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Experimental Genital Mycoplasmosis: Time of Infection Influences Pregnancy Outcome†

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Genital infection of rats with *Mycoplasma pulmonis* causes adverse pregnancy outcome and can result in in utero spread of infection to the fetus. The current study was designed to determine whether the stage of pregnancy when infection occurs influences pregnancy outcome. Rats were inoculated with 3×10^7 CFU of *M. pulmonis* at 10 days prior to breeding (−10) or at gestational day (gd) 11 or 14 and were necropsied at gd 11, 14, or 18 or within 24 h of parturition (term). Control rats received sterile broth. *M. pulmonis* was isolated from the placenta, amniotic fluid, or fetal tissues only from rats infected prior to breeding ($P < 0.001$). All infected rats had significantly more loss of pups than did control rats ($P < 0.006$), but rats infected prior to breeding or at the beginning of the third trimester (gd 14) were much more likely to have fetal losses. Rats infected in the early second trimester after implantation (gd 11) did not experience severe losses. Litter sizes, total litter weight, and individual pup weight from all infected rats, regardless of gestational stage when infected, were significantly smaller than those of control rats ($P < 0.001$). On the basis of the results of this study, we conclude that the time of infection plays a major role in determination of pregnancy outcome and spread of infection from the genital tract to the respiratory tract.

Mycoplasma pulmonis is a common pathogen of mice and rats in many conventionally maintained colonies (3–5, 14). In addition to its role in respiratory disease, *M. pulmonis* is responsible for genital infections and infertility (5, 7, 17, 18). It has been estimated that *M. pulmonis* infection decreases rat birth rate 50 to 100% (5, 17). We have demonstrated the adverse impact of genital infection on pregnancy outcome in experimentally infected Sprague-Dawley (SD) rats (17). Genital infection prior to breeding resulted in increased fetal resorptions and an increased number of dams with no liveborn pups (17). Furthermore, individual pup weight, litter size, and litter weight were also decreased in infected rats. In a second study, we demonstrated that *M. pulmonis* could invade the placenta, breach the placental barrier, and establish an amniotic fluid infection by gestational day (gd) 14 (18). *M. pulmonis* was isolated from the oropharynx as well as lungs of fetuses at gd 18, confirming in utero transmission (18). Histological evidence was compatible with an active infection characterized by placentitis, amnionitis, and occasional mild fetal bronchopneumonia (18).

M. pulmonis is an ideal candidate for a model of intrauterine infection. First, it is a naturally occurring disease. Second, the infection can be established by intravaginal inoculation, without requiring extensive manipulation of the animal. Third, the natural course of disease in the rat is similar to that predicted for human pathogens, i.e., an ascending infection that breaches the placental barrier and establishes as an amnionitis. Finally, a strong database exists for normal reproductive physiology and pregnancy maintenance in the rat.

Previous studies demonstrating the adverse effects of genital mycoplasmosis on pregnancy outcome were performed with SD rats infected prior to breeding (17, 18). The objective of the present study was to determine the effect of stage of gestation at the time of *M. pulmonis* infection on pregnancy outcome.

MATERIALS AND METHODS

Rats. Specific-pathogen-free (SPF) SD male and female rats were purchased from a commercial vendor (Harlan Sprague-Dawley, Inc., Indianapolis, Ind.). These rats were monitored and maintained free of the following pathogens: Sendai virus, H-1 virus, rat coronavirus, sialodacryoadenitis virus, reovirus type 3, Kilham rat virus, Hantaan virus, *M. pulmonis*, respiratory and enteric bacterial pathogens, endoparasites, and ectoparasites. Females were either nonpregnant breeding age animals when shipped or were shipped to arrive on gd 11 or 14. These rats were shipped in filter containers to protect their SPF status.

Husbandry. All rats were housed in Microisolator cages (Lab Products, Inc., Maywood, N.J.) to maintain the various infection groups in separate isolation. Rats received autoclaved food (Autoclavable Rodent Laboratory Chow 5010; Purina Mills, Richmond, Ind.) and water ad libitum. Hardwood chip bedding and cages also were autoclaved before use. The cages were opened, and rats were handled only with disinfected, gloved hands under a laminar flow hood. Rats were kept at a density of four rats or fewer per cage. Cages were changed twice per week. Approximately 3 days prior to parturition, pregnant dams were placed in individual cages.

Male rats. On the day of arrival, male SPF SD rats were anesthetized with methoxyflurane inhalant anesthesia for ear notching and blood collection. Males were housed with other males when not used for breeding. Male rats were considered infected with *M. pulmonis* after the first exposure to an infected female. Infected males were not used to breed uninfected control female rats and were not housed with any uninfected rat at any time.

Breeding and pregnancy detection. Five to 7 days prior to breeding, female rats were exposed to soiled bedding from the cages of uninfected male rats to synchronize estrous cycles. Vaginal cytology was examined every other day to detect onset of estrus. Females were determined to be ready for breeding when cornified vaginal epithelial cells were first noted. A harem breeding system was used, with two to three females at approximately the late proestrus or early estrus stage of the estrous cycle being housed with one male rat for 3 days, in which time the females would complete one entire estrous cycle. Monitoring of vaginal cytology gave good results for breeding the uninfected rats. However, vaginitis with large numbers of leukocytes in vaginal swabs of infected rats made the vaginal slides difficult to interpret. Therefore, the breeding protocol for infected rats was altered so that harems of two to three infected females were housed with a male rat continuously for 7 days. Within this period, each female was expected to go through two complete estrous cycles. Female rats were given two breeding sessions in which to become pregnant before being judged infertile. Approximately 11 to 14 days after removal of the male, abdominal palpation was performed on the female twice weekly to detect pregnancy. Rats for the 11-day-pregnant groups often were chosen on the basis of breeding records alone, since small litters were difficult to palpate at this stage. By gd 14, fetuses were palpable

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and mammary development had begun. Decisions to rebreed a female were generally made by day 14 after initial separation from the male. Pairings for repeat breedings were made at random.

