Prevalence of *Coxiella burnetii* in women exposed to livestock animals, Denmark, 1996 to 2002

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Q fever is a zoonotic infection which can pose a danger 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 *Coxiella burnetii*. The Danish National Birth Cohort collected blood samples from 100,418 pregnant women in the period 1996 to 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* by commercial enzyme-linked immunosorbent assay. Positive samples were confirmed by immunofluorescence (cut-off titre ≥1:128). The proportion of seropositive women was higher in the occupationally exposed (47.2% seropositive; relative risk (RR): 9.8; 95% confidence interval (CI): 6.4–15.2) and the domestically exposed population (32.2% seropositive; RR: 6.7; 95% CI: 4.3–10.6) than in unexposed women (4.8% seropositive). We found a high prevalence of antibodies to *C. burnetii* among pregnant women with occupational or domestic exposure to cattle and/or sheep compared with 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 particularly high risk often include individuals with occupational exposure to live animals, such as veterinarians, farmers and those living in close contact with domestic livestock. Q fever, caused by *Coxiella burnetii*, is a disease of particular concern for pregnant women because infection in pregnancy is suspected to be a potential cause of foetal morbidity and mortality. French case studies have suggested risk of miscarriage, intrauterine growth retardation, oligohydramnion, stillbirth and premature delivery in untreated pregnancies [2-4]. 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 [5-9]. For healthy humans, Q fever infection often has a mild, influenza-like course, but pneumonia is also common. Immunocompromised patients and patients with pre-existing valvulopathy or vascular defects are at risk of a more severe course of the infection [10,11].

In small ruminants, infection with *C. burnetii* is known to cause miscarriage, retained placenta, endometritis and infertility, and placentas of infected animals contain high numbers of bacteria [12,13]. 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 byproducts during birth, especially placenta [10,11,14]. 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 [15,16].

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 [2]. 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 [17].

When conducting a 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 Denmark in occupationally and domestically exposed women compared with unexposed women sampled from a population based study of pregnant women.

Methods

Study participants

The Danish National Birth Cohort (DNBC), a nationwide cohort of 100,418 pregnant women and their offspring [18], 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 was collected by means of a computer-assisted telephone interview scheduled to take place in gestational week 12. Interviews included data on reproductive history, age, smoking status, domestic contact to animals and very detailed questions regarding occupational exposure to different animals (interview forms are available at the DNBC website).

Women who confirmed having worked on a farm with live animals during their pregnancy or up to three months before becoming pregnant, were further questioned about the type of animals, the size of the herd, occupation, etc. 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 biobank. A detailed description of the cohort can be found elsewhere [18].

We sampled three groups from the DNBC cohort (Figure 1):

- Women with self-reported occupational exposure to livestock (n=195), i.e. veterinarians (n=118) and women who worked on a farm with at least 40 dairy cattle (n=77);
- Women with self-reported domestic exposure to livestock (n=202), i.e. cattle (n=180), sheep (n=22) or both (n=13), who were living on a farm and cohabiting with a farmer, but did not have occupational exposure to these animals;
- A randomly sampled reference group of women (n=461). Two of these were domestically exposed to animals and were consequently reclassified as such, leaving 459 controls.

It was a prerequisite for all three groups that the women had participated in the interview in early pregnancy and had delivered a blood sample to the biobank.

In order to evaluate a possible association between geographic area and seropositivity, the participants were classified using the nomenclature of territorial units for statistics (NUTS3) [19], which divides the regions of Denmark into 11 areas. These were used in a definition of urban versus rural residence.

Detection of antibodies against *C. burnetii*

The diagnosis of Q fever relies upon serology. *C. burnetii* expresses two groups of antigens, phase I and phase II. In acute Q fever, antibodies against phase II antigens are initially elevated, and their titre is higher than that of antibodies against phase I antigens. As with most other infections, IgM antibodies appear first. In chronically infected individuals, especially 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 concluded that phase II IgG antibodies persisted after five and 12 years, respectively [20].

