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# Experimental Inoculation of Male Rats with *Coxiella burnetii*: Successful Infection but No Transmission to Cage Mates

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Beginning in 2007, the largest human Q fever outbreak ever described occurred in the Netherlands. Dairy goats from intensive farms were identified as the source, amplifying *Coxiella burnetii* during gestation and shedding large quantities during abortions. It has been postulated that wild rodents are reservoir hosts from which *C. burnetii* can be transmitted to domestic animals and humans. However, little is known about the infection dynamics of *C. burnetii* in wild rodents. The aim of this study was to investigate whether brown rats (*Rattus norvegicus*) can be experimentally infected with *C. burnetii* and whether transmission to a cage mate occurs. Fourteen male brown rats (wild type) were intratracheally or intranasally inoculated with a Dutch *C. burnetii* isolate obtained from a goat. At 3 days postinoculation, a contact rat was placed with each inoculated rat. The pairs were monitored using blood samples and rectal and throat swabs for 8 weeks, and after euthanasia the spleens were collected. Rats became infected by both inoculation routes, and detection of *C. burnetii* DNA in swabs suggests that excretion occurred. However, based on the negative spleens in PCR and the lack of seroconversion, none of the contact animals was considered infected; thus, no transmission was observed. The reproduction ratio  $R_0$  was estimated to be 0 (95% confidence interval = 0 to 0.6), indicating that it is unlikely that rats act as reservoir host of *C. burnetii* through sustained transmission between male rats. Future research should focus on other transmission routes, such as vertical transmission or bacterial shedding during parturition.

Q fever is a zoonotic disease caused by the bacterium *Coxiella burnetii*. Goats, sheep, and cattle are considered the primary animal reservoirs for zoonotic transmission (2). Urine, feces, and especially the birth material of infected animals are sources for *C. burnetii* contamination of the environment. The bacterium can survive for years in the environment and can travel long distances as aerosols (7). Inhalation of aerosolized bacteria is considered the primary route of infection for humans (2). Although *C. burnetii* is prevalent worldwide, there are remarkable differences in the incidence of human Q fever in different areas of the world, even when rates of infection in animals are comparable (7). In the Netherlands, there has been a sharp increase in the human incidence of Q fever since 2007, with 168, 1,000, 2,354, 504, and 81 reported cases per year from 2007 to 2011, respectively (according to RIVM data [[http://www.rivm.nl/Onderwerpen/Ziekten\\_Aandoeningen/Q/Q\\_koorts](http://www.rivm.nl/Onderwerpen/Ziekten_Aandoeningen/Q/Q_koorts)]). Epidemiological studies indicated intensified dairy goat production as the main source (5, 13) and prompted government actions to reduce *C. burnetii* emission at the herd level. Pest control was added to the mandatory hygiene measures as of February 2009. Because the role of rodents in the epidemiology of *C. burnetii* in domestic ruminants is largely unknown, the impact of this control measure is uncertain. In literature the role of wild vertebrates in the sylvatic cycle of *C. burnetii* is established (1), and it has been proposed that brown rats (*Rattus norvegicus*), as commensal rodents, have a significant role in the dissemination of *C. burnetii* to humans and domestic animals in the United Kingdom (15). A quick scan of the presence of *C. burnetii* in rats in the Netherlands demonstrated the presence of *C. burnetii* DNA (4%) and antibodies (11%) in wild rats captured for pest control at various locations, including, but not limited to, infected goat farms (8). Based on this knowledge, rats represent a likely threat for (re)introduction of *C. burnetii* in

the domestic cycle. However, the presence of *C. burnetii* positive rats could indicate merely spillover because of a very high environmental burden and does not prove the role of rats as an effective reservoir. For rats to act as reservoir hosts, *C. burnetii* should persist in the population through rat-to-rat transmission, i.e., without repeated introduction from an external source. To enable sustained transmission within a population, the basic reproduction ratio ( $R_0$ ) has to be larger than one.  $R_0$  represents the average number of secondary cases caused by one infectious individual during its entire infectious period, in a completely susceptible population (3). To get more insight in the potential role of rats as reservoirs of *C. burnetii*, we investigated whether brown rats can readily be infected and subsequently transmit *C. burnetii* to a cage mate in an experimental setting. The results from the pairwise experiments were used to estimate the  $R_0$  for rat-to-rat transmission of *C. burnetii*.

