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Journal

Infection and Immunity, 87(7)

ISSN

0019-9567

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Publication Date

2019-07-01

DOI

10.1128/iai.00834-18

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BACTERIAL INFECTIONS

Characterization of the Horizontal and Vertical Sexual Transmission of *Chlamydia* Genital Infections in a New Mouse Model

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ABSTRACT *Chlamydia trachomatis* is the most common sexually transmitted bacterial pathogen worldwide, and there is a need to control this epidemic. So far there is no established animal model in which both the horizontal and the vertical transmission of *Chlamydia* can be studied. To implement a horizontal sexual transmission model, male mice were inoculated in the meatus urethra with *Chlamydia muridarum* and they were caged with naive female mice. Urine and vaginal swab specimens were collected for culture. To study vertical transmission, newborns were euthanized and specimens were cultured. As controls, females were mated with sham-infected male mice. All *C. muridarum*-inoculated male mice had positive urine cultures. As determined by serology, all females caged with *C. muridarum*-inoculated males became infected, and 93% of them had positive vaginal swab specimen cultures. More females mated with *C. muridarum*-infected male mice (35%) than females mated with sham-infected male mice (0%) were infertile ($P < 0.05$). Also, *C. muridarum*-infected females delivered significantly fewer pups (3.8 ± 3.2/mouse) than control females (6.3 ± 1.6/mouse) ($P < 0.05$). Of the newborn mice, 32% were *C. muridarum* positive either in the lungs or in the intestines. Female mice housed with sham-infected males had no positive vaginal swab specimen cultures or *C. muridarum*-positive pups. This new mouse model of horizontal and vertical sexual transmission of *Chlamydia* closely parallels *C. trachomatis* sexual transmission in humans and may be a good model system to better understand the pathogenesis of these infections.

KEYWORDS *Chlamydia muridarum*, *Chlamydia trachomatis*, genital infection, infertility, mouse model, neonatal infection, sexual transmission

Chlamydia trachomatis is the most common sexually transmitted bacterial pathogen, annually infecting approximately 130 million individuals worldwide (1–3). Depending on the population, about 5% to 20% of women and 4% to 12% of men are positive for *C. trachomatis* during their reproductive age (2, 4, 5). In the United States, infections with *C. trachomatis* are the most commonly reported to the CDC and affect ~1.7 million patients, including ~100,000 pregnant women, each year (4, 6). Cervicitis/urethritis in females and urethritis in males are the most common acute presentations (7). Severe acute and chronic infections may result in long-term sequelae, including pelvic inflammatory disease (PID), chronic abdominal pain, ectopic pregnancy, and infertility (8–11). If *C. trachomatis* infections during pregnancy are not diagnosed and properly treated, they can affect 70% of newborns, resulting in conjunctivitis, pneumonia, and gastrointestinal colonization (12, 13). Other associated complications include low birth weight, premature birth, premature rupture of membranes, and an increased risk of death (14, 15). Since 50% to 80% of infections are asymptomatic, it is difficult to identify and treat patients (10, 16). Attempts to control chlamydial infections using screening programs and antibiotic treatment have failed, and there is no licensed vaccine (17–19).

Citation Pal S, Tifrea DF, de la Maza LM. 2019. Characterization of the horizontal and vertical sexual transmission of *Chlamydia* genital infections in a new mouse model. Infect Immun 87:e00834-18. https://doi.org/10.1128/IAI.00834-18.

Editor Craig R. Roy, Yale University School of Medicine

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Received 16 November 2018
Returned for modification 6 December 2018
Accepted 19 February 2019
Accepted manuscript posted online 4 March 2019
Published 20 June 2019

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Pathogen survival depends on the ability to spread from one host to another. Thus, an effective way to eradicate an infection is to block transmission. Currently, there are no *C. trachomatis* sexual transmission models in mice. To study chlamydial infections in female mice, animals are inoculated by the vaginal, transcervical, uterine, or intrabursal route (20–25). Rank et al. (26) implemented a model in which male guinea pigs were infected in the meatus urethra with *Chlamydia caviae* and were then caged with naive female guinea pigs. Transmission of *C. caviae* from male to female guinea pigs was characterized, but transmission to their newborns was not reported. Guinea pigs have certain advantages over mice since their estrous cycle is longer (~14 versus ~5 days) and the anatomy of their genital tract better resembles that of humans. However, the longer gestational period of guinea pigs (63 to 68 versus ~21 days for mice), the lack of immunological reagents for in-depth analyses, and the high cost of purchase and maintenance of guinea pigs discourage the use of the guinea pig model. The numerous immunological tools, harem-style breeding, and lower maintenance costs make mice an attractive alternative model for analyzing *C. trachomatis* sexual transmission.

