A Model Aerosol Exposure System for Induction of Porcine *Haemophilus* Pleuropneumonia

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ABSTRACT

One group of six pigs and another group of three pigs were separately exposed in a polyethylene enclosed chamber for ten minutes, respectively, to *Haemophilus pleuropneumoniae* serotype 1 and *Bacillus subtilis* aerosols generated by an ultrasonic nebulizer.

***Haemophilus pleuropneumoniae* and *B. subtilis* were deposited throughout the lungs immediately following aerosol exposure. The number of *H. pleuropneumoniae* and *B. subtilis* deposited varied within and between lungs in each group. The mean numbers of both organisms deposited in the posterior (caudal and accessory) lobes were significantly greater than those in the anterior (cranial and middle) lobes ($P<0.001$). The four principals that received *H. pleuropneumoniae* aerosols and the two contact controls developed fatal fibrinous pneumonia which simulated that seen in natural infections. Since this exposure system consistently resulted in clinical disease it has good potential as a model for the study of pathogenesis of the disease and more specifically for the evaluation of vaccines.**

RÉSUMÉ

Cette expérience consistait à placer un groupe de six porcs dans une chambre tapissée de polyéthylène et à les y soumettre, durant dix minutes, à des aérosols de *Haemophilus pleuropneumoniae* produits par un nébuliseur ultrasonique; elle visait aussi à soumettre un autre groupe de trois porcs à un procédé similaire, mais avec *Bacillus subtilis*. Il s'ensuivit un dépôt diffus de ces microbes dans les parenchyme pulmonaire. Leur nombre varia cependant d'un porc à l'autre et d'un groupe à l'autre. Le nombre moyen de *H. pleuropneumoniae* et de *B. subtilis* déposés dans les lobes pulmonaires caudaux et accessoire s'avéra sensiblement plus élevé que dans les lobes craniaux et moyen ($P<0,001$). Les quatre porcs qui subirent les aérosols de *H. pleuropneumoniae*, tout comme les deux témoins de ce groupe, développèrent une pneumonie fibrineuse mortelle qui ressemblait à celle des cas spontanés de cette maladie. Comme ce système expérimental produisit constamment une maladie clinique, il pourrait constituer un modèle intéressant pour l'étude de la pathogénèse de la maladie et plus spécifiquement pour l'évaluation des vaccins destinés à la combattre.

INTRODUCTION

For the study of experimental disease due to *Haemophilus pleuropneumoniae* the intranasal and intratracheal routes of exposure have been commonly used (1-5). Aerosol exposure has not yet been used to study porcine *Haemophilus* pleuropneumonia (PHP), although in calves it has consistently produced a more uniform exposure of the respiratory tract (6).

An aerosol system, previously used to expose pigs to *Bacillus subtilis* was used in this study to expose pigs to aerosols of *H. pleuropneumoniae* (7).

The present study was initiated in attempts to standardize a model system of exposure of pigs to aerosols of *H. pleuropneumoniae* for possible future detailed investigation of the pathogenesis of and immunity to this infection.

MATERIALS AND METHODS

ANIMALS

A total of 11 pigs (6-15 kg weight) were obtained from a herd known to be free of *H. pleuropneumoniae*, *Mycoplasma hyopneumoniae* and other enzootic respiratory diseases.[1] The pigs were housed in isolation as previously described (7) and were randomly assigned to three separate treatment groups of six, three and two animals that, respectively, received *H. pleuropneumoniae* aerosols, *B. subtilis* aerosols and no treatment.

BACTERIAL SUSPENSIONS FOR AEROSOLIZATION

Haemophilus pleuropneumoniae strain #A79-9 (serotype 1) was

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obtained from chronic lesions of PHP in a commercial pig. The organism was inoculated into 10 mL of sterile pleuropneumonia-like organisms (PPLO) broth (containing 10% horse serum, 5% fresh yeast extract and 0.1% dextrose) and incubated at 37°C under 10% CO_2 atmosphere for ten hours after which the cultures were dispensed into small vials in 1 mL volume and stored at -70°C. Aliquots of broth cultures (0.2 mL) were subsequently inoculated into the yolk sac of seven day old chick embryos and after 48 hours the yolk was harvested, distributed in 1.0 mL volumes in vials and stored at -70°C until required.

