Duration of *Mycoplasma hyopneumoniae* Infection in Gnotobiotic Pigs

N.R. UNDERDAHL, G.A. KENNEDY AND A.S. RAMOS, JR.*

SUMMARY

Sixteen gnotobiotic pigs raised in flexible plastic isolators (four pigs per isolator) were inoculated with a culture of *Mycoplasma hyopneumoniae.* One pig was killed and underwent necropsy at weekly intervals for the following 16 weeks. Macroscopic lesions were observed in the lungs of 13 of 16 pigs and microscopic lesions were found in 14 of 16 pigs. *Mycoplasma hyopneumoniae* was cultured from the trachea or lungs from 10 of the 16 pigs. Scanning electron microscope studies showed areas of damage to the cilia, collections, of leucocytes and mucus, and mycoplasma in the trachea as well as the bronchi. These conditions were found in all the pigs seen at necropsy from nine to 16 weeks postinoculation and there was no evidence of noticeable regression or recovery during this 16 week period.

RÉSUMÉ

Durée de l'infection à *Mycoplasma hyopneumoniae,* chez des porcs gnotoxéniques

Cette expérience consistait à inoculer 16 porcs gnotoxéniques, gardés par groupes de quatre, dans autant d'isoloirs flexibles en plastiques, avec une souche de *Mycoplasma hyopneumoniae.* Il fallait ensuite tuer hebdomadairement un de ces porcs et en effectuer la nécropsie. Treize développèrent des lésions pulmonaires macroscopiques et 14, des lésions microscopiques. On recouvra *M. hyopneumoniae* de la trachée ou des poumons de dix porcs. La microscopie électronique permit de démontrer, tant dans la trachée que dans les bronches, des foyers de cils endommagés, une accumulation de leucocytes et de mucus, ainsi que des mycoplasmes. Ces lésions affectaient tous les porcs dont on effectua la nécropsie, de la neuvième à la 16e semaine après l'inoculation. Aucun des porcs ne manifesta d'amélioration ou de guérison, au cours de cette période de 16 semaines.

INTRODUCTION

Mycoplasmal pneumonia (MP) is considered to be the world's most prevalent swine disease. It has been reported that 35-50% of pigs marketed in the major swine producing countries have pneumonic lesions typical of mycoplasma (5). It has been assumed that mycoplasmal pneumonia is a chronic disease. However, no detailed experiments to determine how long the infectious agent remained in the lung or the time needed to resolve the macroscopic and microscopic lesions have been reported. Livingston *et al* (2) described the loss of cilia and presence of mycoplasmas on the bronchial surface. Scanning electron microscope studies confirmed Livingston's report and also found identical damage in the trachea. Mebus *et al* report that the bronchial changes coincided with the macroscopic pulmonic lesions and that the branches of the bronchus were not equally affected, which accounted for the differences in severity of lobular lesions (4).

In Livingston's report of three pigs killed 42 days postinoculation (PI) only one had severe macroscopic and microscopic lesions. He postulated that in pigs with uncomplicated infection with *Mycoplasma hyopneumoniae* the lesions would regress after a period of five to six weeks.

This study was undertaken with gnotobiotic pigs to determine if there was regression of both microscopic and macroscopic pneumonic lesions five to six weeks following infection with *M. hyopneumoniae*, if the regression could be observed, and if there was a corresponding repair of damage to epithelial lining of the trachea and the bronchi. The scanning electron microscope (SEM) and the light microscope (LM) techniques were used for these studies.