The goal of this study was to implement a new mouse model of horizontal and vertical sexual *Chlamydia* transmission. Male mice, infected in the meatus urethra with *Chlamydia muridarum*, were caged with naive females to analyze horizontal transmission. To study vertical transmission, pregnant females were followed until delivery and their newborns were euthanized at 10 postnatal days (PND). This new model can be used to better understand the immunopathogenesis of these infections and to improve diagnostic, preventative, and therapeutic measures.

RESULTS

Characterization of *C. muridarum* infection and humoral immune responses in male mice. Ten male mice were inoculated with *C. muridarum* in the meatus urethra. As controls, 5 male mice were sham infected with sugar phosphate glutamate buffer (SPG) (Fig. 1A and Table 1). At 1 day after infection, each male mouse was housed with four naive females for 3 weeks. All male mice were euthanized at 3 weeks postmating. All 10 inoculated male mice were culture positive for *C. muridarum* from 4 to 21 postmating days (PMD). The highest number of *C. muridarum* inclusion-forming units (IFU) was recovered between 7 and 11 PMD (median, 1.0×10^6 IFU/ml urine). By 21 PMD, the cumulative median number of *C. muridarum* IFU recovered per mouse was 2,589,520 (range, 353,340 to 22,746,860). When male mice were euthanized at 22 PMD, the urethra and urinary bladder were cultured for *C. muridarum*. All urethrae (10/10) and 70% (7/10) of the urinary bladders were positive. All infected males developed high *C. muridarum*-specific IgG antibody titers in serum (geometric mean titer [GMT], 11,143; titer range, 6,400 to 12,800) (Table 2). None of the sham-infected males had positive urine cultures or *C. muridarum*-specific antibody titers.

Assessment of *C. muridarum* infection in female mice following horizontal sexual transmission. To study horizontal sexual transmission, 40 naive female mice were caged with *C. muridarum*-inoculated males (at a ratio of 4 females to 1 male). As controls, 20 naive female mice were housed with sham-infected males (at a ratio of 4 females to 1 male). To monitor infection, vaginal swab specimens were collected from 4 to 46 PMD. Over the observation period, 93% (37/40) of the female mice had at least one positive result by culture of a vaginal swab specimen (Fig. 1B). The median time of shedding for infected females was 42 PMD (range, 2 to 46 PMD), and the highest rate of vaginal swab specimen culture positivity (53%) was at 21 PMD. Of the *C. muridarum*-positive female mice, 27% (10/37) shed high numbers of *C. muridarum* IFU (≥1,000 total *C. muridarum* IFU shed in 6 weeks), while 73% (27/37) shed low numbers of IFU (<1,000 total *C. muridarum* IFU shed in 6 weeks). For the high-shedding group, at 6 weeks the cumulative median number of *C. muridarum* IFU per mouse was 3,289,572 (range, 4,252 to 16,685,047), while for the low-shedding group, the number was 24 (range, 2 to 134). None of the female controls caged with sham-infected male mice had positive culture results for *C. muridarum*.

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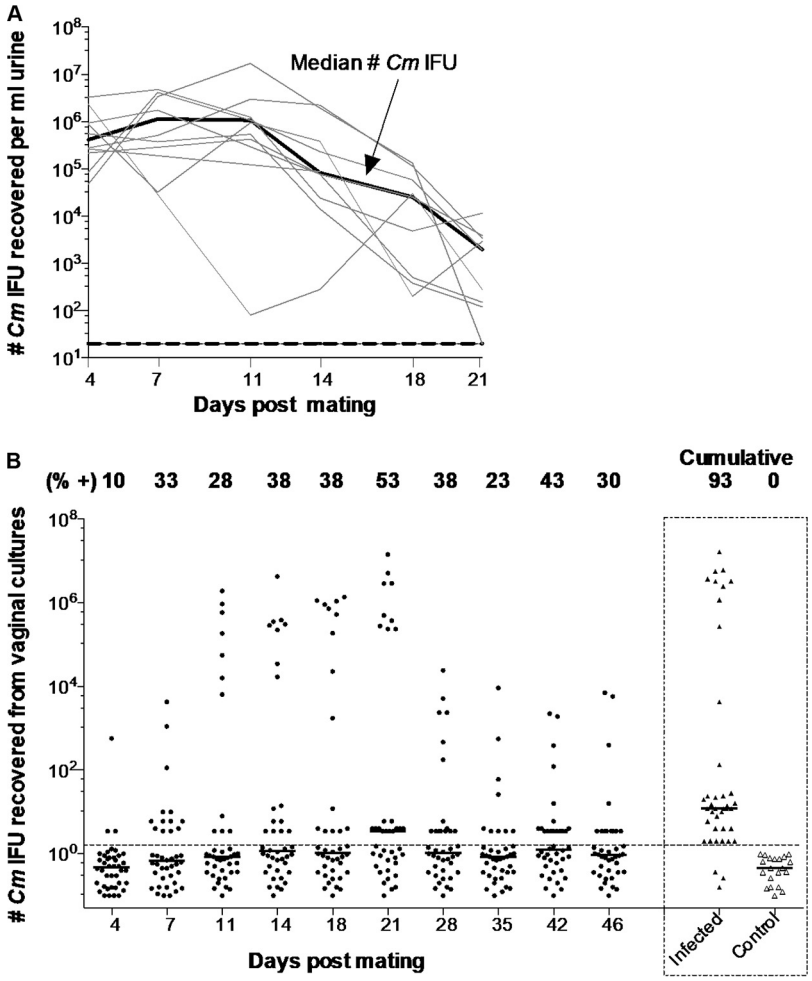