Aliquots of infected egg yolk were inoculated into 10 mL of sterile PPLO broth and incubated as before, after which it was used to inoculate sterile PPLO agar plates (105 × 15 mm, containing 6% horse serum, 5% fresh yeast extract and 0.1% dextrose) and incubated at 37°C in 10% CO_2 for 18 hours. The plate cultures were harvested with sterile physiological saline (SPS) (0.85% NaCl) and centrifuged at 3000 × g for 30 minutes. The resulting pellets were washed twice with 150 mL of SPS and stored on ice. The culture for aerosolization was initially standardized to an optical density of 0.5 at 540 nm and then diluted 1/5 with SPS immediately before use.

The *B. subtilis* culture was processed as before (7). Viable counts of both organisms were determined before and after aerosolization on appropriate media and expressed in colony forming units (CFU). The concentration of organisms aerosolized was calculated by averaging the concentration of CFU before and at the end of aerosolization. The volume of culture aerosolized was obtained by subtracting the volume of culture remaining in the nebulizer chamber after aerosolization from that present before aerosolization.

AEROSOL EXPOSURE

Two separate experiments were conducted. In the first experiment the three pigs (group 1) were exposed to an aerosol of *B. subtilis* for ten minutes, while in the second experiment the six pigs (group 2) were exposed to an aerosol of *H. pleuropneumoniae* for ten minutes according to the protocol described (7). After the exposures all three pigs in group 1 and 2 of the six pigs in group 2 were killed immediately by pentobarbital given intravenously and examined for lesions. The remaining four pigs in group 2 were returned to their isolation unit and observed for clinical signs until their death.

RECOVERY OF *H. PLEUROPNEUMONIAE* FROM THE AEROSOL

Haemophilus pleuropneumoniae culture at about the same concentration (as in exposure of pigs above) was aerosolized for ten minutes into the exposure chamber devoid of pigs. An approximate recovery rate of *H. pleuropneumoniae* from the aerosol in the chamber was obtained by a glass air sampling impinger[2] (for ten minutes) that contained 20 mL PBS (Fig. 1) (8). Plate counts were made as before (9) on duplicate aliquots from a pair of samples for each of two simulated exposures.

QUANTITATION OF AEROSOLIZED BACTERIA IN THE PORCINE RESPIRATORY SYSTEM

Tissue samples were collected from the respiratory tract, associated lymph nodes and tonsils in the same manner as before (7) with the exception that the anterior part of the caudal lobe of the lung was not sampled. The samples were cultured as before except that for attempted isolation of *H. pleuropneumoniae* PPLO agar plates were used. All plates were read after 18-24 hours of appropriate incubation conditions.

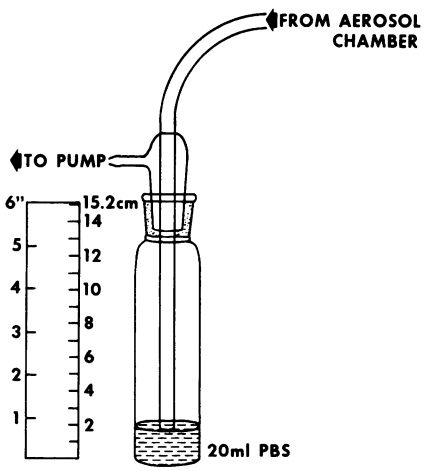

Fig. 1. Liquid impinger sampler.

CONTACT EXPOSURE TO *H. PLEUROPNEUMONIAE*

Control pigs 484L and 485L were put in the aerosol chamber for one hour with pig 482L which was challenged by aerosol of *H. pleuropneumoniae* 24 hours before. Pigs which died were pathomorphologically examined (7). Samples for bacteriological examination were collected as previously described in addition to spleen, liver and pleural exudate.

