In Utero Transmission of *Mycoplasma pulmonis* in Experimentally Infected Sprague-Dawley Rats†

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Genital mycoplasmal pneumonia is important as an animal model for the interaction between infectious agents and the host during pregnancy as well as in its own right as a confounding variable affecting research projects in which the rat is used as a model to study reproductive function and physiology. We report the in utero transmission of *Mycoplasma pulmonis* and the development of placental, amnionitis, and mild fetal bronchopneumonia in Sprague-Dawley rats. A minimum of 10 days prior to breeding, specific-pathogen-free female Sprague-Dawley rats were infected by intravaginal inoculation with $3 \times 10^7$ CFU of *M. pulmonis* X1048 or with an equal volume of sterile broth. Rats and fetuses were subjected to necropsy at days 11, 14, and 18 of gestation. *M. pulmonis* was able to invade the placenta, cross the placental barrier, and establish an amniotic fluid infection by gestational day 14. It was isolated from the oropharynx and lungs of fetuses at gestational day 18. The placenta was more frequently colonized than amniotic fluid, followed by the fetal oropharynx and lungs, supporting an ascending route of infection. Histopathological evidence also support an active infection, with lesions compatible with placitis, amnionitis, and mild fetal bronchopneumonia. *M. pulmonis* can traverse the placenta, resulting in infection of the amniotic fluid and in utero transmission of the microorganism to the developing fetus.

The adverse effects of murine respiratory mycoplasmal pneumonia on biomedical research with rat and mouse models are well documented (1, 2, 6, 7). However, little effort has been spent in understanding the genital form of disease. Genital infection with *Mycoplasma pulmonis* is estimated to decrease the birth rate in rats by 50 to 100%. A natural *M. pulmonis* infection in a colony of cesarean-derived, barrier-maintained LEW/Tru rats was described, in which low levels of infection kept clinical signs of disease masked for months despite positive serologic results (8). When signs of disease did become evident, not only was respiratory mycoplasmal pneumonia seen but also decreased fertility was observed in the breeding females, 80% of which had *M. pulmonis* isolated from the genital tract. This affects research considerably, not only directly in studies of reproductive biology in which rats are used but also on any study involving rats, if only because of less economical production of this animal resource.

We have demonstrated devastating effects of *M. pulmonis* infection on the outcome of pregnancy in experimentally infected rats (14). Significant resorptions were observed in dams at term and during gestation. Litter size, litter weight, and pup weight also were decreased. Thus the genital disease can confound studies which depend on measurement of the very parameters (pup weight, litter size, resorptions) that are likely to be affected by genital mycoplasmal infection.

Recognition of the critical importance of *M. pulmonis*-free rodents to biomedical research has led to the production of cesarean-derived, barrier-maintained colonies which are regularly screened for *M. pulmonis*. Even these efforts have failed to completely eliminate *M. pulmonis* from many of these colonies (8, 11). In conventionally housed rats, the incidence of genital mycoplasmal infection is as high as 40% in some colonies (3, 7, 11); respiratory mycoplasmal infection in conventional colonies approaches 100% (11). The incidence of respiratory mycoplasmal infection in barrier-maintained facilities is much lower but is still a significant problem (4, 11).

Transmission of *M. pulmonis* in utero could be one explanation for the failure of cesarean derivation to completely eliminate this pathogen. There are at least two potential routes of in utero transmission: first, transmission can occur as a result of preexisting uterine infection at the time of implantation, and, second, the microorganism could breach the placental barrier and infect the amniotic fluid and fetuses after implantation. Pathogens that can invade the placenta cannot be eliminated from animal colonies by cesarean derivation; this has a severe impact on management of the disease. *M. pulmonis* has previously been isolated from amniotic fluid of a pregnant rat (12), suggesting that in utero transmission does occur.

The objective of the present study was to determine whether in utero transmission of *M. pulmonis* occurred in experimentally infected Sprague-Dawley rats.