MATERIALS AND METHODS

The 16 neonatal gnotobiotic pigs used were all from one litter obtained from a cross-bred sow (York x Duroc x Landrace). The pigs, obtained by hysterotomy within a sterilized plastic surgical isolator, were transferred to individual cages within a flexible plastic isolator with four pigs in each isolator. Each pig was fed 96 mL of a sterilized canned milk diet[1] twice a day. The diet was gradually increased to 192 mL per feeding towards the end of the experiment. Rectal swabs, collected prior to inoculation and at necropsy, were cultured on blood agar plates and in thioglycolate broth.[2]

The culture of *M. hyopneumoniae* used was Strain NB-12 isolated in this laboratory in 1956. The isolate had been passaged 26 times in colostrum deprived, surgically obtained pigs, and 22 times in broth medium. A pool of cultures of the liquid medium including passages 19-22 was used for inoculation. The pigs were inoculated intranasally with 3 mL of the culture diluted 1:1 with physiological balanced salt solution when the animals were eight days of age. Previous experience with flooding the respiratory tract with the infectious culture had

*Department of Veterinary Science, University of Nebraska, Lincoln, Nebraska 68583. Present address of G.A. Kennedy: College of Veterinary Medicine, Kansas State University, Manhattan, Kansas 66506.

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[1]SPF-Lac, Borden Special Products Company, New York, New York.

[2]Difco Laboratories, Detroit, Michigan.

resulted in 90+ percent of the pigs being infected initially.

At weekly intervals, one pig was randomly selected and euthanasia obtained by exsanguination while under chloroform anesthesia. The chest cavity was opened using sterile technique and the lungs were examined for macroscopic lesions of mycoplasmal pneumonia (MP). A small piece of the affected lung, or the right apical lobe in pigs without lesions, was collected and the anterior 5 cm of the trachea was swabbed for examination by bacteriological culturing. The specimens were cultured for mycoplasma by the method of Goodwin *et al* (1) and for other contaminating bacteria on blood agar plates and in thioglycolate broth.

The larynx, trachea and the lungs were then removed and fixed by introducing 10% buffered formalin into the larynx until the lungs were slightly distended. The lungs were then stored in 10% buffered formalin solution. Samples were collected for SEM from midtrachea, base of trachea, origin of the apical lobe, midapical lobe, origin of cardiac lobe, midcardiac lobe, and origin of diaphramatic lobe. The pieces of tissue were prepared for SEM by the method of Malick and Wilson (3). Sections for LM examination were collected from the trachea, the area with macroscopic lesions or the right apical and cardic lobes in pigs without macroscopic lesions. The tissues were embedded in paraffin by standard methods and stained with hematoxylin and eosin. The specimens were examined by LM and the microscopic lesions were evaluated and graded by the method described by Livingston (2) and is as follows:

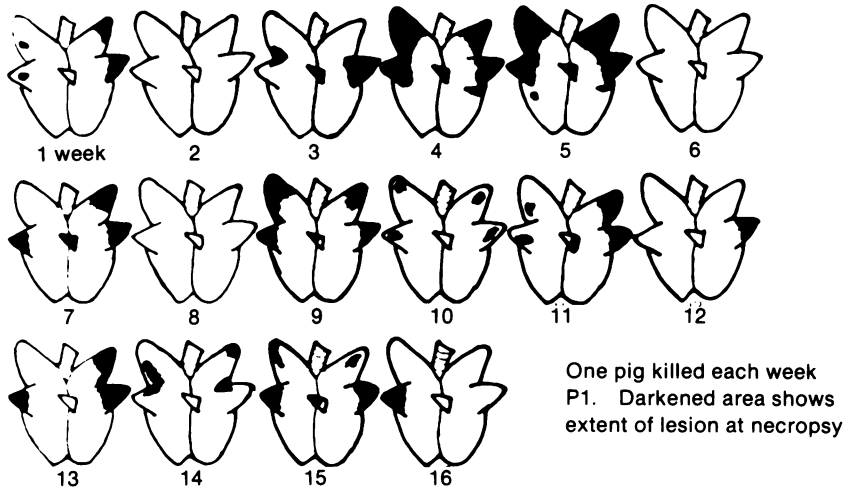

FIGURE 1. Macroscopic lesions noted in lungs from pigs infected with *Mycoplasma hyopneumoniae* and necropsied one to 16 weeks postinoculation. Data from one pig per week.

\+ one or more lymphoid nodules in the submocosa of bronchi or bronchioles but without penetration of the muscularis mucosa.

++ one or more lymphoid nodules extending through the muscularis mucosa. Walls of the alveoli thickened and a few septal cells and neutrophils in the alveoli and bronchi.