STATISTICAL ANALYSIS

The average number of bacteria deposited in the anterior and posterior pulmonary lobes at time 0 were compared by the chi^2 test.

RESULTS

DISTRIBUTION OF *H. PLEUROPNEUMONIAE* AND *B. SUBTILIS* IN LUNGS IMMEDIATELY AFTER AEROSOL EXPOSURE

The average volume and concentration of cultures aerosolized, the number of deaths and the mean number of CFU per gram of lung at zero time are shown in Table I. The regional distribution of organisms in lungs immediately after exposure to aerosols are shown in Figs. 2 and 3. Both *B. subtilis* spores and *H. pleuropneumoniae* were deposited throughout the areas of lungs sampled. The average numbers of *B. subtilis* present per gram of tissue of pigs #1, 2 and 3 were 8.3, 5.6 and 7.5 × 10^3 CFU, respectively, for the lungs and 42, 21 and 66 for the tracheobronchial lymph nodes. The numbers of CFU of *B. subtilis* in wash fluid of rinsed

[2]Supplied by Defence Research Board, Suffield, Alberta.

TABLE I. Results of Experiments on Aerosol and Contact Exposure of Pigs

Exp. No.	Challenge by	Mean concentration of culture aerosolized[a] (volume mL)	Number of pigs: Exposed	Number of pigs: Died	Mean number of bacteria/gram of lung at time 0[b]
#1.	Aerosol of *B. subtilis* spores	2.7×10^7/mL (30)	3	NA	$7.13 \times 10^3 \pm 0.68 \times 10^3$
#2.	Aerosol of *H. pleuropneumoniae*	4.85×10^6/mL (33)	6[b]	4	$1.55 \times 10^3 \pm 21 \times 10^3$
#3.	Contact exposure to *H. pleuropneumoniae* aerosolized pig	NA	2	2	NA

[a]Computed by averaging the concentration of CFU before and after aerosolization
[b]Two pigs were killed at zero time
NA = Not applicable

2.5 cm portion of tracheal mucous membrane from these three pigs were zero, 50 and 31 respectively.

The numbers of *H. pleuropneumoniae* recovered postexposure from pigs #4 and 5 respectively were: lungs, 1.9×10^3 and 1.2×10^3 CFU/gram; turbinates, 30 and 30 CFU/2.5 cm length; tracheobronchial lymph nodes, 60 and 30 CFU/gram and tracheal mucous membrane, zero and 87 CFU/2.5 cm length.