## MATERIALS AND METHODS

**Inoculum.** *C. burnetii* isolate X09003262-001 was selected for these experiments. This isolate was obtained from the placenta of an aborted goat from a farm on which 25% of the goats had aborted during the Q fever

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outbreak in the Netherlands (farm N) (9). The isolate was typed CbNL01, which was the predominant strain in goats during the Dutch epidemic (9). *C. burnetii* was isolated by filtering crushed placenta tissue (FastPrep-24 [MP Biomedicals], two times for 20 s with 6 m/s<sup>2</sup>, with a 5-min break in between) over a rough cell strainer (Nunc), a 1.2- $\mu$ m pore size and finally a 0.45- $\mu$ m-pore-size filter (Pall Corp.). Filtrate was inoculated into buffalo green monkey (BGM) cells (European Collection of Cell Cultures, no. 90092601), followed by incubation for 14 days. Culture medium (Eagle minimal essential medium with 10% bovine serum albumin, 1% nonessential amino acids (NEAA), and 1% GlutaMAX) was refreshed twice a week. The growth of *C. burnetii* was monitored by vacuolization of the BGM cells and confirmed by an immunofluorescence assay using a *C. burnetii*-specific monoclonal antibody (MAb313-Oregon Green; Squarix Biotechnologies, Marl, Germany). A large batch of passage 2 of strain X09003262-001 was stored at  $-80^{\circ}\text{C}$  in aliquots after removal of the cell line debris via a freeze-thaw step and centrifugation for 10 min at  $100 \times g$ . The inoculum was adjusted to  $10^6$  mouse infective dose (MID) per ml. The MID was determined by peritoneal inoculation of 10-fold dilutions into four mice per dilution, and is defined as the lowest concentration still infective for all mice (demonstrated by PCR-based detection of *C. burnetii* in the spleen). In addition, a decimal dilution series of the inoculum was prepared in duplicate and, after DNA isolation (200  $\mu$ l), tested by quantitative PCR (qPCR). Determination of the MID was approved by the ethical committee on animal experiments of the Central Veterinary Institute, a part of Wageningen UR (registration no. 2009082.c).

**Animals.** Adult male wild-type Groningen (WTG) rats were used in the experiments. These rats are bred from originally wild-caught brown rats (*Rattus norvegicus*) and therefore considered the best laboratory-bred representatives of the brown rats present at Dutch farms. However, a wild origin brings on the possibility of prior exposure to *C. burnetii*, and 16 rats from the breeding colony were therefore serologically tested for antibodies to *C. burnetii* by enzyme-linked immunosorbent assay (ELISA) and for *C. burnetii* DNA in the spleen by PCR. Once the absence of *C. burnetii* in this colony had been demonstrated by both methods, 28 male rats were obtained.