FIG 1 (A) Number of *C. muridarum* (*Cm*) IFU recovered from male mouse urine. Urine samples for culture were collected from male mice during the 3 weeks of mating. Each continuous thin line corresponds to a *C. muridarum*-inoculated male mouse, while the thick line represents the median number of IFU recovered. Dotted lines denote the limit of detection (LOD; <20 IFU/ml of urine). All sham-inoculated male mice were negative. (B) Number of *C. muridarum* IFU recovered from vaginal swab specimen cultures. Naive female mice were mated with *C. muridarum*-inoculated male mice, and vaginal swab specimens for culture were collected over a 6-week period. Each closed circle represents one female mouse. The numbers at the top indicate the percentages of mice with positive vaginal swab specimen cultures. The cumulative percentage of positive mice and the number of IFU recovered per mouse are denoted by closed triangles. All cultures of specimens from females mated with sham-infected males were negative (not shown). The dotted horizontal line represents the LOD (<2 IFU/culture).

Humoral and cellular immune responses in female mice following *C. muridarum* horizontal infection. At 65 PMD, female mice were euthanized and humoral and cell-mediated immune (CMI) responses were determined (Tables 2 and 3). All females housed with infected male mice developed high *C. muridarum* IgG antibody titers (GMT, 23,072; range, 3,200 to 102,400), while those caged with sham-infected males were negative for *C. muridarum* IgG. Also, in contrast to females mated with sham-infected male mice, sexually infected females developed high *C. muridarum*-specific T-cell lymphoproliferative responses (change in the counts per minute, 8,283 ± 2,783 versus 562 ± 240; $P < 0.05$). The levels of gamma interferon (IFN-γ) and interleukin-17 (IL-17) in the T-cell supernatants were also significantly higher in *C. muridarum*-infected females than in noninfected females (5,578 ± 148 versus <15 pg/ml, respectively, for IFN-γ and 195 ± 33 versus <4 pg/ml, respectively, for IL-17) ($P < 0.05$). None of the animals had detectable levels of IL-4 (<4 pg/ml).

Western blot analysis of control and *C. muridarum*-infected female, male, and newborn mice. Western blot analyses were performed with serum samples collected

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TABLE 1 Culture results after *C. muridarum* genital infection in male mice

Male mouse group	No. of mice urine positive/total no. of mice (% positive)	No. of urine cultures positive/total no. of cultures (%)	Results for organ cultures at 22 PMD[c]			
			Urethra		Urinary bladder	
			Median (range) no. of *C. muridarum* IFU	No. of organs positive/total no. (% positive)	Median (range) no. of *C. muridarum* IFU	No. of organs positive/total no. (% positive)
C. muridarum inoculated	10/10 (100)[a]	53/54 (98)[a]	520 (20–14,496)[b]	10/10 (100)[a]	25 (<2–6,292)[b]	7/10 (70)[a]
Sham inoculated	0/5 (0)	0/30 (0)	<2	0/5 (0)	<2	0/5 (0)

[a]$P < 0.05$ by Fisher's exact test compared to the sham-infected male mice.
[b]$P < 0.05$ by the Mann-Whitney U test compared to the sham-infected male mice.
[c]PMD, postmating days.

from *C. muridarum*-infected male, female, and newborn mice (Fig. 2). Sera from sham-infected mice were used as controls. Sera from infected male, female, and newborn mice had similar antibody responses, recognizing lipopolysaccharide (LPS; ~10 kDa), a 23-kDa band, the major outer membrane protein (MOMP; 42 kDa), the 60 kDa-cysteine-rich protein and/or the 60-kDa heat shock protein, the 70-kDa heat shock protein, and several high-molecular-mass proteins (100 to 150 kDa), likely corresponding to the polymorphic membrane proteins. As expected, sera from newborn mice reacted weakly, showing reactivity at the same bands as the mothers' sera. Samples from sham-infected animals did not react.

