} percentile were used as reference group.

The relationship between serological status, birth weight and gestational age, respectively, was also evaluated.

We also evaluated late induced abortions and stillbirth.

Detection of antibodies against C. burnetii

C. burnetii expresses two antigens, phase I and phase II. When infected, phase II IgG and IgM antibodies are elevated, and they may remain positive for months to years. A large study from Australia and England found that phase II IgG antibodies persisted after four and 12 - years, respectively [15].

In acute Q fever, primarily antibodies against phase II are raised, and titers are higher than antibodies against phase I. As with most other infections, IgM antibodies appear first.

In chronic forms of the disease, antibodies against phase I are elevated.

In order to determine antibodies against *C. burnetii,* we chose a two-step approach. First, all samples were screened in a commercial enzyme-linked immunosorbent assay (ELISA). Positive samples from the ELISA were confirmed with an immunofluorescence antibody test (IFA) which is considered to be gold standard when diagnosing Q fever.

The commercial ELISA kit were purchased from Panbio (Queensland, Australia) (cat. no. E-QFB01G and E-QFB01M) and used according to the manufacturer's instructions with minor modifications; due to low sample volume the samples were diluted differently from what was prescribed in the instructions but the same dilution factors were used.

Samples which were positive for either IgG or IgM antibodies in the ELISA were confirmed with an IFA test from Focus Diagnostics (ca. no. IF0200G and IF0200M). The test was performed according to the instructions provided by the manufacturer, with the following minor modifications: due to a low amount of sample material, the diluted samples 1:10 from the ELISA were used to further dilute the samples as described by the manufacturer. The effect of the dilution in the Panbio buffer was tested prior to the use on patient samples and did not show any influence on the results (results not shown).

A local cutoff adjusted to the Danish population has been defined [16], including negative, equivocal and positive titers. When the ELISA positive samples in our study were reanalyzed using IFA, a modified version of the Danish cutoff was used. A sample was considered IFA positive when any of the phases were 1:128 or above.

For women without animal exposure, only the blood sample from the first trimester was analyzed. In women with contact to livestock, blood samples from the umbilical cord or mid-pregnancy were analyzed initially (n = 361 women) and therefore seroconversion during pregnancy could be monitored.

In order to detect a possible seroconversion throughout pregnancy, our strategy was to initially analyze the last existing blood sample (for 79 women this was the mid pregnancy sample and for 282 it was the umbilical cord sample). If this sample was tested positive in ELISA, the first blood sample from pregnancy week 12–16 was analyzed using ELISA.

In order to select which of the ELISA positive samples from the beginning of pregnancy were to be reanalyzed in IFA, the following criteria had to be met: a change in ELISA from negative in the beginning of pregnancy to positive in the mid-pregnancy or umbilical cord sample or a doubling in the adjusted ELISA OD-value throughout pregnancy.

In analyses of pregnancy outcome, women with seroconversion as well as women who were seronegative in the midpregnancy or in the umbilical cord sample were classified as seronegative.

All serological analyses were performed in a certified laboratory at Statens Serum Institut, Denmark. Laboratory personnel were blinded for exposure status and samples were always analyzed in the same batch of commercial kits.

Statistical analysis

Associations between positive serology (IFA), spontaneous abortion, preterm birth and Small for Gestational Age (SGA) were analyzed by logistic regression. The association between gestational age at birth (which does not follow a normal distribution) and positive IFA serology was tested using a non-parametric (Wilcoxon) test. We examined the association between positive serology (IFA), birth weight and gestational age for children born at term, respectively, by fitting multiple linear regression models.

Maternal age (<25 years, 25–34 years, 35+ years), number of previous pregnancies (0, 1+) and smoking

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during pregnancy (0, 1–10, 11+ cigarettes per day) were *a priori* selected as potential confounders.

All analyses were carried out using STATA statistical software, version 11.

Women enrolled in the Danish National Birth Cohort gave both verbal and written consent to participate. The women gave permission to include interview information, blood samples, and health information from other registers in the Danish National Birth Cohort. This study was approved by the Danish National Birth Cohort, the Danish Data Protection Board, and the Danish Regional Scientific Ethical Committee.

Results

Among the 856 women, antibodies against *C. burnetii* (IFA) were detected in 169, while 687 women were IFA negative. The majority (87%) of the IFA positive women had contact to livestock (Table 1).

Table 1 Maternal characteristics of pregnant women according to Q fever seropositivity in immunofluorescence antibody test (IFA)

	IFA positive (n = 169)	IFA negative (n = 687)
Age:		
<25 years	10 (5.9%)	94 (13.7%)
25 - <35 years	139 (82.3%)	492 (71.6%)
35 years+	20 (11.3%)	101 (14.7%)
Prior pregnancies		
0	57 (33.7%)	250 (36.4%)
1+	112 (66.3%)	437 (64.6%)
Gestational age at recruitment:		
<8	21 (12.4%)	111 (16.2%)
Week 8-12	86 (50.9%)	321(46.7%)
Week 12- < 16	38 (22.5%)	186 (27.1%)
Week 16+	24 (14.2%)	69 (10.0%)
Smoking:		
Non-smokers:	155 (91.7%)	566 (82.1%)
1- < 10 g/day	4 (2.4%)	64 (9.3%)
+10 g/day	8 (4.7%)	48 (7.0%)
Unknown	2 (1.2%)	9 (1.3%)
Animal Contact:		
Occupational or domestic contact to livestock (cattle, goats, sheep)	147 (87.0%)	250 (36.4%)
No contact to livestock	22 (13.1%)	437 (63.6)
Residence:		
Living in rural area	121 (71.6%)	303 (44.5%)
Living in non-rural area	45 (26.6%)	373 (54.3%)
Unknown	3 (1.8%)	8 (1.2%)

IFA positivity

Among the 169 IFA positive women, 159 were positive in IgG phase II; 73 of these were also IgG phase I positive, six were only IgG phase I positive. Seven women were positive in IgM phase II, three in IgM phase I. For six women, there was an overlap in positivity between IgM and IgG phases. Hence, the participants' serology mainly indicated previous infections.

Maternal age was normally distributed and age at recruitment was similar among IFA positive and IFA negative women (mean: 24.7 years (SD: 7.0) vs. mean: 23 years (SD 9.8)). There was no difference in the number of previous pregnancies between the two groups and the IFA positive and IFA negative women were, on average, recruited at the same gestational age (11 weeks 1 day (SD 3.7) vs. 10 weeks 6 days (SD 3.6)). A higher proportion of IFA negative were smokers. Seropositive samples were mainly from women who had contact to livestock during pregnancy or 3 months prior to becoming pregnant (Table 1).

Serology and pregnancy outcome

We found no association between positive serology and risk of spontaneous abortion (adjusted OR: 1.5; 95% CI: 0.3-7.6) or preterm birth (adjusted OR: 0.4; 95% CI: 0.1-1.1) (Table 2).

Infants born by seropositive mothers had a 0.9 day older gestational age than infants born by seronegative mothers, but this difference was not significant (p = 0.06, Wilcoxon non-parametric test). The relation between positive IFA serology and gestational age was also tested in a multiple linear regression model which did not change the results significantly (adjusted difference: 1.2 - days; 95% CI: -0.4 days - +2.7 days, (Table 2)).

When evaluating the birth weight for all newborns, there was a significant weight difference (168 g; 95% CI: 70-267 g) with the IFA positive babies being heavier; results were similar when restricting analyses to term babies (37 completed weeks or more): (134 g; 95% CI: 47-221 g) (Table 3).

We found no association between SGA and seropositivity (IFA) (OR: 0.4; 95% CI: 0.8-1.0) (Table 3).

One IFA negative woman had an induced abortion after pregnancy week 12 due to fetal disease. One preterm birth was a stillbirth in gestational week 23; two women had stillbirths in gestational week 35, all were IFA negative.

To further explore the relationship between contact to livestock, seropositivity and pregnancy outcome, we also examined the pregnancy outcome among IFA positive women with livestock contact compared to IFA negative women with no contact to livestock. We also compared pregnancy outcome among IFA positive versus IFA

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Table 2 Gestational age parameters for Danish pregnant women according to antibodies against *C.burnetii*, immunofluorescence antibody test (IFA)

	IFA positive for *C. burnetii* antibodies (n = 169)	IFA negative for *C. burnetii* antibodies (n = 687)	Measures of association	
			Crude	Adjusted
Spontaneous abortion < 22 weeks (n = 8)	2 (1.2%)	6 (0.9%)	OR: 1.4	1.5 (95% CI: 0.3–7.6)*
Preterm birth (< week 37) (n = 41)	3 (1.8%)	38 (5.5%)	OR: 0.3	OR: 0.4 (95% CI: 0.1–1.1)**
Gestational age (>week 36 + 6) (n = 806****) Median gestational age (interquartile range)	40 weeks 3 days (39 w, 3d; 41 w,1 d)	40 w, 2 days (39w, 3d; 41w,1d)	Mean difference in days*** 0.91 days	1.2 days (−0.4– +2.7)**

*adjusted for age **adjusted for smoking, age and gravidity *** babies of IFA positive mothers were older **** of which 163 were seropositive and 643 seronegative.

negative pregnant women within the groups of women with livestock contact. None of the results showed any significant association between seropositivity and adverse pregnancy outcome (not shown).