**Experimental design and sample collection.** Out of 28 rats, 14 75-day-old animals were randomly assigned to either the intratracheal (i.t.;  $n = 7$ ) or the intranasal (i.n.;  $n = 7$ ) group. Both inoculation methods provide exposure by inhalation, which is assumed to be the most important infection route for rats under natural conditions (15). However, it was not known which method would be most efficient, and i.n. inoculation could provide an easily applicable refinement for future animal experiments, if shown to be equally effective. On day 0, the rats were inoculated by injecting 100  $\mu$ l of the inoculum ( $10^6$  MID/ml) into the trachea (exposed by skin incision) or by pipetting a 100  $\mu$ l of the same suspension into the nose. The rats were housed individually in cages (Macrolon type III) in two biosafety level 3 (BSL3) type isolators (separate isolators for the i.t. and i.n. groups) and observed daily. At 3 days postinoculation (dpi) a contact rat was placed with each inoculated rat. Only eight contact rats of the same age as the inoculated rats were available. The other six contact rats were 20 days younger and divided equally over both groups. Preinoculation samples (blood, throat, and rectal swab) were collected 3 days prior to the inoculation day (day  $-3$ ). Starting at 3 dpi, swabs from the throat and rectum were collected twice a week from all rats and stored frozen in 500  $\mu$ l of phosphate-buffered saline (PBS). Blood samples were collected by orbital puncture at 7, 14, 21, 35, and 49 dpi for the inoculated rats and at 10, 17, 24, 38, and 52 dpi for the contact rats (i.e., 7, 14, 21, 35, and 49 days after introduction into the cage of the inoculated mate). To prevent complete blindness, blood collection was discontinued for rats showing unilateral blindness. Blood samples were left to clot, and centrifuged and serum samples stored at  $-20^{\circ}\text{C}$ . On days 56, 57, and 58, rats were euthanized by cardiac puncture, and blood, spleens, and swabs from the throat and rectum were collected. Inoculation, sample collection, and euthanasia were performed under isoflurane anesthesia. This study was approved by the scientific and animal experiments committee of the

Netherlands Vaccine Institute (DPA 201100047) and was conducted at their facilities.

**Serology.** A human Serion ELISA classic *C. burnetii* Phase II IgG kit (Clindia Benelux B.V., Leusden, Netherlands) was modified to test rat sera as described previously (8). The cutoff value for this ELISA was determined at a  $\log_{10}$ -transformed optical density (OD) of  $-0.957$ , which corresponds to an OD of 0.110.

**DNA isolation.** Throat and rectal swabs (in 500  $\mu$ l of PBS) were incubated at  $100^{\circ}\text{C}$  for 30 min to inactivate *C. burnetii* and enable safe working outside the BSL3 facility. Subsequently, the swabs were vortexed, 200  $\mu$ l of PBS was transferred to a clean tube, and 10  $\mu$ l of a *Bacillus thuringiensis* spore suspension ( $10^7$  spores/ml) was added as a DNA isolation and PCR inhibition control. After storage at  $-20^{\circ}\text{C}$ , DNA isolation was performed using the spin-column "purification of total DNA from animal blood or cells" protocol from a Qiagen DNeasy tissue kit. In the final step, DNA was eluted in 100  $\mu$ l of prewarmed elution buffer.

DNA was isolated from 10 mg of spleen using a Qiagen blood and tissue kit according to the manufacturer's "purification of total DNA from animal tissues" protocol, with an additional inactivation step: the tissue was ground using a pestle under BSL3 conditions. ATL buffer, proteinase K, and 10  $\mu$ l of *B. thuringiensis* spore suspension were added, and the sample was incubated at  $56^{\circ}\text{C}$  overnight. Next, an incubation step at  $100^{\circ}\text{C}$  for 30 min was incorporated to ensure inactivation of *C. burnetii*. Afterward, tubes were transferred outside the BSL3 facility, and the manufacturer's protocol was continued.

**qPCR.** A multiplex qPCR for *C. burnetii* (multicopy target IS1111 and single-copy target *com1*) and *B. thuringiensis* (*cry1b*) was performed as described elsewhere (2a). A positive (*C. burnetii* DNA) and blank (water) control were included on each plate. Crossing point (Cp) values were determined by using the second derivative method with Roche Light-Cycler 480 software.

**Data analyses.** Rats were considered infected if the spleen was positive in PCR. To quantify transmission in the experiment, the reproduction ratio  $R_0$  and 95% confidence interval (CI) were calculated using a final-size method as described by Velthuis et al. (14). In short, the probability that transmission from an infectious rat to a susceptible contact rat takes place ( $P_{\text{inf}}$ ) was calculated by dividing the number of contact rats that became infected during the experiment by the number of successfully inoculated rats. The 95% CI for  $P_{\text{inf}}$  was calculated using a binomial distribution. The resulting values of  $P_{\text{inf}}$  were then translated into a point estimate and 95% CI for  $R_0$  by applying the following formula:  $R_0 = (2 \times P_{\text{inf}})/(1 - P_{\text{inf}})$  (4). This formulation assumes that infectivity shed by the inoculated rat is equally shared by both rats in the same cage.