+++ as above plus extensive perivascular and peribronchiolar lymphoid hyperplasia. Massive accumulation of neutrophils and septal cells present in alveoli and bronchi of affected areas.

++++ as above but involving extensive areas of the lungs as well as proteinaceous fluid in alveoli of affected areas.

TABLE I
RESULTS OF CULTURES AND MICROSCOPIC EXAMINATION OF LUNGS FROM PIGS INFECTED WITH *M. HYOPNEUMONIAE*

Plastic Isolator and Pig No.	Killed Week PI	*M. hyopneumoniae* culture Trachea	Lung	Presence of Mycoplasma by SEM	Macroscopic Lesions	Microscopic Lesions
B304-4	1	0	+	+	+	ND
B305-1	2	0	neg	neg	neg	(±)
B306-2	3	0	+	+	+	+++
B307-4	4	neg	neg	+	+	++
B306-1	5	neg	+	+	+	++++
B304-1	6	neg	neg	neg	neg	(±)
B307-1	7	+	+	+	+	+++
B305-2	8	neg	+	+	neg	+
B306-3	9	neg	neg	+	+	++
B307-3	10	+	+	+	+	++
B304-2	11	+	+	+	+	+++
B305-2	12	+	+	+	+	+
B307-2	13	neg	neg	+	+	++
B306-4	14	+	neg	+	+	+++
B305-4	15	+	+	+	+	++
B304-3	16	neg	neg	neg	+	++

0 - problems with medium
ND - not done

RESULTS

The pigs grew well on the minimum diet. Throughout the experimental period the pigs did not cough, sneeze or show any clinical signs of pneumonia. On necropsy macroscopic lesions typical of MP were seen as early as seven days PI and 13 of the 16 pigs inoculated had macroscopic lesions (Figure 1). The three pigs seen at necropsy on week 2, 6 and 8 PI did not have macroscopic pneumonic lesions. Pigs killed on week 4 and 5 PI appeared to have the more extensive macroscopic lesions, however, macroscopic and microscopic lesions persisted in all pigs necropsied from nine to 16 weeks PI.

Cultures prepared from either the tracheal swabs or lung tissues were positive for *M. hyopneumoniae* in ten of the 16 pigs (Table I). Both positive and negative cultures were obtained from pigs in all four isolators. Mycoplasma was isolated by cultural methods from one of the three pigs without macroscopic lesions. Cultures made of rectal swabs from all the pigs

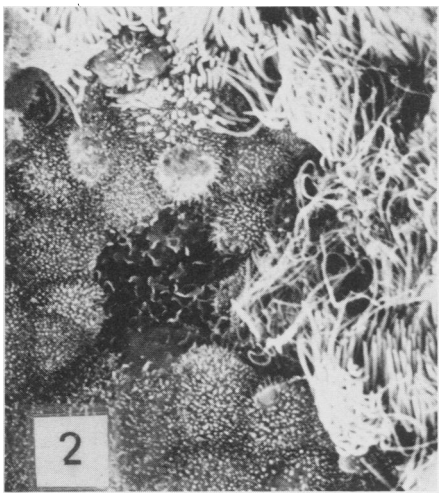

FIGURE 2. Scanning electron micrograph of midtrachea. Some epithelial cells have lost or damaged cilia. Pig B306-3. Nine weeks PI. X2000.

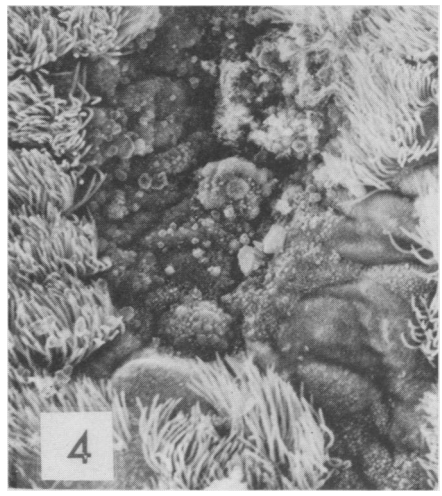

FIGURE 4. Scanning electron micrograph showing loss of cilia. Pig B307-2. Thirteen weeks PI. X1100.