Experimental infection of female rats. All procedures were performed under a laminar flow hood. Control and infected rats were processed on different days. A minimum of five rats per necropsy time point were used in the study. Rats were infected 10 days prior to breeding (−10) or at gd 11 or 14. Rats were anesthetized with 0.8 mg of ketamine (Bristol Laboratories, Syracuse, N.Y.) and 0.8 mg of xylazine (Rompun, Haver-Lockhart, Shawnee, Kans.) intraperitoneally to produce a 30- to 40-min anesthesia period. Females were infected by intravaginal inoculation with 3×10^7 CFU of *M. pulmonis* X1048 (17, 18) in 0.1 ml of Frey's broth. Control females were given an equal volume of sterile broth intravaginally. All females, including the control group, recovered from anesthesia with their pelvises elevated to facilitate establishment of infection in those receiving the *M. pulmonis* inoculum. Once righting reflexes were recovered, two or three rats of the same group were placed into a Microisolator cage with food and water available. No infected female rat was ever housed with an uninfected female.

Necropsy. A minimum of five rats per infection time (−10, day 11, or day 14) were necropsied at gd 11, 14, and 18 and within 24 h of parturition (term). Necropsy procedures were performed as previously described (17, 18). The trachea was excised, with care to prevent blood contamination of the lumen, from the larynx cranially to the tracheal bifurcation caudally. The lumen of the excised trachea was washed with 0.5 ml of sterile phosphate-buffered saline (PBS), which was then cultured as described below. The uterus of each rat, regardless of gestational stage, was removed aseptically. Samples obtained from the uterus and associated fetal units (placenta, intact amniotic sac, and fetus) are described in detail below. The uterus was opened and examined for evidence of fetal resorption. Resorbed fetuses were those that had obviously implanted in the uterine wall but had no remaining recognizable fetal structure compared with other fetal units at the same stage of gestation.

The genital tracts from 11-day-pregnant rats were removed and sagitally sectioned on the midline. One half of the tract was minced in 0.9 ml of Frey's broth and then diluted and plated as described below. Thus, this sample contained both uterine and fetal tissues. In term dams, the uterus was washed by injection of 0.5 ml of sterile PBS into the uterine lumen with a 1-ml syringe and 23-gauge needle and aspiration of the PBS back into the syringe. The uterine wash solution was then cultured as described below.

The umbilicus and placenta from a maximum of four fetuses at both gd 14 and 18 were minced in 0.9 ml of Frey's broth. Placental development was not sufficient in 11-day-pregnant rats to permit dissection and culture. Placentas were not available from term rats.

Amniotic fluid was collected for culture from all rats at gd 14 and 18. In 18- and 14-day-pregnant rats, a maximum of eight fetuses were chosen at random and dissected from the uterus, with the chorioamniotic membranes kept intact. Each fetus was placed in a separate sterile petri dish, and the chorioamniotic membranes were torn away from the fetus, allowing the amniotic fluid to collect in the petri dish. A sterile 1-ml syringe was used to aspirate the amniotic fluid, which was cultured as described below. Amniotic fluid samples could not be obtained for either gd 11 or term dams.

Embryos at gd 11 were too undeveloped to be handled as separate entities. A maximum of four fetuses at gd 14 were minced in 0.9 ml of Frey's broth. Because of their size, no further dissections of these 14-day fetuses were performed. Oropharyngeal swabs were obtained from a maximum of four fetuses at gd 18 and from neonatal pups (<24 h old). In addition, the lungs of both gd 18 fetuses and neonatal pups were excised and minced in 0.9 ml of Frey's broth. All samples were cultured as described below.

Mycoplasmal cultures. All samples were placed on ice and cultured within 10 min of collection. Unless otherwise noted, all samples were diluted 10-fold serially in Frey's broth to 10^{-5} to determine the number of color-changing units (CCU). Unless noted otherwise, 20 μl of the undiluted sample and of each dilution to 10^{-2} was plated in duplicate on Frey's agar to determine the number of CFU. Vaginal swabs were diluted serially 10-fold in Frey's broth to 10^{-5} and streaked on Frey's agar (positive or negative for growth). Tracheal lavage fluids were diluted serially 10-fold in Frey's broth to 10^{-5} and plated (positive or negative for growth).

Broths were incubated at 37°C in ambient air and checked daily for color change indicative of growth. Plates were incubated at 37°C in 5% CO_2 and checked on days 3, 5, and 7 for growth. *M. pulmonis* was identified on the basis of glucose fermentation and morphology. Selected cultures were confirmed by immunoblotting on nitrocellulose (NC).

Antiserum to *M. pulmonis*. Three New Zealand White rabbits were purchased for antibody production. On day 0, a preinoculation serum sample was drawn from the central artery of the ear of each rabbit. An inoculation of 1.15×10^8 CFU of *M. pulmonis*, grown in broth containing rabbit serum, was injected into the marginal ear vein. This inoculation was repeated on day 2. On day 7, 2.3×10^8 CFU of *M. pulmonis* was inoculated intravenously. Serum was drawn on days 14, 16, 21, 25, and 35. Because the anti-*M. pulmonis* titers as determined by enzyme-linked immunosorbent assay in two of three rabbits were under 1:1,000 on day 35, all rabbits were given a booster of 1.15×10^8 CFU of *M. pulmonis* on day 37. On day 42, the rabbits were exsanguinated via cardiac puncture after heavy sedation with an intramuscular injection of ketamine and xylazine. The final serum titer in all rabbits was ≥1:10,000. The sera were stored frozen at −20°C in a manual defrost freezer.