Fertility results. There were fewer fertile females among the females caged with *C. muridarum*-inoculated male mice (65%, 26/40) than among the females mated with sham-infected males (100%, 20/20) ($P < 0.05$) (Table 4). In addition, females mated with *C. muridarum*-infected male mice produced significantly fewer pups than control females mated with sham-infected males (3.8 ± 3.2/mouse versus 6.3 ± 1.6/mouse; $P < 0.05$). However, when comparing the number of pups born from pregnant females, no significant differences were observed between the two groups (5.6 ± 2.1 pups for the *C. muridarum*-infected group versus 6.3 ± 1.6 pups for the sham-infected group; $P > 0.05$). The median number of PMD when the pups were born was not different between *C. muridarum*-infected females (29 PMD; range, 20 to 44 PMD) and sham-inoculated females (26 PMD; range, 20 to 42 PMD) ($P > 0.05$), suggesting that *C. muridarum* infection did not interfere with mating.

Among the female mice shedding *C. muridarum* at high levels, 70% (7/10) were infertile and delivered 2.2 ± 3.6 pups/mouse, while in the group shedding *C. muridarum* at low levels, 26% (7/27) were infertile and delivered 4.1 ± 2.9 pups/mouse ($P < 0.05$) (Fig. 3A). In the group not shedding *C. muridarum*, all mice (3/3, 100%) were fertile and delivered 5.7 ± 1.2 pups/mouse, indicating that mating had occurred.

Vertical *C. muridarum* infection and humoral response of newborn mice. Of the female mice caged with *C. muridarum*-infected males, 65% (26/40) became pregnant, and of these, 88% (23/26) had positive vaginal swab specimen cultures. Sixty-five percent (17/26) of the culture-positive pregnant females transmitted *C. muridarum* to at least one of their pups, while 35% (9/26) did not (Fig. 3B).

When newborn mice were euthanized at 10 postnatal days (PND), 32% (46/144) of the pups born from *C. muridarum*-positive females had positive cultures of tissue specimens from the lungs and/or intestines, 13% (19/144) had positive lung tissue specimen cultures, and 24% (34/144) had positive intestinal tissue specimen cultures

TABLE 2 *C. muridarum*-specific antibody ELISA titers in male, female, and newborn mice

Mouse group	IgG GMT (range) to *C. muridarum* EBs		
	Males (22 PMD[a])	Females (65 PMD)	Newborns (10 PND[b])
C. muridarum inoculated	11,143 (6,400–12,800)	23,072 (3,200–102,400)	3,200 (1,600–6,400)
Sham inoculated	<100	<100	<100

[a]PMD, postmating days.
[b]PND, postnatal days.

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TABLE 3 *C. muridarum*-specific cell-mediated immune responses in sexually infected female mice at 56 PMD[b]

Male mated with female mice	LPA response to *C. muridarum* EBs (change in no. of counts per minute, mean ± 1 SE)	Mean cytokine response (concn [pg/ml], mean ± 1 SE) to *C. muridarum* EBs		
		IFN-γ	IL-17	IL-4
C. muridarum inoculated	8,283 ± 2,783[a]	5,578 ± 148[a]	195 ± 33[a]	<4
Sham inoculated	562 ± 240	<15	<4	<4

[a]$P < 0.05$ by Student's *t* test compared to female mice mated with sham-inoculated male mice.
[b]PMD, postmating days.

(Fig. 4). The median number of *C. muridarum* IFU was 90 (range, 20 to 91,000) from the lungs and 48,320 (range, 200 to 180,000,000) from the intestines. All pups born from infected dams had high anti-*C. muridarum* IgG titers in sera at 10 PND (GMT, 3,200; range, 1,600 to 6,400) (Table 2). All 129 pups born from sham-infected female mice were *C. muridarum* culture and *C. muridarum* antibody negative.

DISCUSSION

Here we describe a new mouse model of *C. muridarum* sexual transmission. With this model, it is possible to characterize horizontal sexual transmission from males to females and vertical transmission from females to their newborns. This mouse model may help to gain a better understanding of the immunopathogenesis of sexually transmitted chlamydial infections and to test preventative and therapeutic protocols to control this pathogen.