**MATERIALS AND METHODS**

*Rats.* Specific-pathogen-free Sprague-Dawley male and female rats were purchased from a commercial vendor (Harlan Sprague Dawley, Inc., Indianapolis, Ind.). The 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. The rats were shipped in filter containers and transferred under laminar-flow conditions to protect their specific-pathogen-free status.

**Husbandry.** All rats were housed in Microisolator (Lab Products, Inc., Maywood, N.J.) cages and handled only under a laminar-flow hood with disinfected, gloved hands. They received autoclaved food (Autoclavable Rodent Lab-
oratory Chow 5010; Purina Mills, Richmond, Ind.) and water ad libitum. They were kept at a density of four rats or fewer per cage. Cages were changed twice per week.

**Breeding and pregnancy detection.** At 5 to 7 days after infection or sham inoculation, female rats to be bred were exposed to soiled bedding from cages housing male rats to synchronize estrous cycles (bedding from infected males was not used in the cages of control females). Vaginal cytologic status was examined every other day to detect onset of estrus. At approximately the late proestrus or early estrus stage of the estrous cycle, two or three females were housed with one male rat for 3 days, in which time the females would complete one entire estrous cycle. Vaginitis with resultant 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 to be 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 14 days of gestation, fetuses were palpable and mammary development had begun, making pregnancy diagnosis easier. Decisions to rebreed a female generally were made by day 14 after initial separation from the male. Pairings for repeat breedings were made at random.

Male rats were processed on the day of arrival. Processing included the use of methoxyflurane inhalant anesthesia to facilitate ear notching for individual identification and blood collection. Males were housed with other males when not used for breeding. A male rat was considered infected with *M. pulmonis* after his first exposure to an infected female. Infected males were not used to breed noninfected control female rats and were not housed with any noninfected rat at any time.

**Experimental infection.** All procedures were performed under a laminar-flow hood. Experimentally infected rats were anesthetized on the day of arrival with 8.0 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 period of anesthesia. Females were infected by intravaginal inoculation with $3 \times 10^{5}$ CFU of *M. pulmonis* X1048 (a gift from M. K. Davidson and J. K. Davis, University of Alabama at Birmingham) in 0.1 ml of Frey's broth and were ear notched with a unique number. Noninfected control females were anesthetized with ketamine and xylazine as described above and given an equal volume of sterile Frey's broth intravaginally. These rats also were ear notched for identification. Each group was processed separately. All rats 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 noninfected female. A minimum of five rats per necropsy time point were used in the study.

**Necropsy.** Five experimentally infected and five noninfected control female rats per time point were subjected to necropsy at days 11, 14, and 18 of gestation. Necropsy procedures were performed as previously described (5). The rats were anesthetized deepy with sodium pentobarbital injected intraperitoneally and exsanguinated by transection of the femoral artery and vein. Fetal rats died in utero on the death of their dams. Vaginal swabs were obtained on all dams, regardless of the stage of gestation, after administration of anesthesia but before exsanguination. After the dam was exsanguinated, the ventrum was swabbed with 70% ethanol. A ventral midline skin incision was made that extended from the pubis to the rami of the mandibles. The abdominal muscles were incised on the linear alba beginning at the pubis and extending cranially to the xiphoid process. The incision was extended through the costochondral cartilages unilaterally and ended at the thoracic inlet. The ventral cervical musculature was bluntly dissected to reveal the trachea. Tracheal lavages were obtained for all dams, regardless of the stage of gestation. The trachea was excised, with care taken 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 0.9 ml of sterile phosphate-buffered saline, and the washings were cultured as described below. The uterus was exposed from the abdominal cavity.

The genital tracts of 11-day-pregnant rats were removed and sagittally sectioned on the midline. Half of the tract was cultured with fetal implantations intact, and the other half was preserved for histopathologic testing. Gross lesions of the genital tract, including resorbed fetuses, were noted. Embryos at this stage were too undeveloped to be handled as separate entities.