To determine antibodies against *C. burnetii*, we used a two-step approach. Initially, all samples were screened in a commercial enzyme-linked immunosorbent assay (ELISA). The commercial ELISA kit (Panbio, Australia, *Coxiella burnetii* (Q Fever) IgG and *Coxiella burnetii* (Q Fever) IgM) were used according to the manufacturer's instructions with minor modifications. Due to small sample size the initial total volume was smaller but same dilution factors were used.

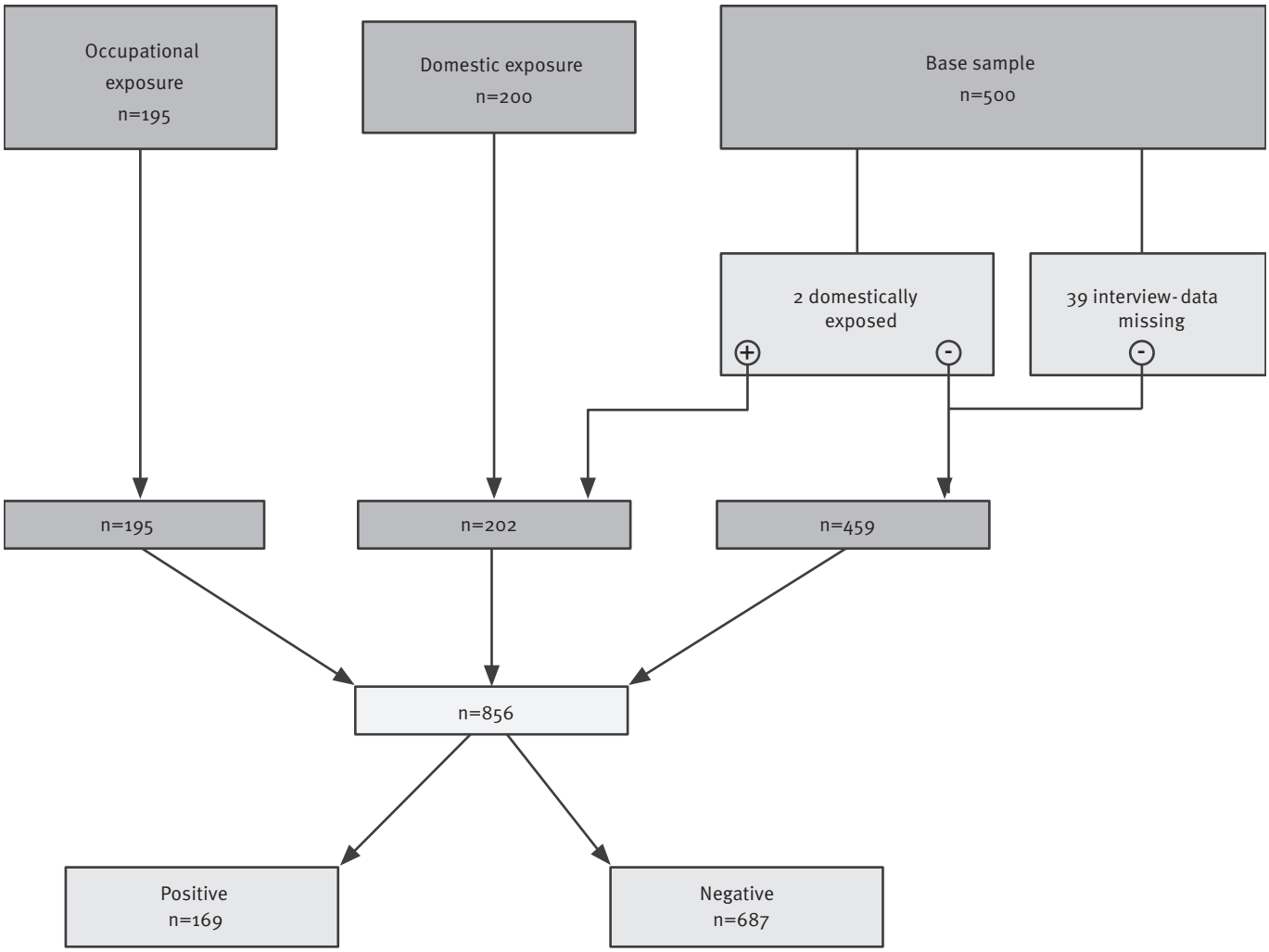
Samples which were positive for either IgG or IgM antibodies in the ELISA were confirmed with an immunofluorescence antibody test (IFA) test. When investigating the association between exposure, Q fever titres and pregnancy outcome, IFA is considered to be the gold standard. The tests (Focus Diagnostics, Q Fever IFA IgG and Q Fever IFA IgM) were performed according to the instructions provided by the manufacturer, with the following minor modifications: due to small sample volume, the 1:10-diluted samples from the ELISA were reused and further diluted as described by the manufacturer. The effect of the initial dilution in the Panbio ELISA buffer was tested on patient samples before the study and did not show any influence on the results (data not shown).