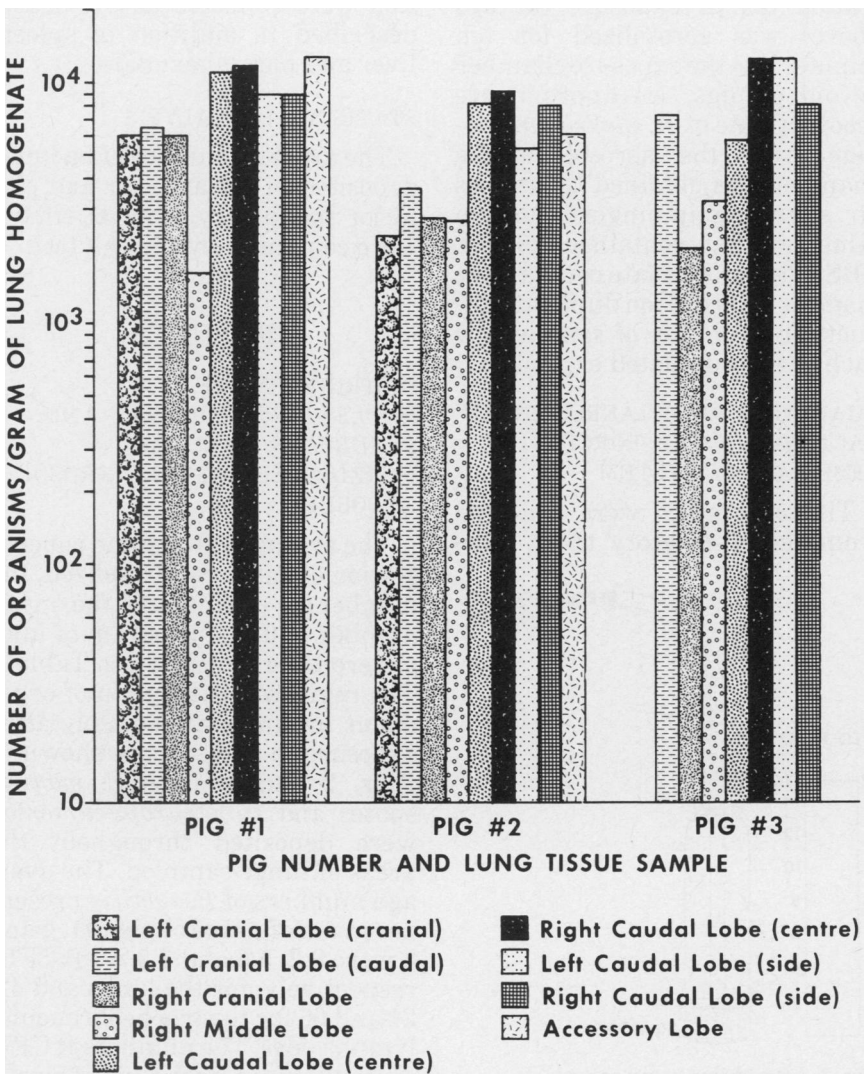

Fig. 2. The distribution of *Bacillus subtilis* in pig lungs immediately after exposure to aerosols.

The mean numbers of *B. subtilis* and *H. pleuropneumoniae* deposited in the anterior (cranial and middle) lobes were significantly less than those in the posterior (caudal and accessory) lobes ($P<0.001$, Table II).

CLINICAL SIGNS AND POSTMORTEM FINDINGS

The four principals and the two contact exposure pigs died within the period of 15 hours to five days after the challenge. After infection, three of the pigs did not develop clinical signs usually observed which were vomiting, polypnea, dyspnea and anorexia. However, all pigs showed epistaxis at or near death and variable cyanosis. Aerosols of *H. pleuropneumoniae* induced disease in all four pigs of group 2 left alive after the challenge. Also the two pigs, exposed to *H. pleuropneumoniae* by contact with the aerosolized pig No. 482L in the chamber, died (Table III).

Postmortem findings were similar for the six pigs necropsied and lesions were confined to the thoracic cavity. All pigs had a hemorrhagic fibrinous pleuropneumonia and varying amounts of blood stained fluid were present in the pleural and pericardial cavities. The bronchial lymph nodes were enlarged and hemorrhagic. The trachea often contained copious blood tinged froth. In most cases fibrin adhered to the affected areas of the lung (Figs. 4 and 5) and adjacent pleura as well as the pericardium (Fig. 5). The pulmonary lesions were either multiple and focal or diffuse with extensive congestion and hemorrhages affecting part or all lobes of the lung with varying severity. The caudal lobes were usually more severely affected than the cranial lobes.

HISTOPATHOLOGICAL FINDINGS

A necrotizing, hemorrhagic fibrinous pleuropneumonia developed in all pigs infected with *Haemophilus pleuropneumoniae*. Congestion, exudation and infil-

TABLE II. Deposition of the Aerosolized Bacteria in Lungs of Zero Hour Pigs

Organism	Average number of organisms per gram in Anterior lobes[a]	in Posterior lobes[b]
B. subtilis	4200	9300
H. pleuropneumoniae	1400	1700

[a]Anterior lobes = cranial and middle lobes
[b]Posterior lobes = caudal and accessory lobes

TABLE III. Pleuropneumonia and Survival Time of Pigs after Aerosol or Contact Exposure to *H. pleuropneumoniae*

Pig No.	Time to death (hours)	Necropsy finding	Microscopical finding	Isolation of *H. pleuropneumoniae* from lungs
A. Aerosol challenge				
471L	15	+	+	+
731L	15	+	+	+
758L	24	+	+	+
482L[a]	36	+	+	+
B. Contact infection				
484L	48	+	+	+
485L	120	+	+	+

