Detection of \textit{Coxiella burnetii} DNA on Small-Ruminant Farms during a Q Fever Outbreak in the Netherlands

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During large Q fever outbreaks in the Netherlands between 2007 and 2010, dairy goat farms were implicated as the primary source of human Q fever. The transmission of \textit{Coxiella burnetii} to humans is thought to occur primarily via aerosols, although available data on \textit{C. burnetii} in aerosols and other environmental matrices are limited. During the outbreak of 2009, 19 dairy goat farms and one dairy sheep farm were selected nationwide to investigate the presence of \textit{C. burnetii} DNA in vaginal swabs, manure, surface area swabs, milk unit filters, and aerosols. Four of these farms had a positive status during the 	extit{Coxiella burnetii} bulk milk monitoring program in 2009 and additionally reported abortion waves in 2008 or 2009. Eleven farms were reported as having positive bulk milk only, and five selected (control) farms had a bulk milk-negative status in 2009 and no reported Q fever history. Screening by quantitative PCR (qPCR) revealed that on farms with a history of abortions related to \textit{C. burnetii} and, to a lesser extent, on farms positive by bulk milk monitoring, generally higher proportions of positive samples and higher levels of \textit{C. burnetii} DNA within positive samples were observed than on the control farms. The relatively high levels of \textit{C. burnetii} DNA in surface area swabs and aerosols sampled in stables of bulk milk-positive farms, including farms with a Q fever-related abortion history, support the hypothesis that these farms can pose a risk for the transmission of \textit{C. burnetii} to humans.

\textbf{MATERIALS AND METHODS}

\textbf{Farm selection.} All goat farms selected for this study participated in the larger integrated human-veterinary Q-VIVE study, described previously by Schimmer et al. (19). In that study, methods regarding human and animal serology used in our study are described in more detail.

All dairy goat farms had the Dutch white dairy goat as the main breed, sometimes in combination with smaller numbers of other breeds of goat. The sheep on the dairy sheep farm are of a relatively rare breed, Dutch Friesian sheep.

Farming procedures varied little among the dairy farms selected, with at least one lambing season per year, animals kept indoors in deep-litter stables, and automated milking procedures performed daily in a separate stable compartment.

The selection of \textit{C. burnetii}-positive dairy goat farms in the current study was based on the following criteria: reported abortion waves due to \textit{C. burnetii} among goats or sheep in 2008 or 2009 (category A) and a positive status in the voluntary (2008) or mandatory (2009) PCR-based bulk milk (tank) screening survey (category B). Farms that tested positive by bulk milk screening and that additionally experienced \textit{C. burnetii}-related abortion waves were classified as farm cate-

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TABLE 1 Characteristics of farms investigated and serology of farm animals and occupants

<table>
<thead>
<tr>
<th>Farm</th>
<th>Farm category</th>
<th>Farm area (m²)</th>
<th>Date of 1st vaccination (day-mo-yr)</th>
<th>Date of sampling (day-mo-yr)</th>
<th>Date of culling (day-mo-yr)</th>
<th>Distance to nearest bulk milk-positive farm (m)</th>
<th>% positive milk samples</th>
<th>No. of positive samples/total no. of samples</th>
<th>Animal serology</th>
<th>Human serology of farmers and family</th>
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<tr>
<td>B</td>
<td>AB</td>
<td>3–40</td>
<td>5-5-2009</td>
<td>12-11-2009</td>
<td>17-2-2010</td>
<td>792</td>
<td>42.9</td>
<td>9/21</td>
<td>R P P</td>
<td>R P P P</td>
</tr>
<tr>
<td>C</td>
<td>AB</td>
<td>NA</td>
<td>18-6-2009</td>
<td>22-10-2009</td>
<td>4-2-2010</td>
<td>12.470</td>
<td>NA</td>
<td>NA</td>
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<tr>
<td>D</td>
<td>AB</td>
<td>1–27</td>
<td>12-4-2010</td>
<td>19-4-2010</td>
<td>23-1-2010</td>
<td>14.505</td>
<td>80.0</td>
<td>52/65</td>
<td>R R</td>
<td>R R</td>
</tr>
<tr>
<td>F</td>
<td>B</td>
<td>1–18</td>
<td>7-7-2009</td>
<td>20-11-2009</td>
<td>10-2-2010</td>
<td>2.613</td>
<td>71.4</td>
<td>15/21</td>
<td>P P</td>
<td>R R</td>
</tr>
<tr>
<td>G</td>
<td>B</td>
<td>1–27 and 40–52</td>
<td>19-3-2010</td>
<td>21-5-2010</td>
<td>15-2-2010</td>
<td>755</td>
<td>9.5</td>
<td>2/21</td>
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<td>R R</td>
</tr>
<tr>
<td>H</td>
<td>B</td>
<td>8–52</td>
<td>7-4-2010</td>
<td>21-5-2010</td>
<td>11-2-2010</td>
<td>1.284</td>
<td>41.9</td>
<td>13/31</td>
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<td>R P R P</td>
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<td>I</td>
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<td>63.6</td>
<td>56/88</td>
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<td>13–21</td>
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<td>15-4-2010</td>
<td>16-2-2010</td>
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<td>45.8</td>
<td>11/24</td>
<td>P P R</td>
<td>P R</td>
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<td>L</td>
<td>B</td>
<td>3–21</td>
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<td>19-5-2010</td>
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<td>92.3</td>
<td>12/13</td>
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<td>R P</td>
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<tr>
<td>O</td>
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<td>3-6-2010</td>
<td>2-2-2010</td>
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<td>10.059</td>
<td>0</td>
<td>0/22</td>
<td>N N/P</td>
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</table>