In 14-day-pregnant rats, up to eight fetuses were chosen at random and dissected from the uterus, keeping the chorioamniotic membranes intact. Each fetus was placed in a separate sterile petri dish, and the amniotic fluid was collected aseptically. The amniotic fluid from all fetuses (maximum of eight) was cultured. The corresponding placenta from these fetuses were either cultured (maximum of four) or taken for histopathologic testing (maximum of four). Individual fetuses were too small for dissection; therefore, the entire fetus (maximum of four) was minced for culture.

Amniotic fluid and placental samples from day 18 fetuses were obtained as described for the day 14 fetuses. In addition, oropharyngeal swabs were obtained from up to four fetuses and cultured for *M. pulmonis*. Lungs from these four fetuses were removed and cultured for mycoplasmas. Fetal lungs from the remaining four fetuses were not cultured but were processed instead for histopathologic testing.

**Mycoplasmal cultures.** All samples collected from all rats and fetuses were cultured within 10 min of collection. Vaginal and oropharyngeal swabs were placed in 0.9 ml of Frey's broth and serially diluted 10-fold to a dilution of $10^{-5}$ in Frey's broth. Tracheal and uterine lavage samples (0.1 ml) from each rat were serially diluted 10-fold to a dilution of $10^{-5}$. Uterine tissue (11-day gestation), placental tissue (14- and 18-day gestation), fetal lung tissue (18-day gestation), and tracheal tissue (18-day gestation) were minced in 0.9 ml of Frey's broth. Tissue to be minced was placed in a sterile petri dish containing 0.9 ml of Frey's broth. Two sterile scalpel blades were used to finely chop the tissue and mix the particles with the broth. The broth containing the minced tissue was transferred via a pipette to a sterile tube. The minced samples were serially diluted 10-fold to a dilution of $10^{-5}$ in Frey's broth. Agar plates were streaked with each undiluted swab, lavage, and tissue homogenate for qualitative isolation. For all tissues except the vaginal swab and dam tracheal lavage, 0.025 ml of each dilution was plated in
duplicate to determine the CFU. Plates were incubated at 37°C under a 5% CO₂ atmosphere and checked on days 3, 5, and 7 for growth. After 1 week, the colonies were counted and the CFU was determined. Broth cultures were incubated at 37°C in ambient air and checked daily for color change, indicative of growth. The reciprocal of the last dilution to show growth was deemed the color-changing unit. When a color change occurred, the broth was subcultured to agar.

*M. pulmonis* was identified as previously described (3).

**Histologic testing.** All tissues were fixed in 10% neutral buffered formalin. Tissues were paraffin embedded, sectioned, stained with hematoxylin and eosin, and examined for microscopic evidence of lesions. Uteri from rats at day 11 of gestation were sectioned sagittally through the implantation sites. Fetuses at day 14 of gestation and placental tissue were sectioned sagittally for examination. The 18-day fetuses with individual placentas were fixed in 10% neutral buffered formalin. After fixation, the trachea, lungs, and stomach were dissected from 18-day fetuses, sectioned sagittally embedded in paraffin, and sectioned onto a glass slide.

**Statistical analysis.** Statistical differences in numbers of microorganisms isolated from different sites were analyzed by analysis of variance if more than two sites were involved or by Student’s *t* test if only two sites were involved (11-day-pregnant group). If the *P* value for analysis of variance indicated a significant difference, multiple mean comparisons were made by Fischer’s protected least significant difference (PLSD) method. For comparisons involving nominal data (positive versus negative isolation of *M. pulmonis* from a given site), χ² analysis was used to determine significance. A probability (*P*) of ≤0.05 was considered significant.