[a]Pig #482L served in the contact experiment (#3)

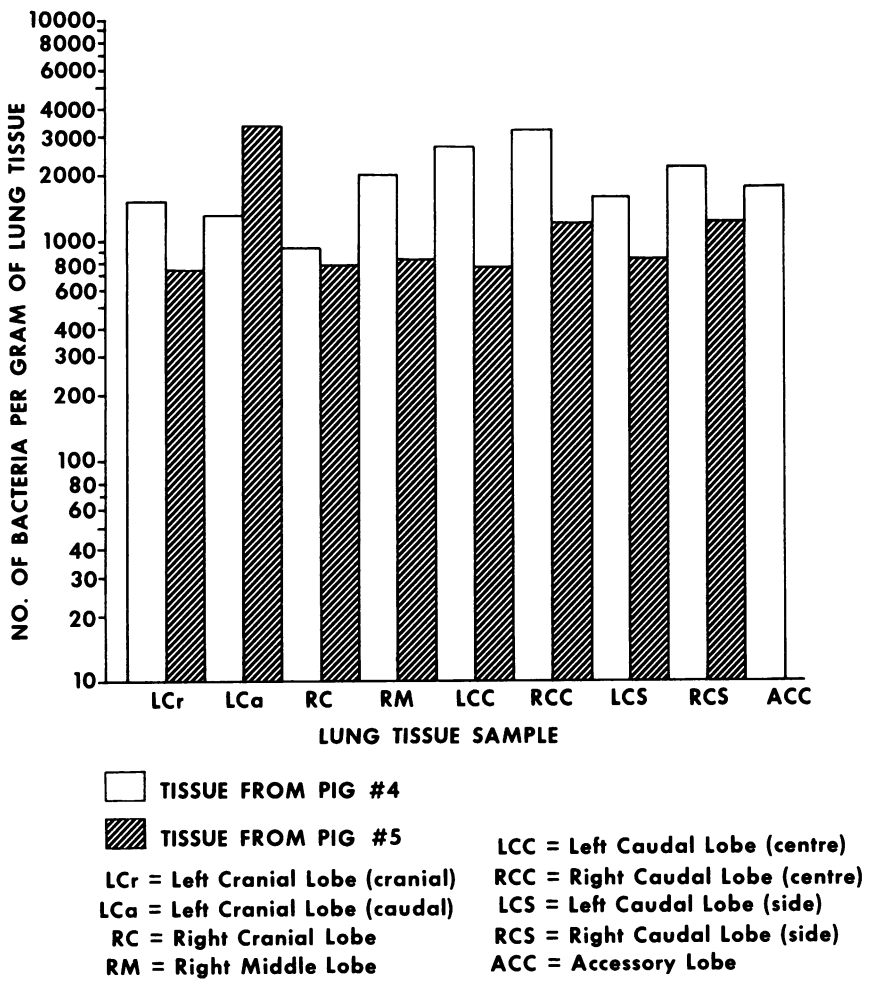

Fig. 3. The distribution of *Haemophilus pleuropneumoniae* in pig lungs immediately after exposure to aerosols.

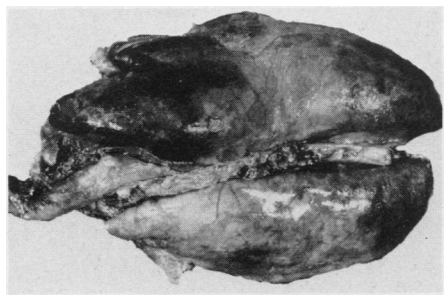

Fig. 4. Acute hemorrhagic fibrinous pleuropneumonia in a pig that died 48 hours following exposure to a pig with clinical signs of PHP. The right anterior lobes are more severely affected than the others.