Seroconversion and pregnancy outcome

A total of 14 women met the criteria for seroconversion during pregnancy in ELISA. These were confirmatory tested in the IFA; 10 of them seroconverted during pregnancy as defined by the modified Danish cutoff. All had occupational or domestic contact to livestock. All gave live birth at term, however, two newborns were SGA (birth weight: 2110 g and 2236 g, respectively) (Table 4).

None of the seroconverters reported episodes of fever during pregnancy at the interview by the beginning of third trimester.

Discussion

We hypothesized that being seropositive in pregnancy would be associated with adverse pregnancy outcome, potentially mediated by reactivation of a latent infection [9-11]. We also hypothesized that acute infection during pregnancy would be related to adverse pregnancy outcome. Neither of these hypotheses were confirmed as no increased risk of adverse pregnancy outcome was found in women with verified exposure to *C. burnetii*.

To our knowledge, this is the first population-based seroepidemiologic study evaluating pregnancy outcome in women with serologically verified exposure to *C. burnetii* against a comparable reference group of seronegative women.

When diagnosing Q fever, a variety of serological methods are available; the Panbio ELISA kit has previously been showed to be superior to other methods [18] and suitable for large-scale screening [17,19]. The micro immunofluorescence antibody test (IFA) is regarded as the gold standard [20] because it is capable of determining both phase I and II antibodies simultaneously by the use of two different antigens in a single sample. We have previously demonstrated coherence between ELISA and IFA [21].

Villumsen et al. established a national, very restrictive cutoff in order to obtain a high specificity and a high predictive value of a positive result [21]; this decision was based on the assumption that Q fever was sporadic in Denmark. However, particularly in rural populations of Denmark, Q fever is more widespread than previously considered [7,8] and one may now argue that the cutoff may be too conservative.

Consequently, in the present study, we decided to use a modified version of the Danish cutoff. A more conservative interpretation of the serological values (theoretically leading to a lower positive prevalence and higher

Table 3 Birth weight parameters for Danish pregnant women according to antibodies against *C.burnetii*, immunofluorescence antibody test (IFA)

	IFA positive for *C. burnetii* antibodies	IFA negative for *C. burnetii* antibodies	Measures of association	
			Crude	Adjusted
Birth weight all (n = 842) Median Birth weight (interquartile range)	166	676	Mean difference in gram*: 204 g	168 g (95% CI:70–267 g)**
	3780 g (3480 g; 4085 g)	3600 g (3260 g; 3997 g)		
Birth weight children born at term (n = 803***) Median Birth weight (interquartile range)	163	640	Mean difference in gram*: 160 g	134 g (95% CI: 47–221 g)**
	3790 g (3490 g; 4090 g)	3650 g (3309 g; 4000 g)		
Small for gestational age term pregnancies (week 37+ (n = 82) Median Birth weight (interquartile range)	9	73	OR: 0.5	0.4 (95% CI: 0.8–1.0)**
	2820 g (2275 g; 3010 g)	2850 g (2350 g; 3030 g)		

*IFA positive babies are heavier **adjusted for smoking, age and gravidity.
*** of which 163 were seropositive and 640 seronegative.

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Table 4 Immunofluorescence antibody test (IFA) titres at beginning and end of pregnancy for the 10 seroconverted pregnancies

	IFA blood sample beginning of pregnancy				IFA blood sample umbilical cord				Pregnancy outcome (all live singletons)	
Patient	IgG phase II	IgG phase I	IgM phase II	IgM phase I	IgG phase II	IgG phase I	IgM phase II	IgM phase I	Gestatio-nal age, weeks	Birth weight
#1	Neg	Neg	Pos: 1:128	Neg	Pos: 1:2048	Pos: 1:1024	Neg	Neg	38	3190 g
#2	Neg	Neg	Neg	Neg	Pos: 1:256	Pos: 1:128	Neg	Neg	38	4200
#3	Neg	Neg	Neg	Neg	Pos: 1:1024	Pos: 1:512	Neg	Neg	42	2110 g
#4	Neg	Neg	Neg	Neg	Pos: 1:128	Pos: 1:128	Neg	Neg	41	4220 g
#5	Neg	Neg	Neg	Neg	Pos: 1:128	Pos: 1:128	Neg	Neg	41	3430 g
#6	Neg	Neg	Neg	Neg	Pos: 1:128	Pos: 1:128	Neg	Neg	42	3400 g
#7	Neg	Neg	Neg	Neg	Pos: 1:128	Pos: 1:128	Neg	Neg	40	3330 g
#8	Neg	Neg	Neg	Neg	Pos: 1:8192	Pos: 1:256	Neg	Neg	39	2236 g
#9	Neg	Neg	Neg	Neg	Pos: 1:128	Pos: 1:128	Pos: 1:256	Pos: 1:128	40	3520 g
#10	Neg	Neg	Neg	Neg	Pos 1:512	Pos:1:128	Neg	Neg	41	3950 g

predictive value) did not reveal any associations between seropositivity and adverse outcome of pregnancy.

Finally, we also acknowledge that the cutoff applied in our study is high compared with some other studies. However, in a seroepidemiologic study including healthy individuals, our priority was to maintain a high predictive value for a positive result. The application of a lower cutoff would have falsely classified additional women as seropositive and lead to misclassification and thus a higher risk of overlooking a potential association between (true) seropositivity and adverse outcome of pregnancy.

Most of the seropositive women had markers of previous infections, but ten met the criteria for IFA seroconversion. It is worth to note that two out of these women gave birth to infants that were SGA. We cannot draw any conclusions on the risk of adverse pregnancy outcome from 10 cases and the low number of seroconverters is a limitation to this study. Hence, we cannot make an inference with respect to pregnancy outcome in women with acute and, in particular, symptomatic infections.

The risk of reactivation of latent infection leading to adverse pregnancy outcome has been reported [9,10]. However, the IgG positive women in our study had a similar proportion of previous spontaneous abortions as the seronegative women, and overall, reactivation of latent infections leading to adverse pregnancy outcomes was not observed in this population.

Detailed information on previous preterm births was not available, and we chose adjustment for prior pregnancies regardless of pregnancy outcome.

In women with contact to livestock, we had the opportunity to evaluate seroconversion throughout pregnancy; in women with no contact to livestock we only had blood samples from beginning of the pregnancy. This could potentially bias data as the women without animal contact were assumed to be negative throughout pregnancy when, theoretically, they could be infected later in their pregnancy. This is why women with seroconversion as well as women who were seronegative in the mid-pregnancy or in the umbilical cord sample were classified as seronegative in analyses of pregnancy outcome. Also, stratified analysis on contact to livestock and pregnancy outcome (spontaneous abortion and preterm birth), irrespective of titer status, showed no significant difference between the groups (results not shown).

A high seroprevalence of *C.burnetii* accompanied by few clinical symptoms in farmers and veterinarians has been found in Denmark as well as abroad [7,8,22]. We evaluated pregnancy outcome in seropositive versus seronegative women who had occupational, domestic, or no exposure to livestock (as stated in the methods section). The vast majority of the seropositive women were exposed to animals (Table 1). Due to few unexposed, seropositive women we are unable to study adverse pregnancy outcome in this group of women or clarify whether the dynamics of infection differ in unexposed women compared to women heavily exposed to *C.burnetii*.

The evidence of the impact of Q fever on pregnancy outcome mainly originates from French case studies of referred infected pregnant patients and pregnancies with Q fever diagnosed retrospectively after an adverse pregnancy outcome [7,8]. The authors conclude that there is a link between placentitis and obstetric complications. However, in a recent study by Angelakis et al., [23] a study of 30 pregnant women with acute infection in pregnancy, no placentitis or isolation of *C.burnetii* is found in 14 available biopsies. 17 of the women were asymptomatic, but only two of these had an uncomplicated pregnancy illustrating the difficulty in segregating harmless seroconversion from infection threatening

maternal and foetal health. In that study, genotyping showed that QpDV plasmid was present in 4 of 7 *C. burnetii* strains isolated from infected women with miscarriage. Apart from differences in study design, numbers of pregnancies included, selection bias and cutoffs, the disagreements between the French, the Dutch and our studies could be related to strain specificity. Risk assessment and management of Q fever in pregnancy may therefore benefit from further clarification of the role of strain differences and virulence factors.