Immunoblotting. Identification of *M. pulmonis* isolates was made by an immunobinding assay. Selected primary or subcultured colonies were blotted onto NC from Frey's agar. The NC was handled with forceps for all steps. After removal from the agar, the NC was placed in Tris-buffered saline (TBS; 0.1 M Tris in 9% NaCl [pH 7.6]) for 5 min. The NC was blocked in 0.1 M Tris in 9% NaCl containing 10% horse serum and 0.2% Tween 20 for 30 min. After blocking, the NC was washed 5 min in TBS three times. Polyclonal rabbit serum diluted 1:1,000 in TBS was used as the antiserum. The NC was incubated in this antiserum for 30 min at room temperature. Three washings in TBS were repeated. The NC was then incubated at room temperature for 30 min in alkaline phosphatase-labeled goat anti-rabbit immunoglobulin G (IgG) antiserum diluted 1:1,000 in TBS. Ten microliters of BCIP (0.2 mg of 5-bromo-4-chloro-3-indolyl phosphate per ml in 100 μl of dimethyl sulfoxide) was added to 10 ml of nitro blue tetrazolium (NBT) solution containing 2 μg/ml in 100 μM Tris hydrochloride (pH 9.7) to make NBT/BCIP alkaline phosphatase substrate. The NC was incubated in the substrate until color developed (3 min or less).

Statistical analysis. Data were analyzed by analysis of variance. If the analysis of variance indicated significance, then Duncan's multiple means test was used to determine the significant differences among the means. Linear regression analysis was performed to determine the relationship between length of infection and pregnancy outcome, with the *F* test applied for determination of significance. Where appropriate, chi-square analysis was used for frequency comparisons. $P \leq 0.05$ was considered significant.

RESULTS

Isolation of *M. pulmonis*. Isolation of *M. pulmonis* from experimentally infected dams is shown in Table 1. No differences in numbers of mycoplasmas isolated at gestational times other than term were observed. At least 50% of the rats tested at each time point remained infected in the vagina. For rats necropsied at term, significantly lower ($P = 0.005$) numbers of *M. pulmonis* were recovered from the vaginas of rats infected after conception, even though rats infected during pregnancy did not appear to clear *M. pulmonis* from the lower genital tract (vagina) as effectively as rats infected prior to breeding (Table 1). The infection was cleared from 43% of the rats infected prior to breeding. Rats which were colonized in the uterus were also colonized in the vagina; however, some rats were colonized in the lower genital tract (vagina) only. A somewhat surprising finding was that rats infected during pregnancy, with the exception of rats infected at gd 14 who went to term, did not appear to show significant spread to the trachea (Table 1). However, in rats infected prior to breeding, spread to the trachea did occur to some extent at all times, with the most dramatic colonization of the lower respiratory tract occurring at term, where both the percentages of rats infected in the trachea and the numbers of *M. pulmonis* cells isolated were significantly higher than those in other groups ($P = 0.0001$). *M. pulmonis* was not isolated from any control rat at any time.

The isolation of *M. pulmonis* from fetuses and 24-h-old rat pups is shown in Tables 2 and 3. *M. pulmonis* was isolated from the placenta, amniotic fluid, or fetal tissues only from rats infected prior to breeding ($P < 0.001$). A limited number of rat pups were colonized in the oropharynx and lung (Table 3). Isolation rates from both the oropharynx and lung at gd 18 were higher for rats infected prior to breeding than at either gd 11 or 14 ($P < 0.001$). *M. pulmonis* was not isolated from any control fetuses or pups at any time.

Impact on pregnancy outcome. Time of infection influenced litter size, litter weight, and pup weight (Table 4). Litter size was determined as the number of term, liveborn pups or as the number of fetal implantation sites of normal size and appearance for gestational age. At gd 18, litter sizes of rats infected at −10 or gd 14 were significantly smaller than those of control rats or rats infected at gd 11 ($P < 0.004$). At term, litter sizes from all infected rats regardless of gestational stage when infected were significantly smaller than those of control rats ($P < 0.001$). Decreases in litter size were seen at gd 18 and at term

TABLE 1. Frequency and numbers of *M. pulmonis* organisms isolated from experimentally infected, full-term SD dams

Pregnancy date when infected (*n*)[a]	Date (gd) when necropsied[b]	Mean CCU ± SD (% positive)[c]		
		Vagina	Uterus[d]	Trachea
−10 (5)	11	4.0 ± 2.2 (80)	4.0 ± 2.0 (80)	1.4 ± 2.2 (40)
−10 (6)	14	2.0 ± 2.4 (50)	ND	0.3 ± 0.8 (17)
11 (5)	14	3.5 ± 2.4 (80)	ND	0 ± 0 (0)
−10 (5)	18	3.8 ± 2.2 (80)	ND	1.6 ± 0.5 (40)
11 (5)	18	3.8 ± 2.2 (80)	ND	0 ± 0 (0)
14 (6)	18	5.0 ± 0 (100)	ND	0 ± 0 (0)
−10 (6)	Term	1.2 ± 2.2* (57)	2.0 ± 2.4 (38)	3.8 ± 1.5* (100)
11 (5)	Term	4.6 ± 0.5† (100)	2.0 ± 1.2 (80)	0 ± 0† (0)
14 (8)	Term	4.1 ± 1.5† (100)	1.8 ± 1.8 (75)	0.25 ± 0.5† (25)