**RESULTS**

*M. pulmonis* was not isolated from any site of any control rat at any time point. *M. pulmonis* was able to invade the placenta, cross the placental barrier, and establish an amniotic fluid infection by gestational day 14 (Table 1). Fetal tissues were infected as well, indicating in utero transmission of *M. pulmonis*. Not surprisingly, in dams which had cleared *M. pulmonis* from the lower genital tract, no bacteria were isolated from tissues in the upper genital tract. The number of dams which were infected with *M. pulmonis* in placentas, amniotic fluids, and fetal tissues was not statistically different (χ² analysis) at 14 and 18 days of gestation (Table 1).

The actual numbers of positive sites and CFU of *M. pulmonis* isolated from each site are shown in Table 2. No significant differences in numbers of *M. pulmonis* cells isolated from the placenta or amniotic fluid were noted between gestational days 14 and 18. At gestational day 14, three rats were positive for *M. pulmonis* in the vagina (Table 1). All isolations of *M. pulmonis* from day 14 fetal units (placenta, amniotic fluid, and fetus) (isolation data shown in Table 2) were obtained from two of these three rats. Each of these two rats had *M. pulmonis* isolated from all amniotic fluids (eight per rat), placental tissues (four per rat), and fetal tissues (four per rat) sampled (Table 2). The remaining rat which was colonized in the lower genital tract did not appear to be infected in the upper genital tract. No isolations were obtained in the upper genital tract from any day 14 rats which had cleared *M. pulmonis* from the vagina. There were no differences in the numbers of mycoplasmas isolated from any site at gestational day 14.

*M. pulmonis* was recovered at gestational day 18 in larger numbers from (in descending order) the placenta, amniotic fluid, fetal oropharynx, and fetal lungs (Table 2). A statistically significant difference was seen between numbers of mycoplasmas recovered from the sites at gestational day 18 (*P* < 0.007). Larger numbers of mycoplasmas were isolated from the placenta than from either the fetal oropharynx or fetal lungs. Larger numbers were isolated from amniotic fluid than from fetal lungs. No other differences were significant. Four rats were infected in the vagina; all isolations of *M. pulmonis* shown in Table 2 were from these rats.

At gestational day 18, only one rat had cleared the infection from the vagina (Table 1); this rat was infected in the trachea (data not shown). As expected, this rat was negative for *M. pulmonis* at the other genital sites tested. The isolation of *M. pulmonis* from different sites of the four gestational-day-18 rats which were colonized in the vagina by *M. pulmonis* is shown in Fig. 1. *M. pulmonis* was recovered from at least one placenta from all four rats. *M. pulmonis* was isolated from only one of four placentas cultured from one rat (Fig. 1, rat A). No other isolations from other upper genital tract sites were obtained in this rat, suggesting that the spread of infection was minimal. In the other rats more extensive involvement was seen. All placentas from all remaining rats (Fig. 1, rats B to D) were colonized by *M. pulmonis*. The results for rat B demonstrate

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**TABLE 1. Isolation of *M. pulmonis* from experimentally infected Sprague-Dawley dams at different stages of gestation**

<table>
<thead>
<tr>
<th>Site</th>
<th>Isolation* at gestational day:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>11 (Isolation %)</td>
</tr>
<tr>
<td>Vagina</td>
<td>4/5 (80%)</td>
</tr>
<tr>
<td>Uterus</td>
<td>4/5 (80%)</td>
</tr>
<tr>
<td>Placenta</td>
<td>ND</td>
</tr>
<tr>
<td>Amniotic fluid</td>
<td>ND</td>
</tr>
<tr>
<td>Fetus</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Results are expressed as the number of dams with at least one positive isolation of *M. pulmonis* from a given site/total number of dams; the percentages are given in parentheses.
* ND, not determined.
* Isolations of amniotic fluid, and fetal tissue could not be cultured on gestational day 11 because of lack of development at that stage.