The present study is subject to some limitations.

Due to the design of the study, it was not possible to include early miscarriage as an outcome. Only few participants were included prior to 8 weeks of gestational age (Table 1). It is possible that the study population is biased towards a "healthy pregnant population". An increased risk in early pregnancy may in our study be reflected by a "protective" effect in later pregnancy.

Also, maternal IgM cannot be detected in umbilical cord blood, meaning that theoretically we could miss a narrow window of acute infections in very late pregnancy with positive IgM but before IgG phase II elevation; the potential effect on pregnancy outcome from this is, however, speculative.

The French recommendation regarding treatment with cotrimoxazole throughout pregnancy in seropositive women [9,10,23] is widely practiced, but has recently been questioned [24]. However, the number of acute infections in our study is too small to impact these recommendations.

Overall, our findings are in line with two new studies from The Netherlands, a country that recently saw the world's largest Q fever outbreak [25]. One study included serum samples from early pregnancy of 1174 pregnant women living in the high-risk area and found no association between positive Q fever serology and adverse pregnancy outcome [13]. The other study was a randomized controlled trial with 1229 women split into a screening group and a control group; no difference in pregnancy outcome was found between the two groups [12].

Conclusion

Seropositivity was not associated with adverse pregnancy outcomes as this study did not find a higher risk of spontaneous abortion, preterm birth, or low birth weight among pregnancies positive for *C. burnetii* compared to seronegative Danish pregnant women.

Abbreviations

SGA: Small for Gestational Age; ELISA: Enzyme-Linked Immunosorbent Assay; IFA: Immunofluorescence Antibody test; DNBC: the Danish National Birth Cohort.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SYN initiated the study, coordinated all blood sample analysis, performed the statistical analysis and drafted the manuscript. AMNA participated in the design of the study and supervised the data collection and management. KRM participated in the design of the study and helped drafting the manuscript. BJK supervised all the ELISA and IFA analysis. KAK helped conceive the study. NHH participated in the design of the study. TBH participated in the design of the study and supervised the statistical analysis. All authors read and approved the final manuscript.

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Presence of Antibodies Against *Coxiella burnetii* and Risk of Spontaneous Abortion: A Nested Case-Control Study

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Abstract

Background and Aims: Q fever is a bacterial zoonosis caused by infection with *Coxiella burnetii*. It is well established that Q fever causes fetal loss in small ruminants. The suspicion has been raised that pregnant women may also experience adverse pregnancy outcome when the infection is acquired or reactivated during pregnancy. The purpose of this study was to assess the potential association between serologic markers of infection with *C.burnetii* and spontaneous abortion.

Methods: A nested case-control study within the Danish National Birth Cohort, a cohort of 100,418 pregnancies recruited from 1996–2002. Women were recruited in first trimester of pregnancy and followed prospectively. Median gestational age at enrolment was 8 weeks (25 and 75 percentiles: 7 weeks; 10 weeks). During pregnancy, a blood sample was collected at gestational week 6–12 and stored in a bio bank. For this study, a case sample of 218 pregnancies was drawn randomly among the pregnancies in the cohort which ended with a miscarriage before 22 gestational weeks, and a reference group of 482 pregnancies was selected in a random fashion among all pregnancies in the cohort. From these pregnancies, serum samples were screened for antibodies against *C. burnetii* in a commercial enzyme-linked immunosorbent assay (ELISA). Samples that proved IgG or IgM antibody positive were subsequently confirmatory tested by an immunofluorescence (IFA) test.

Results: Among cases, 11 (5%) were *C. burnetii* positive in ELISA of which one was confirmed in the IFA assay compared to 29 (6%) ELISA positive and 3 IFA confirmed in the random sample.

Conclusions: We found no evidence of a higher prevalence of *C.burnetii* antibodies in serum samples from women who later miscarried and the present study does not indicate a major association between Q fever infection and spontaneous abortion in humans. Very early first trimester abortions were, however, not included in the study.

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Introduction

Q fever, a zoonotic infection caused by *Coxiella burnetii*, has previously been considered a rare, imported infection in Denmark, but recent studies have found antibodies against *C.burnetii* in a large percentage of Danish dairy herds and among individuals exposed to livestock animals [1–3].

In cattle and small ruminants Q fever is known to cause abortions, retained placenta, endometritis and infertility, and placentas of infected animals contain a high number of organisms [4,5]. The bacteria remain viable for months in the environment and the most important route of transmission to humans is inhalation of contaminated aerosols.

For otherwise healthy people, Q fever infection is often asymptomatic or with a mild, flu-like course, but may also cause severe pneumonia. Pregnant women, immunocompromised patients and patients with pre-existing cardiac valve or vascular defects are at risk of a severe course of the infection [6,7], [8].

The precise mechanisms by which the infection compromises pregnancy are largely unknown, but adverse pregnancy outcome has been reproduced in BALB/c mice in which infection followed by repeated pregnancies resulted in spontaneous abortion and perinatal death [9].

C. burnetii is an intracellular pathogen, but the cell types infected by *C.burnetii* in humans are unknown. A recent study used a human trophoblast cell line and found that *C.burnetii* infected and replicated within trophoblastic cells but the bacteria seemed unable to interfere with development of a normal pregnancy.

The study suggested that normal development of pregnancy may be impaired by the cooperation of trophoblasts and placental immune cells responsive to *C.burnetii* within the placental tissue [10].

Present evidence mainly originates from French case studies of referred pregnant women in which infection resulted in spontaneous abortion, intrauterine growth retardation, oligohydramnion, stillbirth and premature delivery in untreated pregnancies. One series of 53 cases demonstrated obstetric complications in 81% of Q fever positive cases not receiving long-term antibiotic treatment [11]. Infection in pregnancy is often asymptomatic but may imply an increased risk of chronic infection [8]. A risk of reactivation of a past infection in subsequent pregnancies has been described and infection in 1st trimester may constitute a specific risk of spontaneous abortion [11–13].

Due to the sparse literature on Q fever in pregnancy, unbiased estimates of the risks of adverse pregnancy outcome among infected women remain largely unknown, and even though Q fever is endemic worldwide many obstetricians know little about the infection. The incidence of Q fever among pregnant women may therefore be underestimated [8].

The objective of the present study was to compare the prevalence of antibodies to *C.burnetii* in a random sample of pregnancies terminated by spontaneous abortion to the prevalence in the background population.

Materials and Methods

Ethics statement

Women enrolled in the Danish National Birth Cohort gave both verbal and written consent to participate. The women gave permission to include interview information, blood samples and health information from other registers in the Danish National Birth Cohort. The study was approved by the Danish National Birth Cohort, the Danish Data Protection Board, and the Danish Regional Scientific Ethical Committee.

Participants

The study was based on interview data and blood samples from the Danish National Birth Cohort (DNBC), which is a nationwide cohort of 100,418 pregnant women and their offspring.

Enrolment in the DNBC took place between 1996 and 2002, and the women were recruited in connection with the first antenatal visit to the general practitioner. Gestational age at enrolment was scheduled to be 10 weeks. The median gestational week of enrolment was 8 weeks (25 and 75 percentiles: 7 weeks; 10 weeks), but some women were enrolled as early as in week 4 and as late as gestational week 27.

The percentage of pregnancies that resulted in a spontaneous abortion in the entire cohort was 4.7%. Foetal life table analysis has estimated the proportion of spontaneous abortions from gestational week 6 to be 11% in the DNBC [14].

Information on exposures before and during the early part of pregnancy was collected by means of a computer assisted telephone interview scheduled to take place in gestational week 12 or as soon as possible thereafter. In case of fetal loss before this interview, participants were offered a similar interview as soon as possible after the fetal loss (interview forms available at www.bsmb.dk).

During pregnancy, two blood samples were collected; one around gestational week 6–12, the second in gestational week 24. A sample was also drawn from the umbilical chord.

The interviews were not performed if the women were not reached within four phone calls, or did not wish to participate.

A more detailed description of the cohort can be found elsewhere [15].

This study was designed as a nested case-control study. A number of 200 pregnancies were randomly selected from the 4740 participants who experienced a miscarriage before 154 gestational days (22 gestational weeks), and for whom a serum sample was taken at the first antenatal visit at the GP and stored in a bio bank.

The case definition was miscarriage, defined as fetal loss before 154 days (22 weeks) after the self-reported first day of the last menstrual period.