[a] Rats were infected intravaginally with 3×10^7 CFU of *M. pulmonis* 10 days prior to breeding (−10) or at gd 11 or 14 or were sham inoculated with sterile broth prior to breeding (control). *M. pulmonis* was not isolated from any site of any control rat (data not shown).
[b] Rats were necropsied at gd 11, 14, or 18 or within 24 h of parturition (term).
[c] The mean CCU is the mean of the logarithm of the last dilution which showed a color change indicative of growth. The highest possible CCU was 5.0. All groups with different superscripts are statistically different; groups with the same superscript are not statistically different. For vaginal CCU at term, $P = 0.005$; for tracheal CCU at term, $P = 0.0001$. No other differences were significant.
[d] Uterine lavage samples were not obtained (ND, not done) at gd 14 and 18 because of the size of the fetal units.

for rats infected prior to breeding or at gd 14 (Table 4). Decreases in litter size did not occur until term for rats infected at gd 11.

Total litter weight and individual pup weights were determined within 24 h of parturition (term). Weights of stillborn pups were excluded. Litter weights from all infected rats, regardless of infection date, were significantly lower than those of control rats (Table 4 [$P < 0.001$]). The most dramatic decrease in litter weight was seen in rats infected prior to breeding; this group had the smallest number of liveborn pups per litter. Individual pup weights for liveborn pups were determined within 24 h of parturition. Pup weights of all infected rats were significantly lower than those of controls ($P < 0.0001$), regardless of time of infection. Pup weights of rats infected at gd 14 were significantly lower than those infected at gd 11 or prior to breeding ($P < 0.03$). No other differences were noted.

The stage of pregnancy at which infection occurred had a significant impact on the number of viable pups (Fig. 1). Pup loss is defined as the total number of resorptions, stillborn pups, or in utero deaths. All infected rats had significantly more loss of pups than did control rats ($P < 0.006$). Among infected rats, the most severe loss of viable pups was seen in rats infected prior to breeding ($P < 0.02$). No stillbirths occurred in rats infected at gd 11. However, five of eight (62%) rats infected prior to breeding and four of eight (50%) rats infected at gd 14 had stillborn pups. In utero deaths with retained fetuses at parturition were observed in one of eight (12%) rats infected prior to breeding.

The majority of pup loss was attributable to resorptions (Fig. 2). At gd 11, one rat infected prior to breeding had two resorptions compared with control rats, which had no resorptions (data not shown in Fig. 2). By gd 14, 83% of rats infected prior to breeding and 40% of rats infected at gd 11 had one or more resorptions compared with control rats, which had no resorptions ($P = 0.01$) (Fig. 2A). At gd 18, 80% of rats infected prior to breeding, 40% of rats infected at gd 11, and 100% of rats infected at gd 14 had one or more resorptions compared with 20% of control rats ($P = 0.04$). At term, the percentage of infected rats with one or more resorptions was not statistically different among the infection times ($P = 0.5$); however, only 20% of control rats experienced resorptions. The greatest mean number of resorptions per rat occurred in rats infected prior to breeding (Fig. 2B), although only 62% of these rats had ≥1 resorption. Rats infected at gd 11 remained stable in both the mean number of resorptions and percentage of rats with resorptions throughout the pregnancy.

Impact of infection on fertility of females and on male rats used for breeding. Of 34 female rats infected prior to breeding, 10 were infertile. Severe pyometria was observed in 6 of these 10 rats; all but two of the infertile female rats were positive for *M. pulmonis* in the genital tract. Three of 11 male rats used to breed infected females also experienced reproductive problems and were judged infertile. All 11 male rats had clinical signs of respiratory disease as evidenced by rales; two of these had severe dyspnea. Three of 11 male rats had otitis media, as indicated by head tilt; one of these rats died spontaneously. The mean number of days from time of exposure to infected females and necropsy of males was 39 ± 19 days.

TABLE 2. Frequency of isolation of *M. pulmonis* at gd 14 and 18 from the placenta and amniotic fluid of SD rats experimentally infected prior to breeding or at different stages of pregnancy

Pregnancy date when infected[a]	Date (gd) when necropsied	No. of sites positive (%)/no. of sites cultured[b]		
		Placenta	Amniotic fluid	Fetus
−10[c]	14	8/24 (33)	16/40 (40)	8/26 (31)
11	14	0/20 (0)	0/40 (0)	0/20 (0)
−10[c]	18	10/16 (62)	15/32 (47)	9/17 (53)
11	18	0/20 (0)	0/40 (0)	0/20 (0)
14	18	0/20 (0)	0/40 (0)	0/20 (0)

[a] Rats were infected intravaginally with 3×10^7 CFU of *M. pulmonis* 10 days prior to breeding (−10) or at gd 11 or 14 or were sham inoculated with sterile broth prior to breeding (controls [not shown]). *M. pulmonis* was not isolated from any site of any control rat (data not shown). Rats were necropsied at gd 14 or 18.
[b] Results are expressed as the number of sites positive for *M. pulmonis* over the total number of sites cultured. At the gd 14 necropsy, fetuses were minced in their entirety and cultured. For gd 18 fetuses, the site sampled was the oropharynx.
[c] Isolation rates from all sites at both the gd 14 and 18 necropsies were higher for rats infected prior to breeding than at either gd 11 or 14 ($P < 0.001$ [chi-square analysis]).