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**TABLE 2. Isolation of *M. pulmonis* from the placenta, amniotic fluid, and fetus of experimentally infected Sprague-Dawley rats**

<table>
<thead>
<tr>
<th>Gestation date</th>
<th>Site</th>
<th>No. positive/no. tested (%)</th>
<th>No. of CFU (mean ± SD)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>14 days</td>
<td>Placenta</td>
<td>8/20 (40)</td>
<td>5.9 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>Amniotic fluid</td>
<td>16/40 (40)</td>
<td>6.1 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>Fetus</td>
<td>8/20 (40)</td>
<td>5.4 ± 1.3</td>
</tr>
<tr>
<td>18 days</td>
<td>Placenta</td>
<td>10/17 (59)</td>
<td>6.0 ± 1.8</td>
</tr>
<tr>
<td></td>
<td>Amniotic fluid</td>
<td>15/32 (47)</td>
<td>5.8 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>Fetal oropharynx</td>
<td>9/17 (53)</td>
<td>3.6 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>Fetal lung</td>
<td>7/17 (41)</td>
<td>2.9 ± 0.7</td>
</tr>
</tbody>
</table>

* No significant differences were noted among numbers of *M. pulmonis* cells isolated from different sites at gestational day 14. At gestational day 18, numbers of *M. pulmonis* cells isolated from fetal lungs were significantly smaller (*P* < 0.007) than those isolated from amniotic fluid or placenta. All isolations were obtained from rats which were positive for *M. pulmonis* in the lower genital tract (Table 1).
* Fetal size at 14 days was too small to allow dissection; therefore, the entire fetus was minced for culture.
* Fetuses collected for histopathologic testing had amniotic fluid cultured.

No other tissues from these fetuses were cultured.

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the ascending route of infection. Five of six amniotic fluid samples tested for this rat were positive for *M. pulmonis*. Unfortunately, the one negative amniotic fluid sample was part of a placental unit which was chosen for histopathologic testing and was not available for culture. Because fetal tissues and placentas chosen for histopathologic testing were kept as a unit, the fetal tissues were unavailable for culture as well. Of the four fetuses chosen for culture from rat B, all were colonized in the oropharynx but only two were colonized in the lungs. Two rats (rats C and D) had *M. pulmonis* isolated from all sites tested. In no instance was *M. pulmonis* isolated from the fetal lungs without also being isolated from the oropharynx, amniotic fluid, and placenta. Similarly, *M. pulmonis* was not isolated from the amniotic fluid without also being isolated from the placenta. In some cases, the placenta was used for histopathologic testing and was not available for culture.

Tissue from control rats had no evidence of inflammation (Fig. 2). The lesions in the infected rats at 11 days of gestation consisted of a moderate to severe multifocal necrotis and placentitis. An inflammatory infiltrate, consisting predominantly of polymorphonuclear neutrophils (PMN) with fewer macrophages and lymphocytes, was present within the uterine lumen and was infiltrating the endometrium (Fig. 3). Scattered glandular crypts were dilated and contained PMN and necrotic debris. The decidual and basal layers of the maternal placenta also contained accumulations of PMN; however, the fetal membranes and the developing embryos appeared within normal limits (Fig. 4).

At 14 days of gestation, a suppurrative inflammatory infiltrate with scattered necrotic foci was present in the decidual layer of the placenta, and the amniotic membranes contained PMN (results not shown).

Normal placental degeneration was observed in the control animals from gestational day 18 (9). These degenerative changes consisted of small scattered necrotic cells and occasional PMN. The suppurrative placentitis characteristic of the infected rats from gestational days 11 and 14 was not present in rats from gestational day 18 with the exception of one placenta from an infected macerated fetus. Fetuses at 18 days of gestation had well-developed lungs and airways. Fetuses from infected rats had evidence of mild bronchopneumonia, characterized by slightly increased numbers of macrophages and occasional PMN.