To characterize horizontal transmission from males to females, male C3H/HeN mice were inoculated in the meatus urethra with 10^6 *C. muridarum* IFU/mouse (20 times the 50% infective dose [ID_{50}]), a dose that should result in infection of all the urethras, 80% of the urinary bladders, ~60% of the epididymides, and ~20% of the testes (27). This

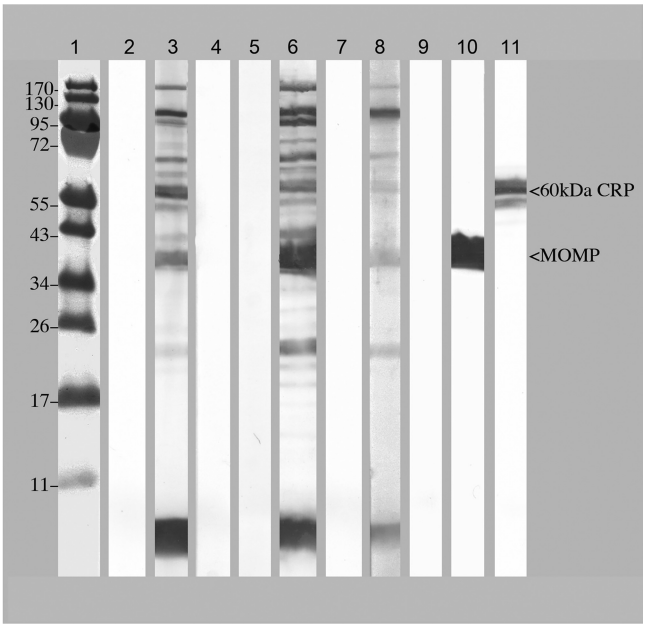

FIG 2 Western blot of serum samples from *C. muridarum*-infected and sham-infected male, female, and newborn mice. *C. muridarum* EBs were run on a 10% SDS-PAGE gel and blotted onto nitrocellulose paper. The EBs were probed with pooled sera collected from *C. muridarum*- and sham-infected animals. Lane 1, molecular weight standards. Sera from female mice: lane 2, preinfected; lane 3, at 65 postmating days with *C. muridarum*-infected male mice; lane 4, at 65 postmating with control sham-infected male mice. Sera from pups: lane 5, born from control sham-infected dams; lane 6, born from *C. muridarum*-infected dams. Sera from male mice: lane 7, preinfected; lane 8, *C. muridarum* infected at 22 days postmating; lane 9, control sham infected at 22 days postmating. Lanes 10 and 11, monoclonal antibodies to MOMP and the 60-kDa cysteine-rich protein of *C. muridarum*, respectively.

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TABLE 4 Fertility results

Male mated with female mice	Results for all female mice		Results for all pregnant mice	
	No. of pregnant mice/ total no. of mice (%)	Mean ± 1 SD no. of pups born/mouse	Median (range) PMD[c] when pups were born	Mean ± 1 SD no. of pups born/pregnant mouse
C. muridarum inoculated	26/40 (65)[a]	3.8 ± 3.2[b]	29 (20–44)	5.6 ± 2.1
Sham inoculated	20/20 (100)	6.3 ± 1.6	26 (20–42)	6.3 ± 1.6

[a]$P < 0.05$ by Fisher's exact test compared to the female mice mated with sham-inoculated male mice.
[b]$P < 0.05$ by Student's *t* test compared to the female mice mated with sham-inoculated male mice.
[c]PMD, postmating days.

dose therefore corresponds in humans to severe *C. trachomatis* infections in which males may present with epididymitis and/or orchitis (28, 29).

This mouse model bears similarities with horizontal human sexually transmitted *C. trachomatis* infections. Two concordant studies reported ~70 to 80% transmission rates in females from *C. trachomatis*-positive males (30, 31). For example, Quinn et al. (30) studied 494 sexual partner couples using urethral and endocervical swabs for the detection of *C. trachomatis*. In this study, 27 female partners of 47 infected males (57%) were culture positive. The transmission rate increased to 70% (53/76) when nucleic acid methods were used for diagnosis. In a study by Schillinger et al. (31), based on culture results, ~80% concordance between female and male sexual partners was found. Rank et al. (26) infected the meatus urethra of male guinea pigs with 10^7 IFU of *C. caviae* and caged them with naive females. In their study, 62% of the female guinea pigs shed *C. caviae*. In our study, we found that 93% of female mice housed with *C. muridarum*-inoculated males had at least one positive vaginal swab specimen culture over a 6-week period, and all of them had positive serological results. The higher transmission rate observed in this study could be the result of several factors, including the high level of susceptibility of C3H/HeN mice to *C. muridarum* infection, the high inoculum used to infect males, and/or the sexual behavior of male and female mice versus that of humans or guinea pigs.