TABLE 3. Isolation of *M. pulmonis* from gd 18 or term pups born to SD rats experimentally infected with *M. pulmonis* prior to breeding or at different stages of pregnancy compared with that from pups from uninfected control rats

Pregnancy date (gd) when infected[a]	Date (gd) when necropsied[b]	No. positive (%)/no. cultured[c]	
		Oropharynx	Respiratory tract
−10[d]	18	9/17 (53)	7/17 (41)
11	18	0/20 (0)	0/20 (0)
14	18	0/24 (0)	0/24 (0)
Control	18	0/20 (0)	0/20 (0)
−10	Term	2/10 (20)	2/10 (20)
11	Term	1/20 (5)	0/20 (0)
14	Term	0/24 (0)	1/24 (4)
Control	Term	0/20 (0)	0/20 (0)

[a] Rats were infected intravaginally with 3×10^7 CFU of *M. pulmonis* 10 days prior to breeding (−10) or at gd 11 or 14 (11 and 14, respectively) or were sham inoculated with sterile broth prior to breeding (control). *M. pulmonis* was not isolated from any site of any control rat (data not shown).

[b] All rats were necropsied at gd 18 or within 24 h of parturition (term).

[c] Results are expressed as the number of pups which were positive for *M. pulmonis* over the total number of pups cultured.

[d] Isolation rates from both sites at gd 18 were higher for rats infected prior to breeding than at either gd 11 or 14 ($P < 0.001$ [chi-square analysis]).

TABLE 4. Litter size, term litter weight, and individual term pup weights in SD rats experimentally infected with *M. pulmonis* prior to breeding or at different stages of pregnancy compared with those of uninfected control rats

Pregnancy date when infected (n)[a]	Length of gestation (days)	Litter		Pup wt (g)[d] ± SD (n)
		Size[b] ± SD	Wt (g)[c] ± SD	
−10 (5)	11	9.2 ± 6.0		
−10 (6)	14	12.3 ± 1.4		
−10 (5)	18	7.2 ± 1.5		
−10 (8)	Term	5.8 ± 5.3	37 ± 35	6.39 ± 1.16 (46)
11 (5)	14	13.2 ± 2.5†		
11 (5)	18	13.4 ± 0.9†		
11 (5)	Term	8.2 ± 2.6*	53 ± 14	6.43 ± 0.52 (41)
14 (5)	18	8.2 ± 1.0		
14 (8)	Term	9.8 ± 2.8	59 ± 14	6.09 ± 0.58 (78)
Control (5)	11	7.8 ± 6.5*		
Control (6)	14	13.3 ± 1.5†		
Control (5)	18	12.8 ± 1.5†		
Control (5)	Term	13.8 ± 1.9†	99 ± 14	7.19 ± 0.77 (68)

[a] Rats were infected intravaginally with 3×10^7 CFU of *M. pulmonis* 10 days prior to breeding (−10) or at gd 11 or 14 or were sham inoculated with sterile broth prior to breeding (control). Rats were necropsied at gd 11, 14, or 18 or within 24 h of parturition (term).

[b] Litter size was determined as the number of term, liveborn pups or as the number of fetal implantation sites of normal size and appearance for gestational age. At day 18, litter sizes of rats infected at −10 or gd 14 were significantly smaller than those of control rats or rats infected at gd 11 ($P < 0.004$). At term, litter sizes from all infected rats, regardless of gestational stage when infected, were significantly smaller than those of control rats ($P < 0.001$). For a group of rats infected at the same gestational stage and necropsied at different times, necropsy values with different superscripts are statistically different ($P < 0.003$ and 0.05 for gd 11 infected and control rats, respectively); groups with the same superscript are not statistically different.

[c] Total litter weight was determined within 24 h of parturition (term). Stillborn pups were not included. Litter weights from all infected rats, regardless of infection date, were significantly lower than those of control rats ($P < 0.001$). No other differences were noted.

[d] Individual pup weights were determined within 24 h of parturition (term). Stillborn pups were not included. Pup weights of all infected rats, regardless of time of infection, were significantly lower than those of controls ($P < 0.0001$). Pup weights of rats infected at gd 14 were significantly lower than those infected at gd 11 or prior to breeding ($P < 0.03$). No other differences were noted.

Relationship between length of infection and pregnancy outcome. In rats infected at −10, both the number of days required to breed the rat and the length of time from infection to necropsy were analyzed for impact on recovery of *M. pulmonis* and for impact on pregnancy outcome. No association was observed between the number of days required for successful breeding and isolation of *M. pulmonis* from the vagina, trachea, or uterus ($P = 0.62$, 0.90, and 0.77, respectively [data not shown]). The litter size decreased ($P = 0.01$, $R^2 = 0.25$) and the number of resorptions increased ($P = 0.02$, $R^2 = 0.25$) as the number of days required to breed an infected rat increased (data not shown).

The length of infection (total number of days infected) did influence isolation of *M. pulmonis* in the lower genital tract and trachea. Length of infection was inversely related to isolation of *M. pulmonis* from the vagina ($P = 0.006$, $R^2 = 0.158$ [data not shown]) and directly related to isolation of *M. pulmonis* from the trachea ($P = 0.0005$, $R^2 = 0.224$ [data not shown]). The length of infection for rats infected during pregnancy did not show an association with litter size (Fig. 3A [$P = 0.17$, $R^2 = 0.073$]) or resorptions (Fig. 3B [$P = 0.63$, $R^2 = 0.009$]). However, in rats infected at −10, there was a demonstrable, direct association between length of infection and both litter size and resorption (Fig. 4). Litter size was decreased as length of infection increased (Fig. 4A [$P = 0.01$, $R^2 = 0.27$]). As would be expected, fetal resorptions also increased with length of infection (Fig. 4B [$P = 0.01$, $R^2 = 0.27$]).