*Farms are categorized as farms with a C. burnetii-related abortion history (category AB), farms with a bulk milk-positive status in 2009 (category B), or control farms (category N). All farms were dairy goat farms, except for farm D, which was a dairy sheep farm. F, farmer; S, spouse/partner; C, child of farmer (can be an adult child); O, other family member or employee; P, past infection (IgG phase II level of >1:32, either with or without an IgG phase I level of >1:32, and IgM phase I and II negative); R, recent infection (IgM phase II level of >1:32, either with or without IgG phase I, and/or IgG phase II levels of >1:32); N, negative; NA, information not available (no farm questionnaire or no participation in animal or human sampling).
added to a 250-ml bottle (VWR International, Netherlands) with 50 ml of NucliSens lysis buffer. The bottles were placed onto a horizontal shaker and homogenized overnight at 50 rpm. NucliSens lysis buffer was transferred from the petri dishes into 15-ml Greiner tubes.

As an internal control, 50 μl of DNA extract, obtained from animal and environmental matrices, was tested in triplicate undiluted and in 10-fold dilutions.

The proportions of positive samples per matrix were compared among the three farm categories (category AB versus category B, category AB versus category N, and category B versus category N) by using Fisher exact tests. For comparisons of the Cq values obtained by qPCR among the three farm categories, a Wilcoxon rank sum test was used.

RESULTS
Serology of ruminants and farm family members at participating farms.
Based on serology, the percentages of positive small ruminants were on average 64.8% for the farms with a *C. burnetii*-related abortion history (category AB), 55.9% among the bulk milk-positive farms (category B), and, by preselection, 0% among the control farms (category N). There was a significant difference in the percentages of positive small ruminants between farms with an abortion history and bulk milk-positive farms (P < 0.05 by Fisher exact test).

The risk of transmission to humans, measured as the proportion of farms with at least one household member or worker with a recent infection according to their serological profile, was found to be related to the Q fever status of the farm. Recent infections were found at all 3 farms (100%) with a *C. burnetii*-related abortion history (one category AB farm did not participate in the human component of the study), in comparison to 8 out of 11 (73%) bulk milk-positive farms (category B) and none (0%) of the 5 control farms (category N). In more detail, of the eight sera taken at farms in category AB, 50% had a profile matching a recent infection according to their serological profile, was found to be related to the Q fever status of the farm. Recent infections were found at all 3 farms (100%) with a *C. burnetii*-related abortion history (one category AB farm did not participate in the human component of the study), in comparison to 8 out of 11 (73%) bulk milk-positive farms (category B) and none (0%) of the 5 control farms (category N). In more detail, of the eight sera taken at farms in category AB, 50% had a profile matching a recent infection, and 50% had a profile matching a past infection. For the 27 sera taken from farms in category B, these rates were 37% and 63%, respectively. For the 13 sera from farms in category N, these figures were 0% and 62%, respectively, with the remaining 38% testing negative.

The presence of *Coxiella burnetii* DNA in animal and environmental matrices. The presence of *C. burnetii* DNA in animal and environmental samples obtained from the 19 dairy goat farms and 1 dairy sheep farm was summarized in Table 2.