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 [21]. A local cut-off adjusted to the Danish population has been defined, including negative, intermediate and positive titres [22] (Table 1). The intermediate zone was defined in order to address people with an a priori elevated risk of Q fever (such as veterinarians, farmers etc.), with intermediate titres in samples from these high-risk groups considered to be probably positive. When the ELISA-positive samples in our study were reanalysed using IFA, a modified version of this Danish cut-off was used. A sample was considered IFA-positive when antibody titres against 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

Figure 1

Sampling of pregnant women from the Danish National Birth Cohort, Denmark, 1996–2002 (n=856)



status, and samples were always analysed in the same batch of commercial kits.

We have conducted another study assessing pregnancy outcome in women with antibodies to *C. burnetii* compared to seronegative women [9]. This and the present study in part use the same material since the blood samples from the Danish national birth cohort is a precious commodity. However, the studies are independent studies with different study designs and objectives.

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 who reported occupational exposure to cattle in the occupationally exposed group. Power calculations were based on the literature and the first Danish data [23] with 11% of 1,613 people tested positive. It was assumed that the prevalence among exposed women would be 10% and 2% in the background population. A sample size of 200 exposed and 200 unexposed would yield an odds ratio of 5 that could be detected by a power of 88% at a two-sided significance level of 0.05. However, as we also wanted to use the sample for another study which required approximately 500 controls, it was decided to use all available blood samples from the reference group in both studies. 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. The median age among occupationally exposed women was 31 years (interquartile range: 28–33 years), compared with 30 years (interquartile range: 27–33 years) in domestically exposed women, and 29 years (interquartile range: 26–32 years) in the unexposed.

Table 1

Cut-off values immunofluorescence antibody test as applied in Denmark

	Negative	Intermediate	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	≥1,024

Source: [22].
In the present study, a cut-off of 1:128 was used for all phases.

When looking at age and seropositivity, the smallest proportion of IFA-positive women were found in the age group younger than 25 years (13.5% seropositives); findings from other age groups, 25 to 34 years and 35 years and older, were similar to each other (22.7% and 18.1% seropositives, respectively). There was no correlation between age and seropositivity.

Figure 2 illustrates the relationship between IgG phase II-positive ELISA and IFA results. Positive IFA results were more frequent in samples with high adjusted optical density values (OD, measuring antibody concentrations) in the ELISA.

In the confirmatory IFA analysis, 92 (47.2%; 95% confidence interval (CI): 40.0–54.4) occupationally and 65 (32.2%; 95% CI: 25.8–39.0) domestically exposed women were *C. burnetii* antibody-positive in IFA, compared with three (4.8%; 95% CI: 3.0–7.1) in the unexposed group. The risk difference between the occupationally exposed and unexposed women was 42% (95% CI: 35–50), and the occupationally exposed had a 9.8 times higher risk of being seropositive than the unexposed women (relative risk (RR): 9.8; 95% CI: 6.4–15.2). The risk difference between the domestically exposed and unexposed women was 27% (95% CI: 0.2–0.3), and the domestically exposed had a 6.7 times higher risk (95% CI: 4.3–10.6) of being seropositive than the unexposed women (Table 3).