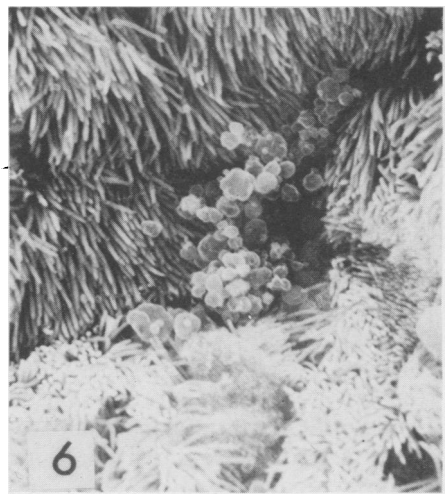

FIGURE 6. Scanning electron micrograph of midtrachea showing collection of mycoplasmas amongst normal appearing cilia. Pig B307-3. Ten weeks PI. X2100.

prior to necropsy contained Gram positive cocci and/or rods. These organisms were also isolated from the larynx or the upper trachea from four of these pigs, but were not isolated from the lungs.

Microscopic lesions were found in the lungs of all pigs with macroscopic lesions, and were evaluated as being moderate to severe (Table I). Of the three pigs without macroscopic lesions one had the early formation of lymphoid nodules in the submocosa and the other two had equivocal lesions.

In the examination by SEM of lungs from infected pigs, many areas in the trachea and the bronchi had cilia that were damaged or missing and the microvilli exposed (Figures 2-5). Mycoplasma could be found scattered (Figure 3) in small clumps (Figure 6), and in other areas large numbers were entrapped in the remaining cilia (Figure 7). Collections of leukocytes and mucus were seen in many areas and many leukocytes appeared to have mycoplasma adhering to the cell surface (Figure 8). These changes were found in all pigs with macroscopic or microscopic lesions. Patches of normal tissue could also be found in the trachea and the bronchi of all pigs (Figures 9 and 10). In some areas mucus was evident (Figure 11). The damage to cilia, collection of leukocytes and mucus, and mycoplasma were found to be as extensive in pigs seen at necropsy 14, 15 and 16 weeks PI as in those pigs seen at necropsy during the first few weeks following exposure.

DISCUSSION

The lesions observed by SEM were similar to those described by Mebus *et al* (4) with loss of cilia, exposure of microvilli and entrapment of numerous mycoplasmas. The damage to

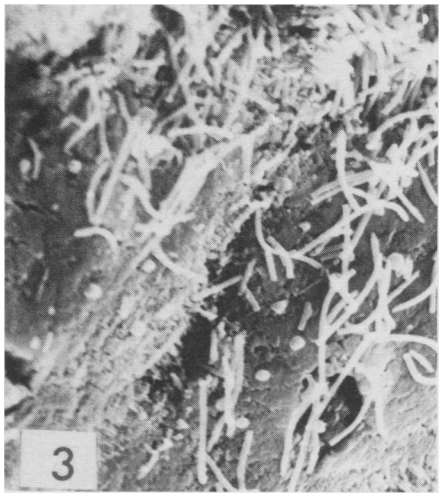

FIGURE 3. Scanning electron micrograph of midtrachea showing loss and damage to cilia with scattering of mycoplasma. Pig B307-3. Ten weeks PI. X3000.

FIGURE 5. Scanning electron micrograph of posterior section of trachea showing loss of cilia and mucus. Pig B305-2. Eight weeks PI. X2000.

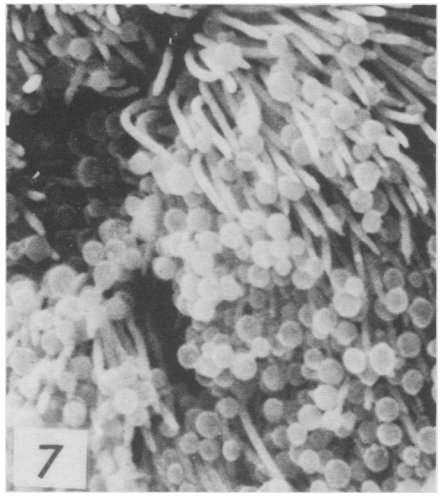

FIGURE 7. Scanning electron micrograph of the posterior section of trachea showing large numbers of mycoplasma. Pig B306-4. Fourteen weeks PI. X5100.