## RESULTS

**Clinical signs.** The most apparent clinical sign was not due to the infection but to orbital puncture: in the course of the trial four rats developed unilateral blindness. Two of these were contact rats (2C and 12C), and two were inoculated rats from the i.n. group (10I and 11I). Other signs observed were: inactivity (12 occasions, 8 rats: 2 contact and 4 inoculated rats from the i.t. group and 2 inoculated rats from the i.n. group), diarrhea (observed at sampling on 10 occasions, 10 rats: 3 contact and 4 inoculated rats from the i.t. group and 3 inoculated rats from the i.n. group), and redness of the skin incision (2 inoculated rats from the i.t. group).

**Serology.** All rats were negative for antibodies against *C. burnetii* prior to inoculation. All but one (12I) of the inoculated rats showed a clear rise in antibody titer, with seroconversion detectable from 15 or 21 dpi (Fig. 1 and 2). For rat 12I (i.n. group), the OD was only slightly above the cutoff value (0.110) from 35 dpi onward. Contact rats 8C, 9C, 10C, 11C, 13C, and 14C from the i.n. group occasionally scored just above the cutoff value at initial testing, but these responses did not increase or persist (data not

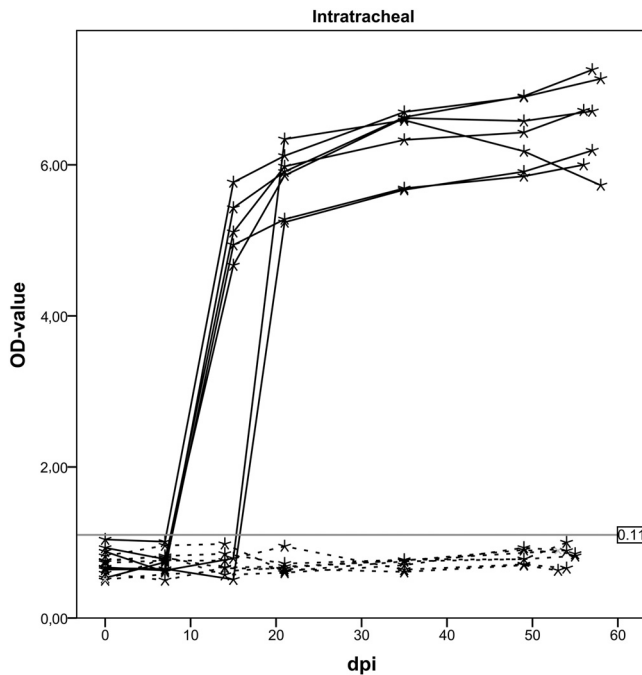


FIG 1 OD values in *C. burnetii* phase II IgG ELISA for seven pairs of an i.t.-inoculated rat (lines) and a contact-exposed cage mate (dashed lines). The OD values are plotted at the day postinoculation or, for contact rats, at the day after introduction in the cage with the inoculated rat (dpi). The cutoff value is indicated (the horizontal line labeled 0.11).

shown). Retesting showed that none of the contact rats could be considered seropositive.