Reporting the IFA results according to the Danish cut-off with intermediate titres classified as negative (Table 1), 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 with unexposed women (0.7% seropositive).

Figure 3 shows the distribution of positive IgG phase II titres in the three groups and illustrates that unexposed women had mainly titres at the lower end of positivity, whereas the higher titres were primarily found in the two groups of exposed women.

Previous versus recent infection

Among the occupationally exposed women, 89 were phase II IgG-positive, 43 were phase I IgG-positive, and 41 of them were positive in both. Three women's IgM titres against phase II antigens were positive, one of them was also positive for IgG against phase II, and another in IgG against both phases. None was phase I IgM-positive. Among the domestically exposed women, 59 were phase II IgG-positive, 30 were phase I IgG-positive, and 26 of them were positive in both phases. Three were phase II IgM-positive, with one of them also being positive for IgM against phase I, and two for IgG against phase II. One was only phase I IgM-positive. Among the unexposed women, 21 were positive for IgG against phase II, six of them were also phase I IgG-positive. One was positive for IgM against phase I as well as IgG against phase II, and one was phase II IgM-positive but negative in all other phases.

Table 2

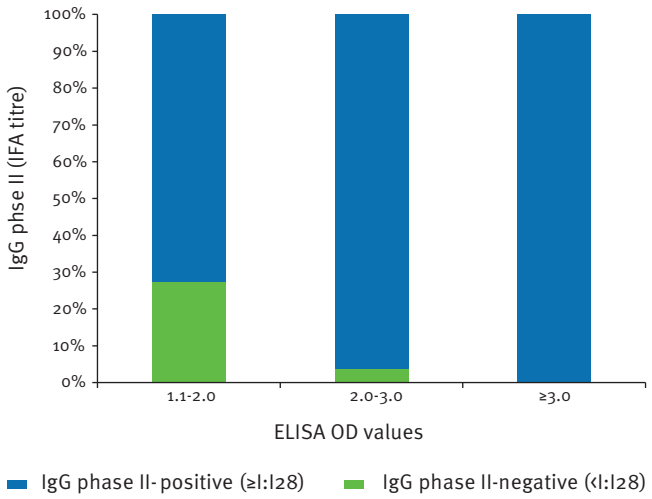
Distribution of selected characteristics among pregnant women sampled from the Danish National Birth Cohort, Denmark, 1996–2002 (n=856)

	Occupationally exposed (n=195)	Domestically exposed (n=202)	Unexposed reference (n=459)
Age (n=856)			
‹25 (n=104)	13 (6.7%)	26 (12.9%)	65 (14.2%)
25–34 (n=631)	148 (75.9%)	140 (69.3%)	343 (74.7%)
≥35 (n=121)	34 (17.4%)	36 (17.8%)	51 (11.1%)
Area of residence			
Rural (n=427)	113 (58.5%)	163 (81.9%)	151 (33.3%)
Urban (n=418)	80 (41.5%)	36 (18.1%)	302 (66.7%)

Data on area of residence not available for all participants.

Figure 2

IgG phase II antibodies against *Coxiella burnetii* in pregnant women, immunofluorescent antibody titres in relation to enzyme-linked immunosorbent assay, Denmark, 1996–2002 (n=856)



ELISA: enzyme-linked immunosorbent assay; IFA: immunofluorescence antibody test; OD: optical density.

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 five worked with sheep. All 202 women domestically exposed were living on a farm and cohabiting with a farmer; 193 of these lived on farms with cattle, 22 on farms with sheep, and 13 on farms where cattle as well as sheep were kept.

Analyses based on specific animal contact according to IFA status showed that 23 of the 31 veterinarians working with cattle were seropositive, and that the risk of being IFA positive were 2.7 times higher in veterinarians who worked with cattle compared to those who did not (RR: 2.7; 95% CI: 1.8–4.0). The positive predictive value of being seropositive being 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 it was only 9.2% for domestic exposure to sheep.