DISCUSSION

Like most mycoplasmas, *M. pulmonis* has a predilection for mucosal surfaces. In conventionally housed rats, prevalence of respiratory mycoplasmosis approaches 100% (2, 14), and the incidence of genital mycoplasmosis can be as high as 40% in some colonies (2, 5, 7, 15). In naturally infected rats, the respiratory tract appears to be the preferred site of colonization; thus, it was not surprising to see that infection spread from the site of inoculation (vagina) to the respiratory tract. However, spread to and colonization of the respiratory tract were influenced by the gestational stage when rats were infected. Rats exposed to *M. pulmonis* prior to breeding were most likely to become infected in the trachea, especially in rats which underwent parturition. Rats infected prior to breeding had a minimum of 21 days (10 days of infection plus 11 days of pregnancy if impregnated on day 1 of exposure to the male); term rats would have an additional 10 days minimum of infection. This time frame was more than adequate for the microorganism to spread to the respiratory tract. Time of infection alone could not explain the spread to the respiratory tract, since no rats infected at gd 11 had spread to the respiratory tract, whereas rats infected 3 days later at gd 14 did have colonization of the trachea.

The mechanisms for spread to the respiratory tract in rats infected prior to breeding and those infected during pregnancy could be different. Rats infected prior to pregnancy may have developed respiratory mycoplasmosis due to differences in exposure and behavior during breeding. Males used to breed vaginally infected females have been shown to rapidly develop signs of mycoplasmal respiratory disease (17). Intranasal inoculation probably occurred in the males as a result of increased grooming and exploring of the vulva of estrus females as well as postcopulatory grooming (11) after breeding with genitally

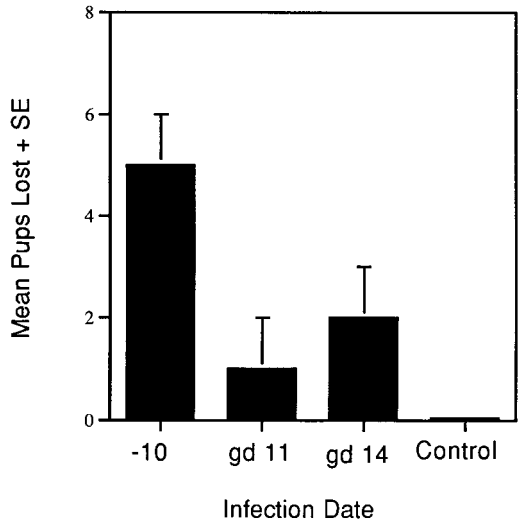

FIG. 1. Loss of viable term pups in SD rats experimentally infected with *M. pulmonis* prior to breeding or at different stages of pregnancy compared with that of pups from uninfected control rats. Pup loss is defined as the total number of resorptions, stillborn pups, or in utero deaths. Rats were infected intravaginally with 3×10^7 CFU of *M. pulmonis* 10 days prior to breeding (−10) or at gd 11 or 14 or sham inoculated with sterile broth prior to breeding (control). Rats were necropsied within 24 h of parturition. Values are expressed as the mean number of losses + standard error (SE). Infected rats had significantly more losses than did control rats ($P < 0.006$); within the infected rat groups, more severe loss was seen in rats infected prior to breeding ($P < 0.02$).

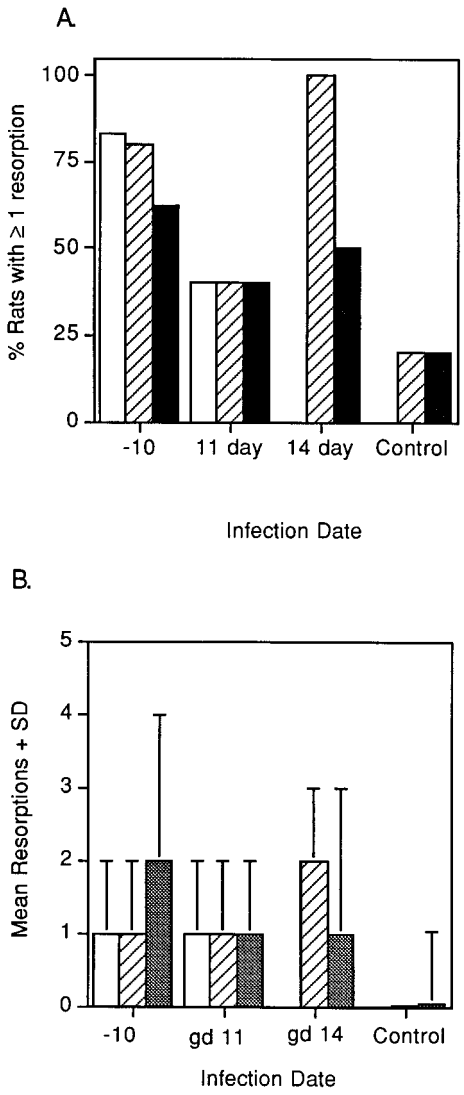

FIG. 2. Resorptions in SD rats experimentally infected with *M. pulmonis* prior to breeding or at different stages of pregnancy compared with number of resorptions from uninfected control rats. Rats were infected intravaginally with 3×10^7 CFU of *M. pulmonis* 10 days prior to breeding (−10) or at gd 11 or 14 or sham inoculated with sterile broth prior to breeding (control). Rats were necropsied at gd 11, 14 (open bars), or 18 (hatched bars) or within 24 h of parturition (term [shaded bars]). (A) Results expressed as the percentage of rats with at least one resorption. (B) Results expressed as the mean number of resorptions per rat + standard deviation (SD).

infected females. Other females may show similar attention and grooming behavior to estrus females. Once one rat per cage has developed respiratory mycoplasmosis, it can become a source of aerosolized *M. pulmonis*. This complex cycle of behavioral exposure might not be as active in cages of pregnant rats not undergoing the estrous cycle and breeding. Female rats infected during pregnancy were not exposed to male rats after infection.