***C. burnetii* DNA in swabs from the throat and rectum and in spleens.** The numbers of *C. burnetii* DNA-positive swabs and rats with at least one positive swab are shown in Table 1. Detailed results for the multicopy target (*IS1111*) are available in Table S1 in the supplemental material for throat swabs and in Table S2 in the supplemental material for rectal swabs. Occasionally, positive results were obtained for swabs from throat or rectum of contact rats; however, the proportion of swabs testing positive was significantly higher for inoculated rats than contact rats in both the i.t. and i.n. groups ( $\chi^2$  test,  $P < 0.05$ ). Comparison of the inoculation routes demonstrates that the proportion of swabs testing positive was higher in the i.t. group than in the i.n. group for both inoculated and contact rats ( $\chi^2$  test,  $P < 0.05$ ). The lowest Cp value for the *IS1111* target was 27.63 for throat swabs (3I at 10 dpi) and 32.94 for rectal swabs (1I at 17 dpi). Based on the lines fitted to the PCR results for the decimal dilutions of the inoculum (Fig. 3), corresponding MID can be calculated from Cp values using  $\log(\text{MID}) = -0.2862C_p + 11.15$  for the *IS1111* target and  $\log(\text{MID}) = -0.2951C_p + 12.99$  for the *com1* target. The lowest Cp values for the throat and rectal swabs thus correspond to 1,747 and 52 MID, respectively. The spleens of all inoculated rats tested positive for *C. burnetii* *IS1111* DNA, and those of six i.t.- and two i.n.-inoculated rats also tested positive for *com1*, whereas none of the spleens of contact rats tested positive for either target. The mean Cp value (*IS1111* target) for the spleens of i.t.-inoculated rats (29.9; 95% CI = 28.5 to 31.4) was significantly lower ( $t$  test,  $P = 0.018$ ) than the mean Cp value for the i.n.-inoculated rats (31.8; 95% CI = 31.0 to 32.7).

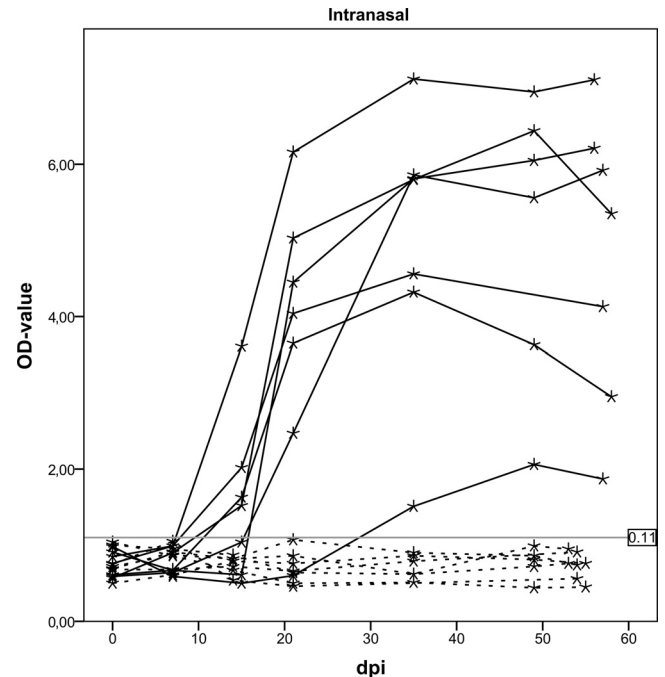


FIG 2 OD values in *C. burnetii* phase II IgG ELISA for seven pairs of an i.n.-inoculated rat (lines) and a contact-exposed cage-mate (dashed lines). The OD values are plotted at the day postinoculation or, for contact rats, at the day after introduction in the cage with the inoculated rat (dpi). The cutoff value is indicated (the horizontal line labeled 0.11).

**Transmission.** Based on the negative PCR results for the spleen tissues of the contact rats, supported by the negative serological results, all contact rats were considered uninfected. Thus, no transmission was observed in this experiment, and the point estimate of  $R_0$  was 0. The upper limit of the 95% CI for  $R_0$  was 0.6.

## DISCUSSION

It has been postulated that rats play a role in the epidemiology of *C. burnetii*, since infected rats have been detected in the field (8). To provide an efficient reservoir, a rat population must sustain *C. burnetii* transmission without repeated introduction. Therefore, rat-to-rat transmission of *C. burnetii* was studied experimentally.

The detection of *C. burnetii* DNA in the spleen and the detection of antibodies against *C. burnetii* in serum demonstrate that rats became infected after i.n. and i.t. inoculations. Differences between the inoculation routes consist of a higher bacterial load in

TABLE 1 Swabs from the throat and rectum that tested positive in *IS1111* and *com1* *C. burnetii* qPCR analyses

Swab	Test	Group <sup>a</sup>			
		i.t. inoculated	i.t. contact	i.n. inoculated	i.n. contact
Throat	<i>IS1111</i> positive	101 (7)	2 (2)	28 (6)	2 (2)
	<i>com1</i> positive	55 (7)	2 (2)	6 (4)	0 (0)
Rectum	<i>IS1111</i> positive	68 (7)	17 (7)	11 (6)	3 (3)
	<i>com1</i> positive	1 (1)	0 (0)	0 (0)	0 (0)

<sup>a</sup> The number of swabs from the throat and rectum that tested positive in *IS1111* and *com1* *C. burnetii* qPCR by group (119 swabs per group) is given. The number of animals with at least one positive swab is indicated in parentheses (seven animals per group).