Urban versus rural area

Among 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 for women living in rural areas (RR: 2.6; 95% CI: 1.9–3.5). Of the unexposed women, 151 (33%) lived in rural areas. Eleven (7.3 %) of them were seropositive, compared with 11 (3.6 %) seropositive among the unexposed women 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.

In general, a higher seroprevalence has been found in studies evaluating groups handling livestock, especially veterinarians, than in studies of the background population [24-30]. In one Dutch study on veterinary students, 18.7% were seropositive [31]; in another, 65% of 189 veterinarians and veterinary students were seropositive. Greater number of hours with animal contact per week, greater number of years since

Table 3

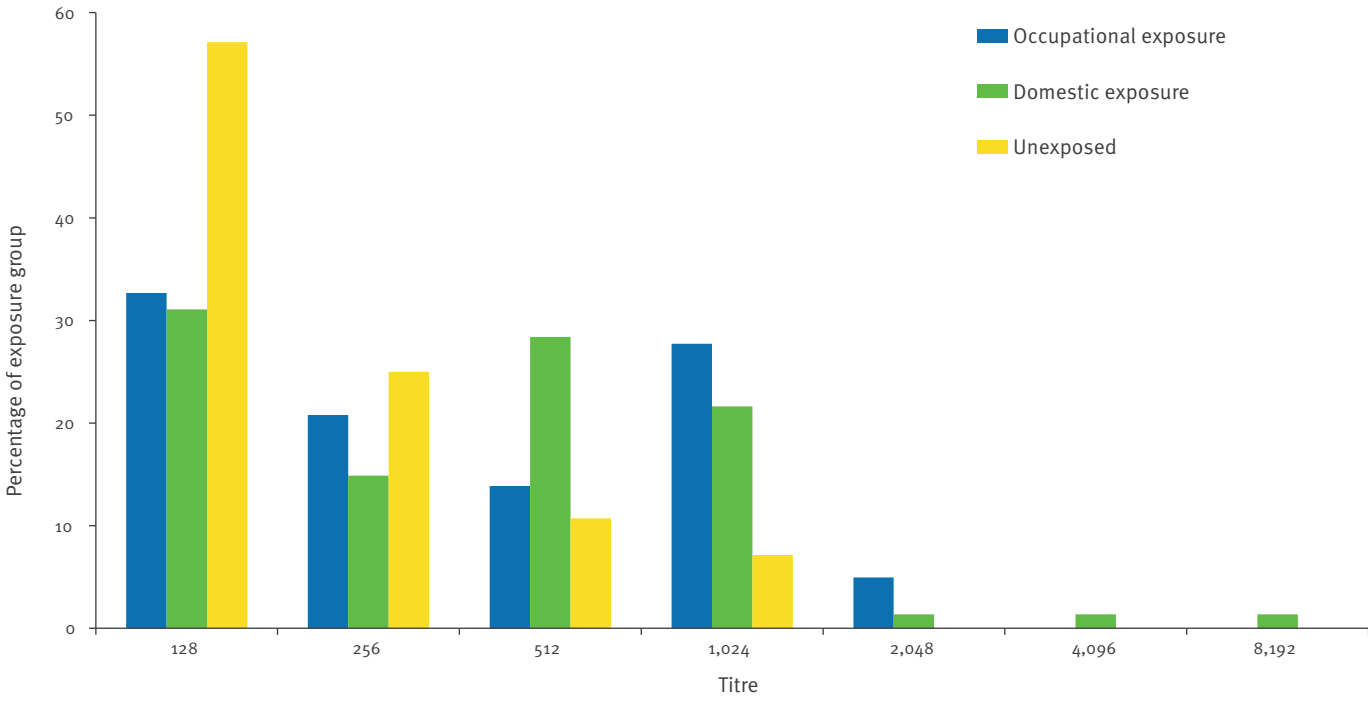
Risk difference and relative risks for pregnant women occupationally and domestically exposed to *Coxiella burnetii*, versus unexposed, Denmark, 1996–2002 (n=856)

	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)	Reference
RR (95% CI)	9.84 (6.37–15.20)	6.71 (4.26–10.57)	Reference

CI: confidence interval; IFA: Immunofluorescence assay; RD: risk difference; RR: relative risk.