The hormonal fluctuations of the reproductive cycle and gestation are known to influence the immune system and resistance to disease (16, 21). In rats and humans, progesterone, estradiol, and testosterone levels are much higher in pregnancy than at any other point in the reproductive cycle. Colonization of the genital tract also may be influenced by hormones. Conventional female rats who were androgenized at 3 days of age, resulting in a state of constant estrus at sexual maturity, showed evidence of oophoritis and metritis with *M. pulmonis* isolated from the genital tract at necropsy, while no lesions were observed in normal controls (13). Furr and Taylor-Robinson (10, 20) were able to increase the rate of infection and increase the number of cultivable microorganisms in mice by pretreatment with progesterone prior to experimental inoculation intravaginally with *M. pulmonis* and subsequent maintenance of increased levels of progesterone postinoculation. Even highly passaged *M. pulmonis* organisms (which had greatly decreased hemadsorptive properties and lower infectivity) were able to establish an infection in one progesterone-treated mouse. If hormone influence alone were responsible for observed differences in colonization, the rats in the later stages of pregnancy should be more susceptible. This was not the case, because spread to the respiratory tract did not occur except in term rats infected at gd 14, even though the numbers of microorganisms present in the vagina were similar among the different infection groups.

The last trimester of pregnancy, in particular, is associated with a depression in cell-mediated, but not humoral, immunity (21). Mitogenic response is decreased during pregnancy. Natural killer cell activity decreases as estrogen levels rise; therefore, this protective mechanism would be predicted to be at its lowest efficiency during pregnancy (6, 8, 19). Finally, local secretion of Igs is influenced by estrogen levels. Increased IgG and IgA can be found in the uteri of ovariectomized rats that receive physiologic doses of estrogen, while IgG and IgA in vaginal secretions concurrently decrease compared with those in untreated control rats (16). It is unclear that the depression in these immune functions is strictly due to hormonal influences. Cell-mediated immunity is important in protecting rats from mycoplasmosis, and a decrease in cell-mediated immunity during the last trimester of pregnancy could partially explain the respiratory colonization in gd 14 infected, term-nec-

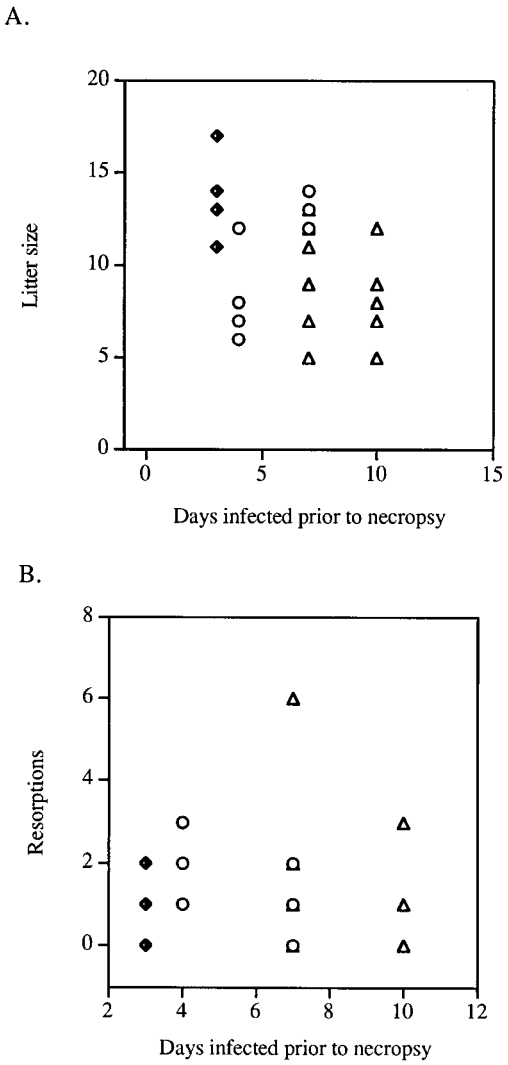

FIG. 3. Association of pregnancy outcome and length of infection in rats infected during gestation. Rats were infected intravaginally with 3×10^7 CFU of *M. pulmonis* at gd 11 or 14. Rats were necropsied at gd 14 ( ) or 18 (○) or within 24 h of parturition (term [△]). (A) Results expressed as the number of pups or fetuses of normal gestational size for each dam. If two or more dams had the same litter size, then the datum points are overlaid and are reflected as a single point. No significant association was found ($P = 0.17$, $R^2 = 0.073$). (B) Results expressed as the number of resorptions for each dam. If two or more dams had the same number of resorptions, then the datum points are overlaid and are reflected as a single point. No significant association was found ($P = 0.63$, $R^2 = 0.009$).