A comparison per matrix among the three farm categories was
based on the number of positive samples, which can be divided into two categories: IS1111 positive or IS1111 and com1 positive. Due to the presence of multiple copies of the IS1111 target within the C. burnetii genome (15), the amplification of this target is expected to occur before the amplification of the single-copy target com1. This was reflected in our data, where for samples showing positive results for both targets com1 and IS1111, Cq values of IS1111 were consistently lower than those of com1. For matrices derived from animal samples such as vaginal swabs, manure droppings, and milk unit filters, the percentage of positive samples was highest for the category AB farms with a C. burnetii-related abortion history, followed by bulk tank milk-positive farms (category B), and was lowest for the control farms (category N). Significant differences in the numbers of positive samples were found primarily between category AB farms and the control (category N) farms and between category B farms and the control (category N) farms. A similar tendency was observed for aerosol samples taken at 500 m in all four wind directions of the farm, although these values were not statistically significant. For aerosol samples taken from the stables, the percentage of positive samples was (almost) 100% for all three farm categories. In addition, the C. burnetii DNA contents in the animal and environmental matrices were compared between farm categories based on averaged Cq values for the targets com1 and IS1111. Significant differences between farm categories were found for both animal and environmental matrices. For vaginal swabs, surface area swabs, and aerosols obtained from the stables, Cq values for the target IS1111 were significantly lower (indicating a high C. burnetii DNA content) on farms with a C. burnetii-related abortion history (category AB) than on farms with a positive status by bulk milk screening only (category B) and on the control farms (category N). No significant differences were observed between farm categories for the manure samples (compared only between category AB and B farms, as category N farms had no positive manure samples), for a single-farm category comparison (farms B to N) for the milk unit filters, and for aerosol samples obtained from a radial distance of 500 m from the farms. Significant differences were found in C. burnetii DNA contents among the three farm groups (categories AB, B, and N) for aerosols obtained in the stables, while the proportions of positive stable aerosol samples were not significant between these farm groups. Finally, based on Cq values for both targets com1 and IS1111, aerosol samples obtained from the stables contained higher levels of C. burnetii DNA than did the aerosol samples obtained at a 500-m distance from the farms (P < 0.01 by Kruskal-Wallis test).

In Fig. 1, the locations of the farms, the farm categories, and the proportions of negative, IS1111-positive, and IS1111- and com1-positive samples per farm are indicated (in green, orange, and red, respectively). Ten dairy goat farms and the dairy sheep farm were located outside the mandatory vaccination area of 2009, and nine dairy goat farms were located within this area.

**DISCUSSION**

In our study, generally both the proportions of C. burnetii-positive animal and environmental samples and the C. burnetii DNA contents within positive samples were highest on farms with a C. burnetii-related abortion history (category AB), followed by the farms with positive bulk milk (category B), and were lowest on the control farms (category N). The higher proportions of positive samples and higher levels of C. burnetii DNA content on the category AB and category B farms suggest that these farms pose an increased transmission risk for humans. This was supported by the serological results obtained from the farm occupants. The only exception to this general rule was found for the aerosol samples. It seems that within stables, C. burnetii is almost always present in aerosols, but levels vary with the Q fever status of the farm. In contrast, aerosols obtained from a radial distance of 500 m from category AB and category B farms were more often found to be positive, but the less common positive samples around negative farms seemed to contain similar C. burnetii DNA levels.

Naturally, it should be noted that especially the aerosol samples obtained outside the stables are greatly influenced by the sampling conditions, such as wind direction, wind speed, humidity, and other sources contaminated with C. burnetii. As samples were taken on a random day, the results might not represent the occurrence over a longer time interval. The results for the C. burnetii DNA contents in aerosols in the vicinity of small-ruminant farms should therefore be interpreted with caution and do not allow firm conclusions.

Although clear differences in C. burnetii DNA contents were
observed among the different farm categories in our study, these differences are thought to be underestimated for several reasons. First, the excretion of *C. burnetii* in vaginal mucus and in milk is intermittent and is known to decrease over time after lambing (7).

The sampling period for farms within the mandatory vaccination area occurred longer after the lambing period than for the farms outside this vaccination area. As these first farms included three of the four farms with abortion histories, the *C. burnetii* DNA contents might have been at a relatively low level at that time of the year for these farms. Second, the reported dates of the actual *C. burnetii*-related abortions at these farms preceded the sampling date by several months to over a year. It is known that levels of *C. burnetii* excretion decrease over time following a Q fever outbreak in animals, again resulting in lower levels on the sampling date (3, 8, 18). Finally, at one of the category AB farms (farm D), the pregnant sheep were already culled before sampling took place, again reducing the number of excreting animals within this farm category. Consequently, true differences are expected to be even greater than those observed in our study.