Figure 3

Immunofluorescence IgG phase II antibody titres against *Coxiella burnetii* in pregnant women, by exposure group, Denmark, 1996–2002 (n=856)



the participants had graduated, living in a rural area, and working as practicing livestock veterinarian were risk factors in that study [32]. An American study found antibodies against *C. burnetii* in 113 (22.2%) of 508 US veterinarians. Compared with veterinarians with a small animal practice, those with a mixed practice for small and large animals and those with a practice for food animals were more likely to be seropositive. Furthermore that study found that having lived on a farm in the past, currently living on a farm, and exposure to ruminants while living on a farm were associated with seropositivity [15].

In Denmark, Q fever became a notifiable disease in animals in 2005. A change in diagnostic practices in cattle and an increasing number of cattle herds testing positive raised the level of awareness among exposed, asymptomatic humans in the period 2006–07. This increased focus on Q fever was thus due to diagnosis and testing rather than to the 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.

The most recent blood samples from our study dated from 2002; since then, two Danish studies have examined the presence of antibodies to *C. burnetii* in humans exposed to animals. In a serological analysis of 1,613 people, tested in 2006¬–07 mainly due to relevant exposure to domestic animals, 177 (11 %) were seropositive and 180 had an equivocal result according to the Danish cut-off [33]. Another study evaluated blood samples from 2008 from people working with domestic animals and found 39 of 359 (11 %) seropositives, with the highest prevalence of antibodies (36%) among veterinarians [34]. Close contact to birth products when performing Caesarean sections and other kinds of veterinary obstetrics is a possible explanation for the higher prevalence of antibodies among veterinarians compared to domestically exposed women found in this study.

According to the authors defining the Danish cut-off [22], high risk groups, such as veterinarians and farmers, with an intermediate 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 cut-off 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 of about 2.4% in the general population in the Netherlands before the outbreak in 2007–10 [35]. This may indicate that *C. burnetii* is generally widespread in Denmark, but could also be an argument in favour of not lowering the cut-off too much and was the rationale behind the cut-off used in this study, which was higher than in other studies [15,25,36,37] .

To our knowledge, human outbreaks of Q fever have only been described to originate from small ruminants. In France, goats and sheep have been the source of infection. The Netherlands experienced the world's largest outbreak of Q fever with more than 4,000 humans infected between 2007 and 2010 [38] and here the source of infection was goats [39].

There are different strains of *C. burnetii*, and, as for other bacteria, and some of the drivers for outbreak potential may be related to the heterogeneity in clinical outcomes, which could arise from differences in virulence and host reservoirs. The presence of strains of different pathogenicity could influence awareness of the disease and therefore partially explain the variation in illness incidence reported from different countries. In the Dutch outbreak, one genotype was suggested be responsible for the human Q fever epidemic, and this was very similar to one of the genotypes found in goats [39]. In comparison to France and the Netherlands, there are few sheep and goats in Denmark; the source of infection here is primarily cattle [40], and as far as we know, Denmark has never experienced a clinically verified Q fever outbreak.

Our study has limitations in that we did not verify positive samples with PCR or culture. But we regard the size of this cohort a major strength of this study. Also, one could argue in favour of testing random negative ELISA samples with IFA, which was not done here. However, the ELISA test was thoroughly investigated before use; the results were published by Kantsø et al [41].

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 women, with the highest prevalence of antibodies found among veterinarians who worked with cattle. Our findings confirm that *C. burnetii* is not a newly emerged pathogen in Denmark and that Q fever is endemic here as probably 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 symptomatic human infection during the recent outbreak in the Netherlands, Q fever should be considered as a possible differential diagnosis in people with close contact to domestic animals, especially veterinarians and women domestically exposed to cattle.

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