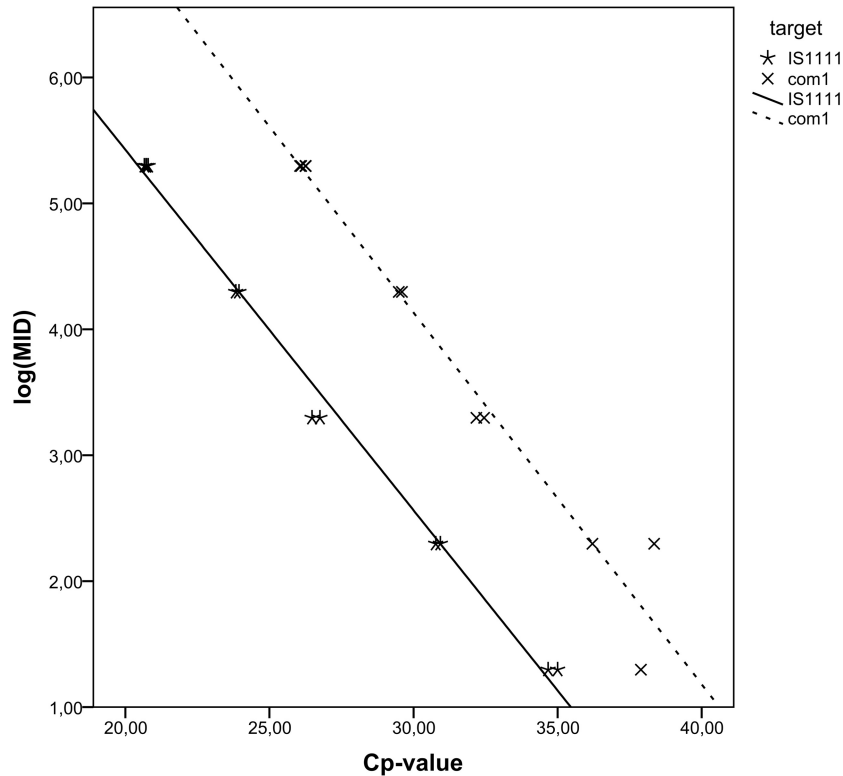


FIG 3 Relation between Cp value in IS1111 (solid line) and *com1* (dashed line) qPCR for *C. burnetii* and  $\log_{10}$ -transformed mouse infective dose [ $\log(\text{MID})$ ] based on the qPCR results (asterisks) for a duplicate decimal dilution series of a *C. burnetii* suspension.

the spleens of i.t.-inoculated rats and more variation in the antibody response for the i.n.-inoculated rats, including a marginal response for one of the i.n.-inoculated rats. In addition, swabs from i.t.-inoculated rats tested positive by PCR more frequently than swabs from i.n.-inoculated rats. In the case of i.n. inoculation, exhalation, sneezing, or swallowing can lead to spilling of part of the inoculum, whereas i.t. inoculation is easier to control. Differences in dose may explain the differences in serological response and spleen bacterial load (as shown, for example, in guinea pigs [10]) and possibly also the differences in frequency of shedding. Nonetheless, all rats were successfully infected by both routes, and all pairs were used in the  $R_0$  analysis. In future experiments the benefits of a more consistent dose should be weighed against the higher pain score for the skin incision needed for i.t. inoculation.