In this model, sexually infected female mice transmitted *C. muridarum* to 65% of their newborns. The rate of vertical transmission of *C. trachomatis* to newborns varies depending on the perinatal care provided. For example, in sub-Saharan Africa and Asia, where women receive no or little perinatal care, it was reported that 33 to 60% of newborns from *C. trachomatis*-infected mothers were culture positive, a rate similar to that found in this study (12, 15, 32, 33). Schachter et al. (12) did a prospective study of

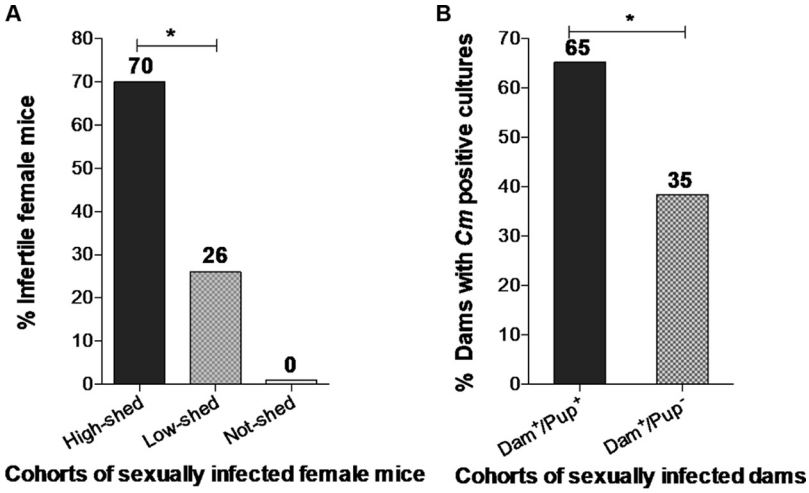

FIG 3 (A) Relationship between vaginal shedding and fertility outcome. The numbers on the bars indicate the percentages of mice that were infertile. *, $P < 0.05$. (B) Correlation between sexually *C. muridarum*-infected pregnant female mice and transmission of the infection to their pups. The numbers on the bars indicate the percentages of pregnant mice that were culture positive. *, $P < 0.05$.

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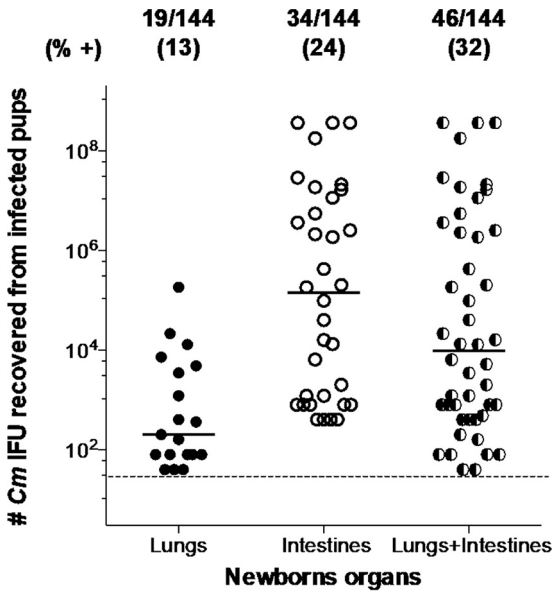

FIG 4 Percentage of newborn mice with positive organ specimen cultures born from *C. muridarum*-infected dams. Each circle represents a single mouse. The horizontal line indicates the median number of *C. muridarum* IFU recovered from the lungs and/or intestines.

perinatal transmission with a group of 5,531 pregnant women in San Francisco, CA, for a 5-year period. Of these patients, 262 (4.7%) had positive *C. trachomatis* cervical swab specimen cultures and 131 of their infants were followed. *C. trachomatis* was cultured from 36% (47/131) of the infants, and 60% (79/131) of the infants had a positive serology. Positive *C. trachomatis* cultures of swab specimens from the eyes, lungs, rectum, and vagina from the newborns were obtained. These vertical transmission rates are similar to those reported in this study. We did not collect ocular swab specimen cultures because *C. muridarum* does not effectively infect the eyes of mice (34).

Here we found that a primary *C. muridarum* infection resulted in decreased fertility. Different factors in males, females, and fetuses may have contributed to the decreased fertility. Acute *C. trachomatis* infection in men may cause epididymitis, which affects sperm production, functionality, and/or transport blockage, leading to a temporary decrease in fertility (32, 35, 36). *C. trachomatis* infections in pregnant females have been associated with low birth weight, premature birth, premature rupture of membranes, and an increased risk of fetal death (15, 32, 37, 38). *C. muridarum* can also produce fetal damage during pregnancy (35). Scarring of the fallopian tube can lead to ectopic pregnancy and infertility (25, 32, 39, 40). In this study, one *C. muridarum*-infected female delivered 5 pups at 20 PMD. Interestingly, at 65 PMD, bilateral hydrosalpinx formation was observed. Therefore, further studies are needed to delineate the exact causes and mechanisms of infertility in this mouse model of sexual transmission.