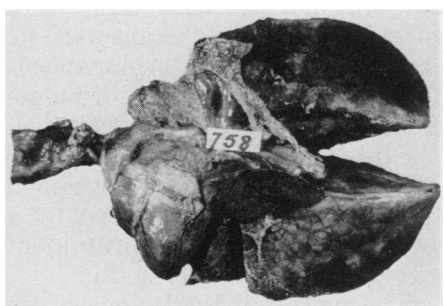

Fig. 5. Fibrinous pericarditis and pleuritis in a pig (Fig. 4) which died 24 hours following exposure to *H. pleuropneumoniae* aerosols.

tration of mononuclear cells occurred in the pulmonary parenchyma. The interlobular septa and their lymphatic vessels were distended by serofibrinous exudate (Fig. 6). Clumps of Gram-negative organisms could be seen occasionally among the fibrin strands in the lymphatic vessels. Although

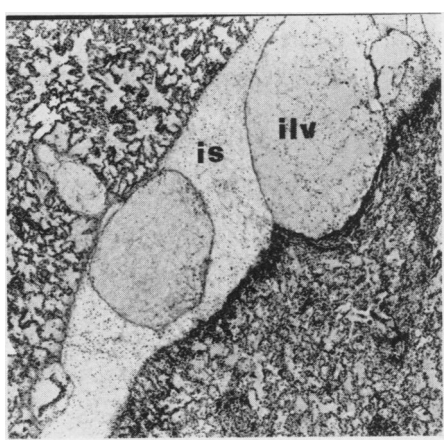

Fig. 6. Section of a pig lung showing distension of the interlobular septa (is) and intraseptal lymphatic vessels (ilv) with edema and fibrin exudate. Death 48 hours post *H. pleuropneumoniae* infection. H & E. X24.

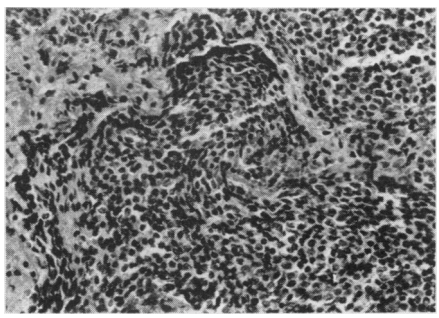

Fig. 7. Swirling mononuclear cells in a pig lung. Death 48 hours post *H. pleuropneumoniae* infection. H & E. X160.

neutrophils were rarely seen in alveoli, mononuclear cells were often arranged in a characteristic swirling pattern in alveolar spaces (Fig. 7). Large numbers of mononuclear cells also accumulated in the interlobular septa. Focal areas of coagulative necrosis developed and were often surrounded by a border of cells with prominent chromatin staining (Fig. 8).

The predominant lesion within the lung was determined by the duration of the clinical disease. Congestion and hemorrhage were more prominent in pigs dying within 24 hours of exposure to aerosols while there was an increased exudation of fibrin, cellular infiltration and necrosis of the pulmonary parenchyma in pigs dying more than 24 hours after exposure.

CULTURAL FINDINGS OF *HAEMOPHILUS PLEUROPNEUMONIAE* IN TISSUES

Haemophilus pleuropneumoniae was isolated in pure culture from lungs, tracheobronchial lymph nodes and blood of pigs with necrotizing hemorrhagic pleuropneumonia. The average concentration of organisms per gram of lung in two pigs (group 2) determined soon after death was 7.7×10^8 CFU which represented a five log increase over the average pulmonary concentration of 1.55×10^3 at zero time. Organisms were usually recovered from the turbinates, tracheal froth, liver and spleen (in five out of six pigs examined) but only occasionally (one out of six pigs) from the tonsils.

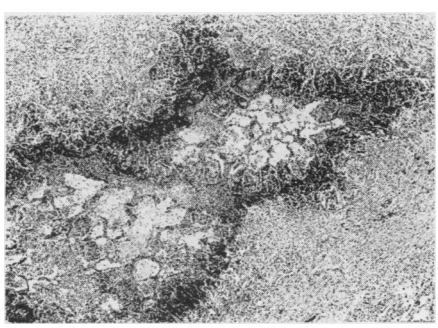

Fig. 8. Section of a pig lung showing areas of coagulative necrosis surrounded by a border of cells with prominent chromatin staining. Death 120 hours post *H. pleuropneumoniae* infection. H & E. X27.