A base sample of 500 non-cases was randomly selected among the 92500 participants with an existing first blood sample from early pregnancy. A total of 18 of the pregnancies in the base sample had spontaneous abortion as outcome and were consequently reclassified as cases.

The random selection of cases and non-cases irrespectively of participation in the scheduled interviews was chosen in order to avoid selection bias.

Serology, specific antibody detection

C.burnetii expresses two antigens, phase II and phase I. In acute Q fever, primarily antibodies against phase II are raised, and their titer is higher than antibodies against phase I. As with most other infections, IgM antibodies appear first.

In chronic forms of the disease, antibodies against phase I are elevated.

When infected, phase II IgG and IgM antibodies are always elevated, and, although declining, they may remain positive for years. A large study from Australia and England found that phase II IgG antibodies persisted after four and 12 years, respectively [16].

The diagnosis of Q fever relies upon serology. In order to determine antibodies against *C. burnetii*, we chose a two-step approach. First all samples were screened in a commercial enzyme-linked immunosorbent assay (ELISA). Positive samples from the ELISA were confirmed with an immunofluorescence antibody test (IFA).

The commercial ELISA kit was purchased from Panbio (Queensland, Australia) (cat. no. E-QFB01G and E-QFB01M) and used according to the manufacturer's instructions with one minor modification. Due to low sample volume the samples were not diluted as prescribed in the instructions but same dilution factors were used.

Samples positive for either IgG or IgM antibodies in the ELISA were confirmed with an IFA test from Focus Diagnostics (ca.no. IF0200G and IF0200M). The test was performed according to the instructions provided by the manufacturer, with the following minor modification: due to low amount of sample material, the diluted samples 1:10 from the ELISA were used to further dilute the samples as described by the manufacturer. The effect of the dilution in the Panbio buffer was tested prior to the use on patient samples and did not show any influence on the results (results not shown).

Also, the IFA cut-off suggested by the manufacturer was not used; since the prevalence of the infection varies between geographic areas, the cut-off suggested by the manufacturer is not necessarily suited for any given area [17]. When reanalyzing

the positive ELISA tests using IFA we used the cut-off adjusted to the Danish population as previously described [18].

In the analyses, the titres have been dichotomized in positive and negative according to the Danish cut-off with the inconclusive results categorized as negative.

A sample was considered positive when IgG titres phase I and II against *C. burnetii* were 1:512 or higher or 1:1024 or higher, respectively. For IgM a sample was considered positive with a titre of IgM phase I of 1:128 or higher or IgM phase II of 1:256 or higher [18].

In acute infection, antibodies against phase II antigens are usually elevated, and a combination of rising antibodies against IgG phase II and IgM usually indicates a present infection.

In a chronic infection, positive antibodies against IgG phase I antigens indicates a possible persisting infection, keeping in mind that diagnosing chronic Q fever requires more than elevated antibodies, including symptoms and supplementary paraclinical tests like PCR and culture of bone marrow.

When investigating the association between Q fever titres and spontaneous abortion we consider IFA to be gold standard. However, as other studies report their results based on ELISA alone, we also report data on ELISA values to ensure comparability with other studies.

All serological analyses were performed according to the manufacturer's instructions in a certified laboratory at Statens Serum Institute, Denmark. Laboratory personnel were blinded for case-status and samples were always analyzed in the same batch of commercial kits.

Specimens

Blood samples from gestational week 6–12 were collected by the general practitioners. Samples were mailed to the Statens Serum Institut where they were stored at -30°C until assayed.

The final data set included 218 cases and 482 non-cases with interview data covering reproductive history, age and smoking status. For the co-variates, age was split into three categories, women $<$25 years, 25–35 and above 35 years. Reproductive history was categorized as previous pregnancies 'yes' or 'no' and smoking status was split into three categories: no smoking, smoking less than 10 cigarettes per day and smoking 10 cigarettes or more per day.

Statistical analysis

Before the sample sizes were decided, power calculations based on the following assumption was made: The risk of spontaneous abortion in DNBC is about 5%. Using 200 cases and 500 non-cases an odds ratio of 3 could be detected by a power of 80% (significance level: 0.05).

The strength of the association between spontaneous abortion and positive serology was expressed as a crude odds ratio. In an adjusted model we controlled for potential confounding using logistic regression. Maternal age ($<$25 years, 25–34 years, 35 years+), gravidity (0, 1+) and smoking during pregnancy (0, 1$-<$10, 10+ cigarettes per day) were *a priori* selected as potential confounders.

Lack of interview data on some participants (table 1) resulted in missing values in the covariates. The missing values were categorized as a separate category for the variable and adjusted analyses were carried out for the entire sample. A subsample of women with complete interview data was also analyzed.

Quantitative analyses on (log transformed) adjusted ELISA OD-values (optical density values measuring antibody concentrations) were also done using linear regression.

All analyses were carried out using STATA statistical software, version 11.

Results

A total of 218 pregnancies that ended in miscarriage and 482 non-cases (pregnancies with no spontaneous abortions until 22 gestational weeks) were included. Maternal age was normally distributed in both groups, but higher in the case group (mean 24.7 years (SD: 9.2) vs. mean 22.6 years (SD: 9.7) in the control group. Cases were, on average, recruited at an earlier gestational age than non-cases (8 weeks 5 days (SD2.1) vs. 11 weeks 1 day (SD 3.6)) (Table 1). Among cases, the median age at abortion was 11.7 weeks (range 6.1 to 20.6 weeks). Three (0.6%) non-cases had delivery before gestational week 32. Furthermore, there were more missing interview data for cases than non-cases and a higher proportion of cases had prior pregnancies or were smokers than non-cases (Table 1).

Detection of antibodies by ELISA

In the initial screening, 11 cases (5.05%) and 29 (6.02%) non-cases were *C. burnetii* positive in ELISA (crude OR: 0.83) (Table 2).

Confirmation by IFA

One (0.46%) case was confirmed positive in IFA (IgM phase II positive). For non-cases three (0.62%) were confirmed positive in IFA (IgM phase II positive, IgG phase II positive and IgM phase I as well as IgG phase II positive, respectively) (Figure 1).

Altogether, three women had serologic signs of acute infection, one had signs of a previous infection; none had evident serological signs of chronic infection.

The prevalence of positive vs. negative Q fever titres in IFA was not significantly higher in women with spontaneous abortion before the end of pregnancy week 22 when compared to the control group. The OR for seropositivity for *C.burnetii* in pregnancies ending with miscarriage as compared to control pregnancies was 0.74 (Table 2).

The OR for IFA seropositivity adjusted for a potential confounding effect of maternal age, previous pregnancies and smoking was 1.19; (CI: 0.12–11.70) (Table 2). Adjusted odds ratios were also calculated using the ELISA results. Results for both were similar to the unadjusted estimates (Table 2). OR's adjusted for gestational age at blood sampling were also similar to the unadjusted estimates (results not shown). In a supplementary analysis consisting only of women with complete interview data results remained unchanged (results not shown).

Even though the microimmunofluorescence antibody test (IFA) was regarded as the gold standard in the analyses, supplementary analyses were carried out based on the ELISA results alone. In an age-adjusted, linear regression analysis on adjusted, log-transformed IgG ELISA OD-values, non-cases had 30% higher OD-values than cases, (95% CI: 19%–42%); $p<0.0005$). Controlling for age in the quantitative comparison did not change the results significantly.

Discussion

To our knowledge, this is the first population based seroepidemiologic study assessing the association between serologic signs of Q fever and spontaneous abortion. We hypothesized an association between serologic signs of Q fever and spontaneous abortion. Our hypothesis was not confirmed.

Previous case series [11,12] have concluded high risks of abortions in infected pregnancies. The cases were mainly clinical with Q fever diagnosed in the French National Reference Centre for Rickettsial Diseases during pregnancy, and the findings reported by Raoult et al. could not be reproduced in the present study.

Table 1. Distribution of selected maternal characteristics.