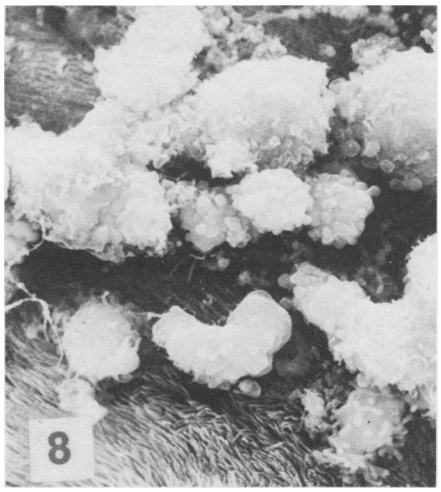

FIGURE 8. Scanning electron micrograph of bronchus show collection of leukocytes with what appears to be mycoplasma adhering to the surface from pig B307-3. Ten weeks PI. X1200.

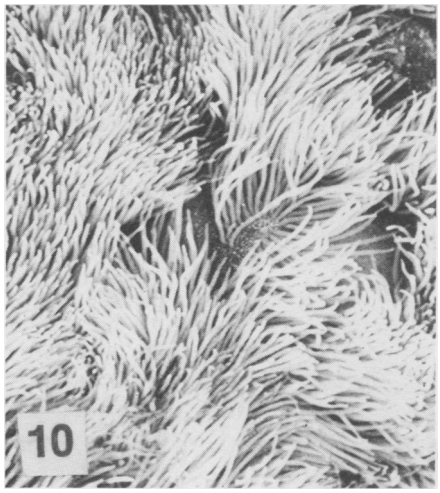

FIGURE 10. Scanning electron micrograph of the lower bronchus showing normal area. Pig B304-4. One week PI. X2400.

epithelial cilia was noted as early as one week PI and was also found in the pigs submitted to necropsy late in the 16 week experimental period. This indicates that M. hyopneumoniae causes a chronic disease and is capable of propagating on tracheal and bronchial surfaces for at least 16 weeks. Although some pigs do not develop macroscopic or severe microscopic lesions they do, however, still become infected and may be capable of shedding mycoplasmas.

This study also demonstrates that when culturing tracheal swabs or lung tissue for mycoplasma, a negative culture does not always indicate freedom from the disease. Of 29 cultures made from tracheal swabs and lung tissues, 14 were negative for mycoplasma. In four pigs the cultures from both the trachea and lung tissues were negative although the lungs had macroscopic and microscopic lesions typical of MP. For the inoculation of mycoplasmal media culture pools made from several areas of the infected lungs or extracts of a larger sample would give a higher percentage of positive cultures. It would indicate that a combination of tests is necessary to diagnose the presence of MP. These tests could include macroscopic and microscopic examination, cultures, serology and the examination of the upper respiratory tree by SEM. In pigs with moderate to severe pneumonic lesions SEM might be the best diagnostic tool, however, more research from field cases of pneumonia is necessary.

The results of this study do not support Livingston's postulate that uncomplicated infection with *M. hyopneumoniae* would regress in five to six weeks. They would also indicate that a large percentage of the infected pigs could become chronic carriers of the infectious agent. From the lesions studied by LM and SEM one could not determine if there was any repair of damaged epithelium as areas of normal or damaged epithelium were found in all the pigs seen at necropsy. The presence of damaged epithelia and mycoplasmal organisms indicated that the infection persisted in pigs for at least 16 weeks.

FIGURE 9. Scanning electron micrograph of normal area taken from posterior section of trachea. Pig B306-2. Three weeks PI. X800.

FIGURE 11. Scanning electron micrograph of the lower bronchus showing mucus. Pig B304-4. One week PI. X2400.

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