The various animal and environmental matrices studied represent the presence of *C. burnetii* DNA on and around small-ruminant farms for different time intervals and with different numbers of ruminants on the farm. Surface area swabs give insight into the presence of *C. burnetii* DNA on a farm over a longer period of time, because (contaminated) dust particles, generated by multiple small ruminants, accumulate on surface areas. Milk unit filters represent a shorter time interval but again represent many goats, as during the passage of the milk, residues accumulate on the milk unit filters. This may explain the relatively high levels of *C. burnetii* DNA in these two matrices.

In contrast, levels of *C. burnetii* DNA in vaginal swabs from individual animals were relatively low. This may be explained by the fact that vaginal swabs provide information on the shedding of *C. burnetii* by individual animals at the moment of sampling only. Also, low levels of *C. burnetii* DNA were found in aerosols, especially in samples taken outside the stables. Suspended dust particles, obtained by aerosol sampling within stables, contained fewer contaminated dust particles than did surface area swabs from those same stables. The level of *C. burnetii* DNA in aerosols obtained from stables represents captured contaminated aerosols at the moment of sampling only, which is influenced especially by dust-producing activities in the stable. Because of these legitimate differences between the matrices, a quantitative comparison of *C. burnetii* DNA contents between matrices within and between farms is difficult. Standardized quantitative sampling of accumulated dust on surface areas (surface area swabs) and dust suspended in air (aerosols) is very difficult. In addition, animal and environmental matrices are known to inhibit the qPCR assay, which complicates the accurate quantitative assessment of *C. burnetii* DNA in these types of matrices.

Our study had several limitations regarding the sampling strategy. First, ideally, all farms should have been sampled at the same time and before vaccination took place to eliminate the effect of vaccination on the study results. Originally, the entire study was planned to start in the fall of 2009, but in March 2009, it was decided suddenly by the Dutch government that the vaccination of small ruminants was to be mandatory in the south of the country. All efforts were then placed on the immediate preparation of the recruitment of farms and retrieval of sera from a sample of the goats and sheep of the small-ruminant dairy farms in the mandatory vaccination area that were willing to participate. Vaccination probably did not affect the results for the farms with abortions to a large extent, since it is known that the vaccine is not effective on ruminants that are already infected (12). However, excretion by goats and sheep at farms with relatively few affected animals, as might have been the situation for farms in category B, was possibly kept at low levels because the vaccine drastically decreased the amount of new infections, and for the few animals infected despite vaccination, excretion levels would have been reduced. Nevertheless, as results for the bulk milk–positive farms more resembled the results for the farms with abortion histories than results for the negative farms, the effect of vaccination on the outcome of our study seems to have been limited.

Second, the detection of *C. burnetii* in our study was based on the amplification of specific targets within the *C. burnetii* genome by qPCR. The viability of the *C. burnetii* organisms within these matrices can therefore not be assessed. The cultivation of *C. burnetii* from complex matrices, such as surface area swabs, manure, and milk unit filters, was not successful. In addition, the cultivation of a large number of samples was not feasible, due to the requirement for biosafety level 3 (BSL3) facilities, and was attempted only for highly positive samples. To date, the cultivation of *C. burnetii* in our laboratory has been successful only with highly positive placenta materials obtained from goats and sheep. However, these materials could not be obtained from the farms included in this study.

From the qPCR results obtained from the various matrices and the circumstantial evidence of the human serology of farm occupants, we conclude that levels of *C. burnetii* DNA in both animal and environmental matrices are high on *C. burnetii*-positive farms and most pronounced on farms with a *C. burnetii*-related abortion wave, which poses an increased risk of infection of humans living on or near these farms. Nevertheless, it should be noted that in addition to the presence of *C. burnetii* in aerosols and other matrices at the farms, local geographical conditions also influence the actual risk of infection for humans living in the vicinity of the farm, as was recently demonstrated (23). This complex interaction between the farm and environment and the risk to inhabitants in the vicinity are currently the subject of more in-depth investigations.

In conclusion, this study supports epidemiological findings suggesting that contaminated dust and aerosols derived from contaminated animal matrices from *C. burnetii*-positive dairy goat farms played a predominant role in the transmission of *C. burnetii* to humans during the outbreaks in the Netherlands (13, 23).

**ACKNOWLEDGMENTS**

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