	Cases (N = 218)	Non-cases (N = 482)
Age in years		
<25	22 (10.09%)	69 (14.32%)
25−<35	148 (67.89%)	358 (74.27%)
35+	48 (22.02%)	55 (11.41%)
Missing	0	0
Gestational age at recruitment		
<8 weeks	88 (40.37%)	90 (18.67%)
Week 8–12	115 (52.75%)	224 (46.47%)
Week 12−<16	13 (5.96%)	118 (24.48%)
Week 16+	2 (0.92%)	50 (10.37%)
Missing	0	0
Gestational age at abortion (cases), delivery or other pregnancy outcome* (non-cases)		
<Week 8	9 (4.13%)	
Week 8–12	107 (49.09%)	
Week 12−<16	78 (35.78%)	
Week 16–22	24 (11.01%)	1 (0.21%)
Week 22–28		2 (0.41%)
Week 28–32		0
Week 32+		479 (99.38%)
Missing	0	0
Gestational age at blood sampling		
<Week 6	27 (12.39%)	40 (8.30%)
Week 6–8	87 (39.10%)	131 (27.18%)
Week 8–10	75 (34.40%)	171 (35.48%)
Week 10–12	25 (11.47%)	90 (18.67%)
Week 12–16	4 (1.83%)	38 (7.88%)
Week 16–28	0	12 (2.49%)
Missing	0	0
Previous pregnancies (N = 617)		
0	48 (22.02%)	169 (35.06%)
1+	110 (50.46%)	290 (60.17%)
Missing	60 (27.62%)	23 (4.77%)
Smoking: (N = 611)		
Non-smokers:	121 (55.5%)	358 (74.27%)
1−<10 cigarettes/day	18 (8.26%)	54 (11.2%)
10+ cigarettes/day	19 (8.72%)	41 (8.51%)
Missing	60 (27.52%)	29 (6.02%)
No interview data available:	60 (27.52%)	23 (4.77%)

*Pregnancy outcome: live born singleton, stillbirth, induced abortion after pregnancy week 12 due to illness in the foetus, live born twins.

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When addressing the Danish population, the selection of diagnosed patients in the French case reports may limit their suitability for assessing the risk of adverse pregnancy outcome among infected, and may give rise to an overestimation of the prevalence of complications.

The Netherlands have recently experienced the world's largest Q fever outbreak [19] and a new Dutch study examined serum samples from 1174 pregnancies with a gestational age of 16 weeks or more from women living in the high-risk area and found no association between positive Q fever serology and adverse pregnancy outcome [20]. However, spontaneous abortion was not the focus of the study.

Table 2. Crude and adjusted odds ratios for seropositivity for *C.burnetii* in pregnancies ending with miscarriage as compared to control pregnancies.

	Crude OR	Adjusted* OR	95%CI
ELISA	0.83	0.94	(0.44–2.02)
IFA	0.74	1.19	(0.12–11.70)

*Adjusted for maternal age (with age 25−<35 as reference group), gravidity and smoking.

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Outbreaks of Q fever have only been described to occur in small ruminants. In France, goats and sheep have been the source of infection and in the recent Dutch outbreak it was goats. Denmark has never experienced a clinically verified Q fever outbreak and the source of infection is assumed to be cows. A partial explanation to the discrepancy in the existing literature might be different strains of the bacteria with varying virulence and predilection for small ruminants; but this remains unknown [21].

We regard the use of ELISA as well as IFA in the analyses a strength in our study.

A variety of serological methods are available; the Panbio ELISA kit has previously been showed to be superior to other and suitable for large-scale screening [17,22]. The microimmunofluorescence antibody test (IFA) is regarded as the gold standard [23] due to the fact that it is capable of determining both phase I and II antibodies simultaneously by use of two different antigens on the single sample.

Some countries have defined their own cut-off while others use the cut-off defined by the manufacturer [18].

The IFA cut-off in Denmark is based on 158 anonymous, healthy blood donors from three city areas of Denmark assumed not to have Q fever. Villumsen et al. have chosen a very restrictive cut-off when defining the local baseline in order to obtain a very high specificity [18]. The use of different cut-offs or criteria for the interpretation of serological results hamper the generalisability of serologic results reported in studies from different countries.

Our supplementary analyses based on ELISA values also facilitates comparison to other studies that only use ELISA or use a different IFA cut-off [24].

It is the high positive OD-values in ELISA that are also positive in IFA which illustrates coherence and supports our choice of strategy in the analyses; using ELISA as seroepidemiologic screening with high sensitivity, and regaining specificity in the confirmatory IFA analyses. The inevitable choices included when defining a cut-off are avoided when doing analyses based on quantitative measures, i.e. the quantitative comparison of ELISA OD-values between cases and non-cases independent of cut-offs further supports the conclusion of our results and enhances how analyses based on ELISA values are a useful supplement to provide additional evidence. However, the quantitative comparison of ELISA OD-values also reveals that the average titer values among non-cases are significantly higher than among cases.

While this finding may be coincidental, a possible causal explanation could be that if Q fever is a risk factor for very early abortion, the exposed participants included in this study constitute a robust survived population of pregnancies that survived the most vulnerable period.

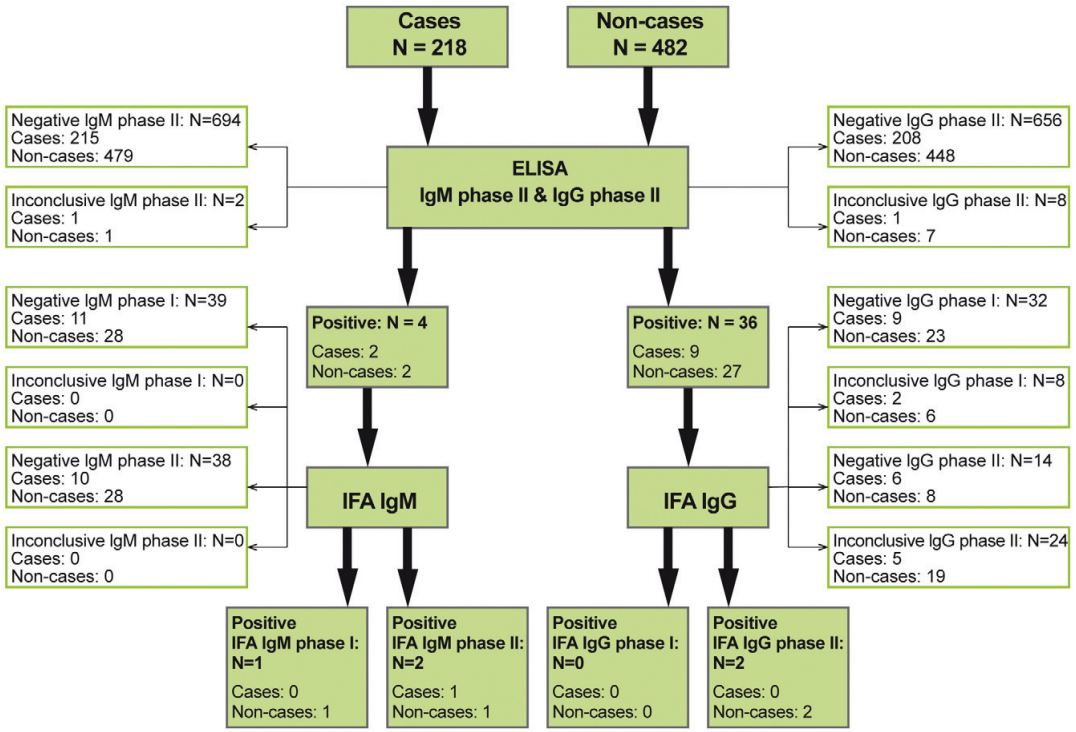

Figure 1. Serologic results: ELISA (using the manufacturer's cut-off) and IFA (using the Danish cut-off) among cases and non-cases. Only assays positive in ELISA were reanalyzed using IFA.
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Using the manufacturer's IFA cut-off in our study would have resulted in a higher seroprevalence in both groups. However, this would not have affected our conclusion.

When the IFA results were reported, the confidence intervals indicate a low precision, and a larger study would be needed to increase statistical power for detection of smaller effects.

The wide confidence intervals also reflect how the power of the study was negatively affected by the lower than expected seroprevalence among women with spontaneous abortion and by the fact that a sample size of only 218 cases was available.

A recent study used a cohort of Q fever patients to compare serological and PCR results. Although the same IFA method was used, there were large discrepancies in the IFA results between three reference laboratories and the authors proposed development of an international standard of Q fever serological investigation [25].

The discrepancy in the results obtained by different centres compromises our understanding of the natural course of Q fever in pregnancy.

Rather than a stand-alone attempt to change previous risk evaluations, our aim was to perform applicable results and to contribute to the sparse literature on Q fever and adverse pregnancy outcome.

This study has some limitations. Due to the gestational age at enrolment into the cohort, the earliest abortions that constitute the largest proportion of miscarriages were not included in this study. Consequently, we cannot exclude a harmful effect of Q fever infection in very early pregnancy, and furthermore our results may reflect a 'healthy pregnant population' due to the fact that the pregnancies have successfully survived through the most vulnerable period. In general, little is known about infections and very early fetal loss. These very early spontaneous abortions are insufficiently registered and thus difficult to approach in research.