In most studies, to increase susceptibility to a *C. trachomatis* or a *C. muridarum* infection, female mice are pretreated with medroxyprogesterone (41). Progesterone treatment pauses the estrous cycle at diestrus and induces a shift toward Th2 immune responses (42–45). Since *C. trachomatis* and *C. muridarum* infections are mainly controlled by Th1 immune responses, switching to a Th2 response makes women and female mice more susceptible to infection and PID (41, 46, 47). The mouse model presented here does not require hormonal pretreatment to increase susceptibility to *C. muridarum* infection and therefore more closely parallels the situation in humans.

Recently, Zhang et al. (48) showed that vaginally inoculated mice disseminated *C. muridarum* into the gastrointestinal tract within a week of the infection and suggested that *Chlamydia* might be using the alimentary canal as a reservoir. Igietseme et al. (34) also reported that *C. muridarum* infection of several mucosal sites, including the genital tract, leads to colonization of the large intestine for at least 8 months. Since the goal of

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this study was to establish a vertical and horizontal sexual transmission model, we did not collect rectal swab specimens, and therefore, we cannot exclude the possibility that coprophagia or grooming resulted in infection of the gastrointestinal tract. However, in a group of sexually infected female mice, we cultured the stools at 65 PMD (data not shown). At that point, the stools of 92% (11/12) of sexually infected mice were *C. muridarum* positive, suggesting that *C. muridarum* had also disseminated to the gastrointestinal tract in this sexual infection model. Further studies are needed to confirm these results and determine the role of gastrointestinal colonization in the pathogenesis of chlamydial infections.

A potential limitation of this study is that outcome events were characterized only during a primary infection. A primary *C. trachomatis* infection in humans can lead to severe bilateral pyosalpinx with tubal dilatation and, therefore, long-term sequelae, including infertility (49). This is not surprising since, due to the lack of adaptive immunity, primary infections are usually more severe than reinfections (50). In addition, young females, the population more likely to have a primary infection, frequently have cervical ectopy, which makes them more susceptible to *C. trachomatis* infection (51, 52). Young female mice are also more susceptible to *C. muridarum* infection and infertility than older animals (53). Likewise, most cases of PID occur within 2 weeks of the diagnosis of an acute *C. trachomatis* infection (54). In the landmark study by Westrom et al. (9), the majority of the infertility cases (56%, 79/141) occurred in women following a single PID episode. Although there is a lack of long-term studies, this evidence suggests that most cases of infertility are due to a severe primary *C. trachomatis* infection. Furthermore, it is important to emphasize that only half of the *C. trachomatis* genital infections in women spontaneously resolve in ~1 year (55). Thus, as in the model described here, a woman can simultaneously become pregnant and infected with *C. trachomatis* and remain infected until delivery. Hence, this model may help characterize severe primary *C. trachomatis* infections during pregnancy.

Another limitation of this study is the use of a high *C. muridarum* dose to infect the male mice (27). In humans, only a small percentage of males infected with *C. trachomatis* have involvement of the upper genitourinary system (56). However, males with severe *C. trachomatis* infections likely cause severe infections and pathological damage to their sexual partners by transmitting a high bacterial load. Here, female mice with high levels of *C. muridarum* vaginal shedding had lower fertility rates and transmitted the infection to more newborns than female mice with low levels of shedding. Future studies using a low *C. muridarum* dose may help answer these questions.

In conclusion, we implemented the first mouse model of horizontal and vertical chlamydial sexual transmission. This mouse model could serve to characterize the immunopathogenesis of chlamydial infections in sexual partners and their offspring. In addition, it could be used to test therapeutic and preventative measures for females, males, and newborns.

MATERIALS AND METHODS

***C. muridarum* stocks.** *C. muridarum* strain NiggII (previously called *C. trachomatis* mouse pneumonitis biovar) was grown in HeLa-229 cells using Eagle's minimal essential medium supplemented with 5% fetal calf serum and cycloheximide (1 μg/ml) (57, 58). Elementary bodies (EBs) were purified as described previously and stored in sugar phosphate glutamate buffer (SPG) at −80°C (21, 58). The number of *C. muridarum* inclusion-forming units (IFU) was determined in HeLa-229 cells (59).