RECOVERY RATE OF *H. PLEUROPNEUMONIAE* AEROSOLS BY THE IMPINGER SAMPLER

A flow rate of 4.72 liters of aerosol per minute was obtained during the sampling period of ten minutes in the simulated exposures. The average volume of culture aerosolized in ten minutes was 30 mL and the average concentration of culture aerosolized was 1.11×10^7 organisms/mL. The percentage recovery rate of the *H. pleuropneumoniae* aerosols was 11% as calculated from the ratio of the calculated chamber aerosol concentration per liter/minute (2.1×10^3) and the estimated concentration of aerosolized culture per liter/minute (2.0×10^4).

DISCUSSION

Recent reports indicate that there is an increasing spread of *H. pleuropneumoniae* infections in the U.S.A. (10) and Canada (11, 12). This has created a definite need for immunoprophylactic control programs. Our experiments were undertaken to characterize the *H. pleuropneumoniae* aerosol infection of pigs as a model for further study of the host parasite interactions in PHP as well as for potential value in evaluating vaccines.

All four pigs challenged with *H. pleuropneumoniae* aerosol, and both pigs exposed for one hour to one of these aerosolized pigs, developed pleuropneumonia, which was similar to that seen in the field (1, 2, 13-16). This consistency suggests an aerosol mode of transmission of *H. pleuropneumoniae* in natural cases of PHP. Both methods of exposure to this bacterium have a potential in producing PHP experimentally.

Of great interest is the distribution of deposited organisms within the lung and development of lesions of PHP. The patterns of distribution of deposited *H. pleuropneumoniae* and *B. subtilis* were similar (Figs. 2 and 3). The density of organisms deposited, which was higher in the lung than in the trachea and turbinates, varied somewhat from area to area of the lung. The relevance of the variations in the numbers of *H. pleuropneumoniae* deposited in the various lobes to the pathogenesis of the lesions is difficult to interpret since only six pigs were studied. Pneumonic lesions occurred more commonly in the caudal lobes (left or right), but also occurred in the anterior lobes (cranial and middle).

Porcine *Haemophilus* pleuropneumonia appeared to result from rapid multiplication of the organism in the lung. The effectiveness of removal of particles deposited in the distal, nonciliated portions of the lung is related to the quantity of particles introduced (17). The development of PHP could be due to the accumulation of unusually large numbers of organisms due to impaired pulmonary clearance mechanisms or because the amount introduced exceeded the ability of the defence apparatus to cope (17-19). Lymphatic transport of particulate matter constitutes part of alveolar clearance (17). However, microorganisms must first penetrate the alveolar membrane or otherwise gain access to the interstitium before they can reach the lymphatic vessels and travel to the bronchial lymph nodes (20, 21). The presence of *H. pleuropneumoniae* in the lymphatic vessels within the interlobular septa might indicate alveolar clearance of the organism *via* lymphatic vessels or entry of organisms into damaged lymphatic vessels.

The apparent recovery rate of *H. pleuropneumoniae* from aerosols as determined by impinger sampling was 11%. Other workers have obtained lower recoveries with *Pasteurella haemolytica* ranging from 0.59-0.94% at differing temperature and humidity (22). The apparent viability of aerosolized organisms is affected by many factors pertaining to the preparation of the bacterial suspension for aerosolization and fluctuating relative humidity and temperature (23). Also, diluent used in the CFU assay of aerosols affect apparent viability of organisms aerosolized and maintained at various relative humidities (24). Under the present experimental conditions, it appears that sufficient *H. pleuropneumoniae* organisms withstood pertinent stresses and survived in the aerosol to be deposited at vulnerable sites in the respiratory tract of pigs.

The consistent production of PHP following exposure to aerosols appears to be a very useful model for further basic and practical experimentation.

ACKNOWLEDGMENTS

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