When studying causes of spontaneous abortions, adjustment for previous miscarriages is controversial [26]. This is why we chose adjustment for prior pregnancies, regardless of pregnancy outcome. Age is an important factor determining miscarriage risk; adjustment for smoking was justified by the inconsistency of previous findings related to smoking and spontaneous abortion [26].

Decline of especially IgM antibodies could also be a limitation in this study since inclusion of this sample took place between June 1997 and September 2002. However, the four IgM antibody titres positive in ELISA are distributed between Dec 1997 and Feb 2002 and the 36 IgG antibody titres positive in ELISA between Aug 97 and June 02; an Irish study used 20 years old blood samples to evaluate the seroprevalence of IgG phase II antibodies [27].

In conclusion, no association between elevated antibody titres against *C.burnetii* and spontaneous abortion after gestational week 8 was found.

The aetiology of spontaneous abortions remains largely unknown with one third of implanted conceptions failing to survive beyond midpregnancy [26]. Some infections are known to increase the risk of spontaneous abortions, but the role of the specific pathogens has been difficult to demonstrate [28]. The size of this study and the fact that very early spontaneous abortions are

not included limit any definite conclusion regarding Q fever infection and risk of early fetal loss.

However, our results suggest that spontaneous abortion associated with *C.burnetii* should not be of great concern among pregnant women.

Regarding directions for future research into *C. burnetii* and spontaneous abortion it would be relevant to study adverse pregnancy outcome in a pregnant population with high-exposed women like veterinarians and female workers with contact to livestock.

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Author Contributions

Conceived and designed the experiments: SYN NHH AMNA KM KAK TBH. Performed the experiments: AMNA BK SYN. Analyzed the data: SYN KM NHH AMNA. Contributed reagents/materials/analysis tools: BK KAK. Wrote the paper: SYN NHH TBH AMNA KM.

Paper V

Risk of adverse pregnancy outcome in women exposed to livestock:
a study within the Danish National Birth Cohort

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Short running head: Animal exposure and pregnancy outcome

SUMMARY

Maternal infection in pregnancy is a known risk factor for adverse pregnancy outcome, and a number of zoonotic pathogens may constitute a risk to pregnant women and their fetuses. With animal contact as a proxy for the risk of zoonotic infection, this study aimed to evaluate pregnancy outcome among women with self-reported occupational or domestic contact with livestock compared to pregnant women without such contact.

The Danish National Birth Cohort collected information on pregnancy outcome from 100418 pregnant women (1996 - 2002) from which three study populations with occupational and/or domestic exposure to livestock and a reference group of women with no animals contact was sampled.

Outcome measures were miscarriage, very preterm birth (before gestational week 32), preterm birth (before 37 gestational weeks), Small for Gestational Age (SGA), and perinatal death.

We found no association between occupational and/or domestic exposure to livestock and miscarriage, preterm birth, SGA or perinatal death.

INTRODUCTION

Maternal infection is a significant risk factor for adverse pregnancy outcomes. It is well established that a number of zoonotic pathogens, including *Toxoplasma gondii*, *Listeria monocytogenes*, certain chlamydiae species, and *Coxiella burnetii*, may constitute a risk for the pregnant woman and her fetus [1-7]. By contrast, pregnancy outcome following other zoonotic infections including salmonella, campylobacter, *Yersinia enterocolitica* and brucella is more sparsely described [8-13].

Concerns about women with occupational contact with livestock and thereby a potential risk of exposure to zoonoses has affected guidelines for pregnant women, but knowledge about zoonoses and pregnancy is limited , and as more studies come out, guidelines have been continually adjusted.

Current Danish guidelines for physicians regarding toxoplasmosis recommend that pregnant women be advised regarding how to prevent exposure to the parasite, whereas neither routine screening nor testing after suspected exposure is recommended. To prevent *Listeria monocytogenes* infection, pregnant women are advised to reduce risk by the safe handling as well as avoidance of certain foods [14]. For *C. burnetii*, screening is recommended for women with

relevant exposure to domestic animals, along with precautions regarding the handling of birth products or assisting deliveries [15].

We previously conducted studies of the seroprevalence of *C. burnetii* in Danish women with exposure to livestock, and evaluated pregnancy outcome among seropositive compared to seronegative women within the Danish National Birth Cohort (DNBC). We found a high prevalence of antibodies to *C. burnetii* among pregnant women with occupational (47.2 %) or domestic exposure (32.2%) to cattle or sheep compared to unexposed pregnant women (4.8% seropositive), but no increased risk of adverse pregnancy outcome in women with verified exposure to *C. burnetii* was found [16].

Q fever is endemic in Denmark [17, 18], and our interest in zoonotic pathogens and their possible impact during pregnancy led us to consider pregnancy outcome in women with animal contact in a broader sense. The DNBC is a large, population-based cohort, and with animal contact as a proxy for the risk of zoonotic infection, we sought to evaluate whether self-reported occupational or domestic animal contact was associated with an increased risk of adverse pregnancy outcome.

METHODS

Participants

Enrolment in the DNBC took place between 1996 and 2002, and the women were recruited in connection with the first antenatal visit to the general practitioner. The median gestational week of enrolment was 10 weeks (25 and 75 percentiles: 7 weeks; 13 weeks), but some women were enrolled as early as in gestational week 5 and as late as 22 gestational weeks.

Information on exposures before and during early pregnancy was collected by means of a computer-assisted telephone interview scheduled to take place in gestational week 12 or as soon as possible thereafter. If fetal loss occurred before this interview, participants were offered a similar interview as soon as possible after the fetal loss. Questionnaires are available at www.bsmb.dk. Among other things, the interviews covered information on reproductive history, smoking status during pregnancy, and domestic contact to animals as well as very detailed information regarding occupational exposure to different animals. A detailed description of the cohort can be found elsewhere [19].

Women representing a total of 92717 pregnancies were interviewed, of which 2552 interviews were carried out after fetal loss. If a woman participated in the cohort with more than one pregnancy, only the first pregnancy was included to avoid non-independent observations, leading to the exclusion of 8704 interviews. Furthermore, 37 were excluded due to lack of information on gestational age at recruitment. Ectopic pregnancies and pregnancies with mola hydatidosa were also excluded (n=44). For the miscarriage analysis, twin pregnancies (n=1804) were included, but for all other outcomes only singleton pregnancies were included. Thus, 83932 pregnancies were eligible for an analysis of miscarriage and 82128 pregnancies for analysis regarding risk of preterm birth and perinatal death. For analyses on small for gestational age (SGA), 881 were further excluded because of missing or implausible birth weight data or a gestational age over 44 weeks, in all 81247 pregnancies.

This study was approved by the steering committee for the Danish National Birth Cohort and the Danish Data Protection Agency, and the data collection was, according to Danish legislation, approved by the Regional Research Ethics Committee, and the women enrolled in the Danish National Birth Cohort gave written consent to participate.

Exposure Measures

The interviews covered specific questions regarding domestic and occupational exposure to livestock during pregnancy and three months prior to pregnancy: women working on farms were asked: “have you worked with farm animal production, that is: with animals?” and “which animals do you work with?” Women with other occupational animal exposure than farming were asked: “which animals do you work with” and “how are you involved in working with live animals” (veterinarians, veterinary nurses, etc.) and for abattoir workers: “are you directly involved in handling animals at the abattoir?” Hence, the women could be occupationally exposed to living as well as dead animals.
These questions enabled us to define occupational exposure as women who had worked with livestock either in an abattoir, on a farm, or in veterinary practice. The animals of interest were dairy cattle, meat cattle, pigs, poultry, horses, sheep and goats.
Likewise, the women who answered yes to living on a farm with livestock were asked: “which species of animals”? Farm animals were defined as cattle, horses, sheep, pigs, poultry, deer, and goat. Exposures to pets were not included.
Self-reported information on exposures during pregnancy as well as three months prior to becoming pregnant enabled us to identify four different exposure groups: Pregnant women with occupational as well as domestic exposure to livestock (n=221), women with occupational but without domestic exposure (n=208), women with domestic but without occupational exposure (n=5248), and a reference group of women with no occupational or domestic contact with

livestock (n=76451).
Exposure to livestock could be further categorized according to specific animal exposure (cattle (n=1381), sheep (n=741), pigs (n=871) and other (n=1040)).

Outcome measures

Pregnancy outcomes of interest were miscarriage, perinatal death, preterm birth, and SGA. Miscarriage was defined as fetal loss before 154 days (22 weeks) after the first day of the last menstrual period, with gestational age estimated from the participants' self-reported last menstrual period. Perinatal death was defined as fetal death after 22 weeks' gestation or infant death within 7 days of birth.
Preterm birth was categorized into very preterm birth (prior to 32 gestational weeks) and preterm births (before 37 gestational weeks). SGA was estimated by an intrauterine weight standard and defined as a birth weight corresponding to -2 standard deviations and below for the specific gestational age (Marsal) [20]. SGA was also estimated as a birth weight below the lowest 10th percentile for gestational age within the present study population, but the external reference was considered the primary analysis of SGA. Data on gestational age (days) and birth weight were obtained from the National Patient Registry.