Sexual infection. Eight-week-old male and female C3H/HeN (H-2^k haplotype) mice were purchased from Charles River Laboratories. The mice were maintained in the vivarium at the University of California, Irvine (UCI), and all experiments were carried out in accordance with NIH and UCI IACUC guidelines. Male mice were infected in the meatus urethra with 10^6 *C. muridarum* inclusion-forming units (IFU; 20 times the ID_{50}) in 2 μl of SPG (27). Sham-infected mice received only SPG. At 1 day after infection, each male mouse was housed with four naive females for 3 weeks. The mice were housed in vented, filtered cages with 0.25-in.-thick corncob bedding (Envigo, Somerset, NJ). The beddings were changed every 2 weeks and also when performing spot checks, if necessary. Urine from male mice was collected twice a week for 3 weeks. Male mice were held over a clean plastic sheet (Parafilm M; Bemis Inc., Neenah, WI) and allowed to spontaneously urinate. The urine was collected with a sterile pipette and mixed with an equal volume of SPG. Urine samples were inoculated into HeLa-229 cell monolayers grown in 48-well plates. Male mice were euthanized at 3 weeks postmating, while female mice were euthanized at 9 weeks postmating. Newborns were euthanized at 10 PND (60). The experiment was repeated.

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Immunoassays. Blood was collected from mice on the day before infection and following euthanasia: serum from male mice was collected at 22 PMD, from female mice at 65 PMD, and from newborn mice at 10 PND. *C. muridarum*-specific antibody titers in serum were determined by an enzyme-linked immunosorbent assay (ELISA) as described previously (59). In brief, 96-well plates were coated with *C. muridarum* EBs, and serum was added to each well in 2-fold serial dilutions. After incubation, the serum was discarded, the wells were washed, and the plates were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG antibodies (Cappel antibodies; ICN). The binding was measured in an enzyme immunoassay reader (Labsystems Multiskan, Helsinki, Finland). Preinfection sera were used as negative controls. The geometric mean titers (GMT) were expressed as the reciprocal of the dilution (59).

Western blotting was performed with *C. muridarum* EBs as published previously (59, 61). In short, following transfer to a nitrocellulose membrane, the nonspecific binding sites were blocked. Serum samples at a 1:100 dilution were incubated overnight. The membrane was washed and incubated with horseradish peroxidase-conjugated goat anti-mouse Ig antibody, followed by visualization of the bands by development with hydrogen peroxide and 4-chloro-1-naphthol.

A T-cell lymphoproliferative assay (LPA) was performed as described previously (59). Briefly, spleens from mice were collected and enriched for T cells by passage over a nylon wool column. Enriched T cells (>95% pure) were counted, and 10^5 cells/well were aliquoted. Antigen-presenting cells (APC) were prepared by irradiating splenocytes with 3,300 rads. UV-inactivated *C. muridarum* EBs were added at a concentration of 5 EBs to 1 APC. Negative-control wells received medium alone, and concanavalin A (5 μg/ml) was used as a positive control. Cell proliferation was measured by addition of 1 μCi of [*methyl*-^{3}H]thymidine per well. The mean count was determined from triplicate cultures. The change in the number of counts per minute was calculated by subtracting the number of counts per minute obtained from T cells stimulated with medium from the number of counts per minute measured from T cells stimulated with *C. muridarum* EBs.

The levels of IFN-γ, IL-4, and IL-17 in supernatants from splenic T cells stimulated as described above were determined using commercial kits (BD Biosciences, San Diego, CA) (62).

Evaluation of *C. muridarum* infection. To monitor infection in male mice, urine was collected twice a week for 3 weeks (limit of detection [LOD], 20 *C. muridarum* IFU/ml of urine). At the end of mating, male mice were euthanized and the urethra and urinary bladder were cultured (LOD, 10 *C. muridarum* IFU/organ). To monitor sexual transmission to female mice, vaginal swab specimens were collected twice a week for 4 weeks and then once a week for three additional weeks (LOD, 2 *C. muridarum* IFU/swab). Newborn mice were nursed by their mothers for 10 days and euthanized, and their lungs and small and large intestines were collected, homogenized, and cultured (LOD, 20 *C. muridarum* IFU/organ) (63). Urine, vaginal swab specimens, and tissue homogenates were cultured in HeLa-229 cell monolayers, and IFU were stained with monoclonal antibody MoPn-40, which recognizes the major outer membrane protein of *C. muridarum* (62).

Statistical analyses. For statistical analyses, the Mann-Whitney U test was used to compare the number of *C. muridarum* IFU, Student's *t* test was used to compare the immune responses and the number of pups, and Fisher's exact test was used to analyze 2-by-2 contingency tables. A *P* value of <0.05 was considered significant.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants AI204286 (to S.P.) and AI092129 (to L.M.D.L.M.) from the National Institute of Allergy and Infectious Diseases.

We do not have any commercial or other association that might pose a conflict of interest (e.g., pharmaceutical stock ownership, consultancy, advisory board membership, relevant patents, or research funding).

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