For all but one (12I) of the inoculated rats *C. burnetii* DNA was detected in swabs collected from the throat or rectum. These results can indicate either active shedding (i.e., excretion of *C. burnetii* after multiplication in the rat) or merely the presence of *C. burnetii* DNA from the environment in the throat and on the anus or, after gastrointestinal passage, in the rectum. All inoculated rats had a PCR-positive spleen, indicating that bacteria entered the host body and may have multiplied; thus, the detection of *C. burnetii* DNA in swabs could at least partly be caused by excretion. That this is likely the case is supported by the observation that the inoculated rats had a higher number of positive swabs than the contact rats.

None of the contact rats tested positive for *C. burnetii* DNA in the spleen, nor did they show an antibody response. Therefore, it can be concluded that, although the prerequisites were available

(infected and excreting cage-mate), no transmission of *C. burnetii* to contact rats occurred in this experiment. Most contact rats incidentally tested positive on rectal or throat swab. We interpreted these positive tests as contamination from the environment or gastrointestinal passage, in contrast to infection, which was defined by a positive PCR result for spleen tissue. Our results point out that PCR results from rectal or throat swabs do not always reflect the infection status.

Based on the results from these experiments the  $R_0$  for rat-to-rat transmission of *C. burnetii* was estimated to be 0, with a 95% CI of 0 to 0.6. This is well below 1, the threshold for an infection to be able to spread and maintain itself within a population (3). Therefore, it is unlikely that a population of rats can maintain *C. burnetii* and act as a persistent reservoir. However, this does not exclude the possibility that rats facilitate transport of bacteria from one farm to another. In light of the conclusion that rats are unlikely to act as reservoir hosts, there are some limitations to our study that should be taken into consideration. First, an important assumption in the used final-size method is that the infection has reached its final size, meaning that the experimental period is long enough either for all contact animals to become infected, or for all infected animals to reach the end of their infectious period. This assumption may not have been met, since swabs from inoculated rats were still positive at the end of the experiment; therefore, the  $R_0$  may be underestimated. An assumption in  $R_0$  estimation is that excreted bacteria are taken up equally by the inoculated rat and the contact rat. This assumption was made because *C. burnetii* is transmitted mainly through the environment, in contrast to,

for example, direct animal contact only. When assuming that all infectivity goes to the contact rat, the upper limit of the CI of  $R_0$  would be reduced by 50%. Another point is that the transmission experiment was carried out with only one isolate of *C. burnetii*. It is possible that use of another strain of *C. burnetii* would have given different results, but the Dutch epidemic showed the CbNL01 strain to predominate in goats (9). The predominant type in humans (type G) is identical to CbNL01 based on the four MLVA markers that were used in both studies (12). The choice for this strain is further supported by recent data that suggest that this strain also predominates in wild-caught rats: *C. burnetii* type A (again identical to CbNL01 based on four markers) was isolated from 5 (3 black, 2 brown) of 11 rats in the Netherlands (A. de Bruin et al., unpublished data). In the experiment, a high inoculation dose ( $10^5$  MID) led to low concentrations of *C. burnetii* DNA subsequently detected on swabs, resulting in no observed transmission. In nature, rats might be exposed to higher or lower doses, which could influence the amount of bacteria excreted and thus the probability of transmission to the next rat. The experiment was limited to one sex to reduce variation, and males were chosen because a higher seroprevalence of *C. burnetii* infection was observed in male wild-caught brown rats in the United Kingdom (15) and because higher bacterial loads were demonstrated in experimentally infected male mice (6). As a consequence, the shedding profile of infected female rats, particularly when reproducing, remains unknown. It is still possible that *C. burnetii* can persist in rat populations through vertical transmission or via bacterial shedding around parturition. Finally, ticks could also facilitate rat-to-rat transmission of *C. burnetii* in nature. However, no *C. burnetii* was detected in 1891 questing ticks collected in the Netherlands between 2006 and 2010 (11).

In conclusion, male brown rats can be infected with *C. burnetii* through i.t. or i.n. inoculation but do not become infected by exposure to an experimentally infected cage mate. The lack of rat-to-rat transmission in our study setup makes an important role for rats as maintenance reservoirs of *C. burnetii* less likely. However, additional research is needed to gain insight into the transmission dynamics of female rats.

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