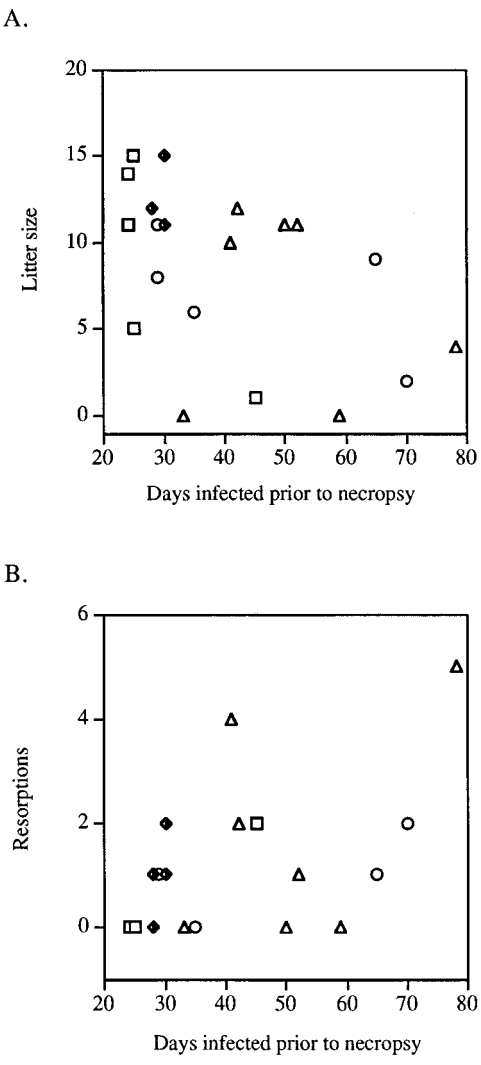

FIG. 4. Association of pregnancy outcome and length of infection in rats infected prior to breeding. Rats were infected intravaginally with 3×10^7 CFU of *M. pulmonis* 10 days prior to breeding (−10). Rats were necropsied at gd 11 (□), 14 ( ), or 18 (○) or within 24 h of parturition (term [△]). (A) Results expressed as the number of pups or fetuses of normal gestational size for each dam. If two or more dams had the same litter size, then the datum points are overlaid and are reflected as a single point. An inverse association was found ($P = 0.01$, $R^2 = 0.27$). (B) Results are expressed as the number of resorptions for each dam. If two or more dams had the same number of resorptions, then the datum points are overlaid and are reflected as a single point. A direct association was found ($P = 0.01$, $R^2 = 0.27$).

ropsied rats. Rats infected at gd 14 may have been immunosuppressed during that stage of pregnancy and therefore were more susceptible to spread of infection than were rats infected at gd 11.

The most severe effects were seen when infection occurred prior to breeding. Most of the fetal losses did not occur until gd 14, even in those animals infected prior to breeding. This implies that the events surrounding implantation may not have been affected. Deleterious effects cannot be related to length of infection alone, because rats infected at gd 14 had more severe disease expression than those infected at gd 11. At gd 14, the fetus is undergoing a period of rapid development (1). The placenta is rapidly developing more intimate contact between the maternal and fetal circulations to support this growth (9). Development of the metrial gland and maximal numbers of pregnancy-associated granulated metrial gland cells also occur around gd 14 (6, 8, 12, 19).

Dams infected at gd 11 had no dramatic results to distinguish them, except for decreased litter size, mean litter weight, and mean pup weight at term. Litter sizes in the groups infected at gd 11 and necropsied at gd 14 and 18 were very similar to those of the uninfected control groups necropsied at these time points. No rat infected at gd 11 had respiratory mycoplasmosis, despite those rats that went to term being infected 10 days prior to necropsy. The failure to cause adverse effects was not a function of inability to colonize the genital tract, because four of five rats necropsied at gd 14 and 18 and all rats necropsied at term had positive vaginal mycoplasma

cultures. If immunosuppression is important in susceptibility, then rats first infected at gd 11 might not be at the stage of gestation when the immune system is maximally suppressed and therefore had a protective response to *M. pulmonis* which minimized spread of the microorganism. The additional influence of hormone levels at different stages of pregnancy might also play a role in colonization.

The most devastating effects of *M. pulmonis* infection on outcome of pregnancy were seen in the rats infected before becoming pregnant. Significant fetal losses were found in dams at term. Litter size, litter weight, and pup weight also were low for this group. Not surprisingly, those detrimental effects were more evident in the rats that did not clear the genital tract infection. We have previously demonstrated in utero transmission of *M. pulmonis* in rats infected prior to breeding (18). In the current study, pups or fetuses from rats infected prior to breeding were culturally positive for *M. pulmonis* at gd 14, 18, and at term. Spread to the fetus was not associated with infections which occurred after implantation, except for in one rat who was infected at gd 14. One pup from this litter had a positive respiratory tract culture, even though the dam was culture negative in the genital tract, suggesting the hematogenous spread of *M. pulmonis* to this pup. Alternatively, the fetus could have been infected by an ascending route prior to clearance of *M. pulmonis* from the lower genital tract.

On the basis of the results of this study, we conclude that the time of infection plays a major role in determination of pregnancy outcome and spread of infection from the genital tract to the respiratory tract. Rats infected prior to breeding or at the beginning of the third trimester (gd 14) were much more likely to experience adverse outcomes, whereas rats infected in the early second trimester after implantation (gd 11) did not experience severe losses. The mechanisms involved in fetal loss (resorptions, stillbirths, and in utero deaths) and failure of the fetus to thrive (low birth weight) may be different, because all infected rats, regardless of time of infection, had decreased pup weights. Among the potential questions which need to be considered to explain these observations are the role of immunomodulation and/or immunosuppression, effects of pregnancy-related hormones, mechanisms of placental invasion, and mechanisms of fetal growth retardation.

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