Statistical analysis

The risk of miscarriage and preterm birth according to animal exposure was estimated as hazard ratios using Cox regression models, with gestational age as the underlying time variable. Using a model for the hazard rate, rather than logistic regression, has a number of advantages. First, gestational age is directly incorporated into the model; second, it makes it possible to take the different gestational durations at entry into the cohort into account.
For miscarriage, time of entry was gestational age at enrolment, and follow-up ended at miscarriage, induced abortion, emigration, or maternal death or at 22 completed weeks of pregnancy, whatever came first. The analyses of miscarriage were repeated on a subsample restricted to women interviewed while still pregnant (prospective data collection) using gestational age at interview as the time of entry.
For preterm birth follow-up ended at 37 weeks' gestation. Women who emigrated or died prior to this gestational age were censored.

The assumption of proportional hazards was checked by using Schoenfeld residuals. In the group with occupational exposure, there were very few miscarriages (n=7), and the assumption was not fulfilled. Analyses of miscarriages and preterm births were repeated by fitting logistic regression models.

The association between exposure to livestock and SGA as well as perinatal death was estimated by logistic regression models.
Furthermore, all analyses were replicated with restriction to pregnant women who reported employment or who had been unemployed for a maximum period of six months prior to becoming pregnant. Analyses restricted to women who were pregnant for the first time and did not have a long time to pregnancy interval (< six months) were also performed.

Maternal age (<25 years, 25-34 years, 35+ years), gravidity (0, 1+), and smoking during pregnancy (0, 1-<10, 10+) were a priori defined to be included as covariates in all statistical analyses made.

RESULTS

Table 1 shows some characteristics of women according to animal exposure. Among the 82128 women, 5830 (6.9 %) reported occupational or domestic contact with livestock in their pregnancy or three months prior to becoming pregnant.

Women with occupational or domestic contact with livestock were recruited at a higher gestational age, were younger, of higher parity, and were more often smokers than women without such contact.

Table 2 presents hazard ratios for miscarriage and preterm birth among women with various animal contacts compared with unexposed women. A total of 2846 pregnancies (3.4%) resulted in miscarriages, and the median gestational age at miscarriage was 12 weeks 6 days (interquartile range (IQR) 10 to 14 weeks) for women with occupational and domestic contact with livestock, compared to 11 weeks 6 days (IQR 10 to 13 weeks) among unexposed women.
Neither occupational nor domestic exposure was found to be associated with miscarriage.
The majority of fetal losses occurred early in pregnancy, and consequently, interview data were obtained after miscarriage for a considerable number of miscarriages in the cohort. However, in the analysis restricted to women who were interviewed while still pregnant, the estimates obtained were essentially the same (results not shown).
For the occupationally exposed group as well as the group with occupational as well as domestic exposure, there were too few events to perform adjusted hazard ratios (HR). For these groups, miscarriage analyses were repeated using logistic regression which did not change the estimates (results not shown).

Among a total of 3936 preterm deliveries, 247 reported animal contact. No increased risk of very preterm or preterm birth was found for any kind of animal exposure (Table 2). Here there were also too few events to perform adjusted HRs for the occupationally exposed group as well as the group with occupational and domestic exposure. For these groups, preterm birth analyses were repeated using logistic regression, which did not change the estimates (results not shown).
In all, 2202 women were SGA, and we found no association between contact with livestock and SGA (Table 3) except for the group with domestic contact OR: 0.8; 95%CI: 0.6-1.0) (p=0.03). However, in analyses repeated on term births only, no association was found (results not shown).
No association between any exposure to livestock and perinatal death (n=570) was found (Table 3).

In the group with domestic exposure to livestock, stratified analyses by different types of animal contact: sheep (n=741), cattle (n=1381), pigs (n=871), poultry (n=1040), and other (n=1364) were performed.
No significant association was found between contact with any of the specific animal types and miscarriage, preterm birth, or perinatal death. However, exposure to pigs was associated with a decreased risk of SGA (OR: 0.5; 95%CI: 0.3-0.9), in analysis restricted to term births, the protective effect was absent.

Analyses restricted to women who reported being employed or having been unemployed for a maximum period of six months prior to becoming pregnant did not change any outcome measures significantly (results not shown). Nor did analyses restricted to women who were pregnant for the first time and did not have a long time to pregnancy interval (< six months) (results not shown).

DISCUSSION

Overall, we found no association between exposure to Danish livestock and adverse pregnancy outcome. Analyses in separate categories for occupational and domestic exposure as well as restricting analysis to women in the labour market failed to change this. Nor did analyses stratified with regard to specific animals.

To our knowledge, this is the first population-based study to address pregnancy outcome among women with self-reported contact to livestock evaluated in separate groups of domestic and occupational exposure, as well as in a group with both exposures.
We assumed that exposure to livestock during pregnancy or during a period of three months prior to becoming pregnant could be a proxy for exposure to zoonotic pathogens and hypothesized that animal contact would be associated with an increased risk of adverse pregnancy outcome. Our hypothesis was not confirmed.
At the time of the study (1996–2002), zoonotic pathogens were common in Danish livestock. For example, in 1998, the prevalence of salmonella was 6.5% in Danish broiler chickens and 3.7% in pigs, whereas the prevalence of campylobacter was as high as 47.1% in broilers and 68.8% in pigs [21].
Between 1994 and 2005, 37 confirmed cases of maternal-fetal Listeria monocytogenes infections were reported in Denmark [22], and a study from 1995 found that 27.4% of 5402 Danish pregnant women had IgG antibodies against *Toxoplasma gondii* [2, 23].
More recent Danish studies on the prevalence of zoonotic pathogens have found that campylobacter is the most frequently reported foodborne pathogen in Denmark. In 2011, the registered number of campylobacter cases was 4068 (73.1 cases per 100000 inhabitants) compared to 1166 salmonella cases (21.0 cases per 100000 inhabitants); it was 224 for yersinia and 49 for listeria [24]. Also, analysis showed a clonal link between Escherichia coli from humans and broiler chicken, broiler chicken meat, pork and pigs, suggesting that production animals may pose a zoonotic risk [25, 26].
Foodborne outbreaks of *Listeria monocytogenes* have been described, and since 2002 the incidence of listeria has increased in Denmark as well as in several other European countries [3, 27, 28]; in 2009, 97 cases were reported in Denmark, compared to 57 in 2008. Fifty of these cases were in females and three were maternal-fetal infections [29].

We find it reasonable to assume that most of the women with domestic or occupational contact with livestock are exposed to zoonotic pathogens, primarily campylobacter and salmonella, but also to ubiquitous agents such as toxoplasma and listeria [2, 28, 29]. In pig farmers and veterinarians, exposure to *Yersinia enterocolitica* is likely, and individuals working with cattle, sheep, or goats would have a risk of VTEC or *C. burnetii* exposure [17, 18, 30].

In our main analyses, we did not differentiate between types of animals because the aim was to address occupational and domestic exposure, rather than to address a possible risk of exposure from specified animals. In order further analyse occupational exposure, we performed analyses restricted to women who were working during or two months prior to pregnancy, but this did not affect any outcome measures significantly.
As indicated above, some zoonotic infections are restricted to specific animal species, whereas others are widespread, which is the justification for looking at individual species. Analyses stratified by type of animal failed to reveal any risk of adverse pregnancy outcome according

to specific animal contact.

It is conceivable that pregnant women, once they know about their pregnancy, modify their behaviour in order to limit contact with livestock and pay increased attention to, for instance, hand hygiene. This may especially be an issue in women with a history of adverse pregnancy outcomes. This change may modify the potential risks from zoonotic infections. There are, however, limited data to quantify the health impact of this possible change in behaviour [31].

This is a very large study. Despite this, some analyses suffered from very low power due to the relative low proportion of exposed women and to the infrequency of the study's outcomes, resulting in few events in some of the analyses. Also, due to the gestational age at enrolment into the cohort, the earliest miscarriages were not included. Consequently, we were unable to reveal a potential harmful effect in the pre-clinical phase of pregnancy.
If women with a history of adverse pregnancy outcome have a tendency to avoid animal exposure, this could introduce behaviour modification bias. However, since analyses restricted to women who were pregnant for the first time and did not have a long time to pregnancy interval (< six months) did not change any estimates significantly; this was not an issue in this cohort.