**DISCUSSION**

We have demonstrated that *M. pulmonis* can cause an ascending infection which traverses the placenta, resulting in infection of the amniotic fluid and in utero transmission of the microorganism to the developing fetus. Isolation of *M. pulmonis* from the amniotic fluid and the placenta at gestational day 18 confirmed in utero transmission. Histopathologic lesions observed included a placenta with unilobular focus of necrosis, occasional PMN, and associated changes consistent with placental inflammation. These changes were similar to those described by the investigators of the Brazilian study (49), which demonstrated that *M. pulmonis* can be transmitted in utero and result in fetal death. It is likely that the fetal deaths observed in this study were due to placental infection and transmission to the fetus. The study also demonstrated that *M. pulmonis* can cause a chronic infection of the maternal genital tract, which can persist for several months in rats.
pulmonis from the placenta, amniotic fluid, and fetal tissues and histopathological evidence both support an active infection, with lesions compatible with placentitis, amnionitis, and fetal bronchopneumonia.

The most likely route of spread is best illustrated by results from individual animals. Four dams had fetuses with mycoplasma-positive placentas. Three of these four also had mycoplasma-positive amniotic fluids. The same three rats had fetuses with positive respiratory tract and oropharyngeal cultures. Therefore, no rat had a lung isolate without a concomitant isolate from the oropharynx, amniotic fluid, and placenta. However, there were fetuses which had oropharyngeal but not lung colonization.

We observed a small but consistent increase in colonization of the placental and fetal tissues as pregnancy progressed, although this was not statistically significant. If all isolations regardless of dam were considered, the tissues cultured at day 18 of gestation had a greater percentage of isolations than did tissues cultured at day 14 (placenta, 40% [8 of 20] versus 59% [10 of 17]; amniotic fluid, 40% [16 of 40] versus 47% [15 of 32]; fetus, 40% [8 of 20] versus 53% [9 of 17] for days 14 and 18 of gestation, respectively). Additional studies with an increased sample size are needed to confirm this observation.

M. pulmonis infection was ascending and persisted throughout pregnancy. Although embryos at day 11 of gestation were not cultured separately from the uterus, histopathologic testing of infected day 11 embryos demonstrated placenta. The results of histopathologic testing and uterine culture at this stage suggest that the microorganisms were gaining access to the embryos. Because of the small size of the fetus at day 14 of gestation, no attempt was made to dissect specific tissues for culture. The entire fetus was minced, and the possibility of contamination from infected amniotic fluid cannot be ruled out. On the basis of the results obtained at gestational day 18, a picture indicative of an ascending infection from the infected genital tract to the fetus emerged. The placenta was more frequently colonized than amniotic fluid, followed by fetal oropharynx and then fetal lungs, strongly supporting an ascending infection which breaches the placental barrier. Placental colonization always occurred if the amniotic fluid was positive for M. pulmonis, further supporting an ascending route of infection. This strongly suggests that the mycoplasmas must first invade the placenta before they can reach the amniotic fluid. The fetus then ingests or inhales the infected amniotic fluid, exposing mucosal surfaces and contaminating the oropharynx. Once the respiratory tract is inoculated by fetal respiratory movements, M. pulmonis can establish infection in the fetal lungs. This hypothesis is supported by mycoplasmal isolation data as well as by histopathological evidence, which demonstrated development of mild bronchopneumonia in infected fetuses.

Infections of the chorioamnion and amniotic fluid have devastating effects on pregnancy outcome in humans. The predominant cause of perinatal morbidity and mortality is prematurity (10, 13, 15). One of the major predisposing factors for preterm labor is intrauterine infection (10, 15). Neonatal infectious morbidity and neonatal respiratory distress syndrome have been associated with amniotic fluid infections (13). Bacterial infections of the chorioamnion are related to prematurity, neonatal sepsis, and neonatal death (10, 13, 15). Because of the intimate interplay of host factors and bacterial virulence factors, the exact mechanisms by which bacterial pathogens cause adverse effects can be addressed best by an animal model of amniotic fluid infection.

M. pulmonis is an ideal candidate for a model of intruter-
ine 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 in similar to that predicted for human pathogens, i.e., an ascending infection that breaches the placental barrier and established an amnionitis. Finally, a strong data base exists for normal reproductive physiology and pregnancy maintenance in the rat.

REFERENCES