We chose to adjust all events for three important risk factors for adverse reproductive outcome. Age is an important factor determining miscarriage risk, and smoking is a well-known risk factor for preterm birth. Adjustment for smoking in the analyses of miscarriages was justified by the inconsistency of previous findings related to smoking and miscarriage [32]. Other confounding factors could be socioeconomic status or strenuous leisure time physical exercise [33]. If socioeconomic status was an essential risk factor for any of the outcomes included in this cohort, it would result in different estimates in sub analyses in women with a connection with the work force. For women living and/or working on farms, with physical activity incorporated into daily routines, a possible effect of leisure time exercise is difficult to quantify.

There could be characteristics entailing different behaviours in women living and/or working on farms that could alter their pregnancy outcome, for instance heavy physical work and perhaps less focus on healthy lifestyle in pregnancy compared to women living in cities. Since our findings are negative, these aspects are probably of minor importance, but should have been taken into account had we found an association between animal contact and adverse pregnancy outcome.
On the other hand, women living in the countryside are less prone to exposure to outdoor air pollution from traffic, which is associated with low birth weight and preterm birth [34]; this could lead to confounding, but is speculative.
A number of women with miscarriage in this study were interviewed after their miscarriage, and recall bias must be taken into account since they may report exposures differently than women who are interviewed while still pregnant, which is why analyses restricted to women

interviewed prior to pregnancy outcome were also performed.

Livestock management practices may change. However, the interplay of causal factors of zoonotic infections in a complex pathway is not new, illustrated, for example, by the recent unprecedented epidemic of Q fever in the Netherlands [35]. With the increasing availability of modern diagnostics and rigorous screening, a higher proportion of test results indicating past or present infections may be detected during pregnancy. Larger seroepidemiological studies on the various zoonoses are needed to further clarify their hazard to human fetal health.
For several infections suspected of or known to constitute a potential hazard to a healthy pregnancy outcome, exposures in professional versus private life are difficult to separate.
For toxoplasmosis, for instance, clinical Danish guidelines for pregnant women have changed in recent years [14]. This is due to the latest research, which does not provide evidence that prenatal treatment – from screening in pregnancy – reduces the risk of mother to child transmission of toxoplasma infection, but also to the fact that detaching occupational from non-occupational exposures has been very difficult; attempts to avoid occupational exposure for veterinarians, for instance, have been deemed useless.

Adverse reproductive outcomes were assessed in four different exposure groups of women with occupational or domestic exposure to livestock. The fact that this large study found no association between exposure to livestock and miscarriage, preterm birth, SGA or perinatal death should diminish general occupational health concerns for pregnant women with general exposures to a range of different farm animals.

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Table 1: Maternal characteristics according to animal exposure (presented for singleton pregnancies) in 82128 women from the Danish National Birth Cohort

	Occupational and domestic exposure to livestock (n=221)	Occupational exposure to livestock (n=208)	Domestic exposure to livestock (n=5248)	Unexposed (n=76451)
Age (years)				
<25	30 (13.6%)	58 (27.9%)	580 (11.1%)	10387 (13.6%)
25 - <35	155 (70.1%)	137 (65.9%)	3847 (73.3%)	57380 (75.1%)
35+	36 (16.3%)	13 (6.3%)	821 (15.6%)	8684 (11.4%)
Gestational age at recruitment (weeks)				
< 8 weeks	20 (9%)	41 (19.7%)	653 (12.4%)	13762 (18%)
8 -11 weeks	95 (43%)	91 (43.8%)	2330 (44.4%)	35604 (46.6%)
12 -15 weeks	75 (33.9%)	54 (26%)	1532 (29.2%)	19280 (25.2%)
16+ weeks	31 (14%)	22 (10.6%)	730 (13.9%)	7790 (10.2%)
Number of previous pregnancies				
0	81 (36.7%)	97 (46.6%)	1515 (28.9%)	29569 (38.7%)
1+	140 (63.4%)	111 (53.4%)	3730 (71.1%)	46849 (61.3%)
Missing	0	0	3 (0.06%)	33 (0.04%)
Smoking				
Non-smokers	201 (91%)	169 (81.3%)	4457 (84.9%)	64222 (84%)
1 - <10 cigarettes/ day	8 (3.6%)	18 (8.7%)	381 (7.3%)	6173 (8.1%)
10+ cigarettes/day	12 (5.4%)	21 (10.1%)	408 (7.8%)	6016 (7.9%)
Missing	0	0	2 (0.04%)	40 (0.05%)
Social status				
Higher grade professionals	32 (14.5%)	65 (31.25%)	758 (14.4%)	18265 (23.9%)
Lower grade professionals	43 (19.5%)	29 (13.9%)	1696 (32.2%)	23551 (30.8%)
Skilled workers	139 (62.9%)	81 (38.9%)	2062 (39.3%)	20807 (27.2%)
Unskilled workers	5 (2.3%)	27 (12.9%)	652 (12.4%)	11092 (14.5%)
Students	2 (0.9%)	5 (2.4%)	37 (0.7%)	1976 (2.6%)
Economically inactive	0	0	32 (0.6%)	590 (0.8%)
Unclassified	0	1 (0.5%)	11 (0.2%)	170 (0.2%)
Employment status				
Working*	220 (99.6%)	206 (99%)	4383 (83.5%)	64641 (84.6%)
Out of work **	1 (0.5%)	2 (1%)	865 (16.5%)	11810 (15.4%)

* or out of work maximum up to 6 months prior to becoming pregnant

** for more than 6 months prior to pregnancy

Table 2: Hazard ratios (HR) of miscarriage, preterm birth and perinatal death according to animal exposure during pregnancy or three months prior to becoming pregnant, with unexposed women as reference group – for 83932 women (miscarriage) and 82128 women (preterm birth and perinatal death) from the Danish National Birth Cohort.

	Miscarriage<22 week				Very Preterm birth 22 -31+6 weeks				Preterm birth 22 -36+6 weeks			
	Miscarriage N(%)	No miscarriage N (%)	HR crude	HR adjusted (95%CI)*	Very Preterm birth N (%)	Not Very Preterm birth N (%)	HR crude	HR adjusted (95%CI)*	Preterm N (%)	Not preterm N (%)	HR crude	HR adjusted (95%CI)*
No livestock exposure	2666 (3.4%)	75436 (96.6%)	1 (ref)		674 (0.9%)	72902 (99.1%)	1 (ref)		3689 (5.0%)	69887 (95%)	1 (ref)	
Domestic exposure to livestock	162 (3%)	5235 (97%)	0.9	0.9 (0.8-1.1)	30 (0.6%)	5077 (99.4%)	0.65	0.66 (0.5-1.0)	232 (4.6%)	4845 (95.4%)	0.9	0.9 (0.8-1.1)
Occupational exposure to livestock	7 (3.4%)	202 (96.7%)	1.0	NA**	1 (0.5%)	200 (99.5%)	NA***	NA***	8 (4.0%)	193 (96.0%)	0.8	NA**
Occupational and domestic exposure to livestock	11 (4.9%)	213 (95.1%)	1.6	NA**	1 (0.5%)	210 (99.5%)	NA***	NA***	7 (3.3%)	203 (96.7%)	0.7	NA**

*Adjusted for maternal age, gravidity and smoking

**Not available due to too few events, but in logistic regression analysis the crude estimates were identical, and adjustment for maternal age, gravidity and smoking did not change the estimates.

***Not available due to too few events

Table 3: Small for Gestational Age and perinatal death parameters (OR) for Danish pregnant women according to animal exposure.

	SGA (gestational week 22 and onwards (external reference)				Perinatal death (gestational week 22 – 7 days post term)			
	SGA N (%)	Not SGA N (%)	OR crude	OR adjusted (95%CI)*	Perinatal death N (%)	No Perinatal death N (%)	OR crude	OR adjusted (95%CI)*
No animal exposure	2080 (2.9%)	70648 (97.1%)	1 (ref)		536 (0.7%)	73040 (99.3%)	1 (ref)	
Domestic exposure to livestock	111 (2.2%)	4934 (97.8%)	0.8	0.8 (0.7-1.0)	30 (0.6%)	5047 (99.4%)	0.8	0.8 (0.6-1.2)
Occupational exposure to livestock	6 (3%)	195 (97.0%)	1.1	1.0 (0.5-2.2)	2 (1%)	199 (99.0%)	1.3	1.4 (0.4-5.6)
Occupational and domestic exposure to livestock	5 (2.4%)	204 (97.6%)	0.8	NA**	2 (1%)	208 (99.0%)	1.3	1.3 (0.3-5.3)

*adjusted for smoking, gravidity and maternal age

**Not available due to too few events