Exposure of Calves to Aerosols of Parainfluenza-3 Virus and *Pasteurella haemolytica*

P.D. Carrière, M.G. Maxie, B.N. Wilkie, M. Savan, V.E.O. Valli and J.A. Johnson*

ABSTRACT

The present study was undertaken to investigate whether sequential exposure to aerosols of parainfluenza-3 virus followed by *Pasteurella haemolytica*, or *P. haemolytica* followed by parainfluenza-3 virus, could lead to the production of pulmonary lesions in conventionally-raised calves. Twenty male calves with low serum antibody titres to both organisms were placed in five equal groups. Synergism of parainfluenza-3 virus and *P. haemolytica* was not demonstrated in any of the sequentially infected groups and pulmonary lesions were mild in all challenged calves. Clinical signs of disease were not present after exposure to parainfluenza-3 virus although the virus was repeatedly isolated from nasal secretions of all inoculated calves. Exposure to *P. haemolytica* produced a transient response which consisted of increased rectal temperatures and respiratory rates, with a mild neutrophilic leukocytosis and a mild left shift present six hours postinoculation and returning to normal within 24 hours. Results from this study suggest, although do not confirm, that reduced pulmonary clearance of inhaled *P. haemolytica* in parainfluenza-3 virus infected calves does not necessarily lead to production of severe pulmonary lesions and that previous exposure to aerosols of *P. haemolytica* may not enhance secondary parainfluenza-3 virus infection.

Key words: Parainfluenza-3 virus, *Pasteurella haemolytica*, aerosolization, pneumonic pasteurellosis, cattle.

RÉSUMÉ

Cette étude visait à déterminer si des contacts séquentiels avec des aérosols du virus parainfluenza-3 et de *Pasteurella haemolytica*, ou *vice versa*, provoqueraient le développement de lésions pulmonaires chez des veaux élevés de façon conventionnelle. Les auteurs formèrent à cette fin cinq groupes de quatre sujets mâles porteurs de faibles taux d'anticorps à l'endroit des deux agents précités. Ils ne réussirent pas à démontrer de synergie entre ces deux agents, dans l'un ou l'autre des groupes infectés de façon séquentielle; les lésions pulmonaires s'avérèrent par ailleurs minimes, chez tous les veaux soumis aux aérosols. Le contact avec le virus parainfluenza-3 ne provoqua pas l'apparition de signes cliniques, même si on l'isola à plusieurs reprises des sécrétions nasales de tous les veaux soumis à ces aérosols. Le contact avec *P. haemolytica* entraîna cependant une réaction transitoire qui se caractérisait par une élévation de la température rectale et du rythme respiratoire qu'accompagnèrent, au bout de six heures, une légère neutrophilie et un léger virage à gauche; en dedans de 24 heures, tout était cependant de retour à la normale. Les résultats de cette expérience laissent croire, sans toutefois le confirmer, que la réduction de la clairance pulmonaire des *P. haemolytica* inhalées par des veaux infectés par le virus parainfluenza-3 ne conduit pas nécessairement à la formation de lésions pulmonaires marquées et qu'un contact préalable avec des aérosols de *P. haemolytica* n'aggrave pas une infection secondaire par le virus parainfluenza-3.

Mots clés: virus parainfluenza-3, *Pasteurella haemolytica*, production d'aérosols, pasteurellose pulmonaire, bovins.

INTRODUCTION

Bovine respiratory disease accounts for serious economic losses throughout the world and is the most frequent cause of sickness and death in feedlot cattle in North America. Parainfluenza-3 (PI-3) virus infection has been suspected for many years to play an important role in the pathogenesis of pneumonic pasteurellosis in cattle (1, 2, 3, 4, 5). Several researchers have reported the appearance of

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more marked signs of respiratory illness in calves experimentally infected with both PI-3 virus and *Pasteurella* spp. than in animals infected with either agent alone (6, 7, 8, 9). However, these dual infection experiments have generally failed to produce a reliable model of experimental disease in cattle until very recently (10). In pulmonary clearance studies, Lopez (5) demonstrated that the four hour pulmonary clearance of *P. haemolytica* in calves was significantly reduced when these calves had been infected with an aerosol of PI-3 virus seven days before the bacterial aerosolization.

We proposed to determine if pulmonary lesions would develop in PI-3 virus infected calves within four days after the *P. haemolytica* aerosolization, that is, could viral-bacterial synergism (11) be demonstrated by the production of overt pulmonary disease? There is also increasing evidence that *P. haemolytica* might be considered a primary bovine pulmonary pathogen based on its unique cytocidal effects on bovine peripheral blood, as well as bronchoalveolar leukocytes (12, 13, 14). Therefore, the second objective of this study was to determine if *P. haemolytica*, by acting alone on the bovine pulmonary defense mechanisms, would enhance a secondary infection of PI-3 virus and perhaps lead to severe pulmonary lesions.

MATERIALS AND METHODS

ANIMALS

Twenty healthy, intact male, conventionally-raised, Holstein-Friesian calves were selected from a group of 45 on the basis of low serum antibody titres to PI-3 virus and to *P. haemolytica*. Calves ranged from eight to 22 weeks of age with a mean of 14 weeks and weighed from 73 to 150 kg with a mean of 102 kg. Nine adult white mice[1] weighing from 28 to 32 g were also used during bacterial aerosolizations.

TITRATION OF SERUM FOR PI-3 VIRUS AND *P. HAEMOLYTICA* ANTIBODIES

The haemagglutination inhibition test (15) was used to titrate serum antibodies to PI-3 virus. The *P. haemolytica* serum antibody titres were obtained by a modification of the microtiter agglutination test developed by Reggiardo (16). Briefly, the antigen employed is a suspension of *P. haemolytica* type 1 grown in brain heart infusion[2] with 0.003% triphenyl tetrazolium chloride, harvested by centrifugation, repeatedly washed with formalinized saline and the concentration adjusted to O.D.1.0 at 525 nm on a spectrometer. Direct microagglutination was performed in "U" bottom microtiter plates[3] and 0.025 mL volumes of stained antigen were incubated overnight at room temperature with 0.025 mL volumes of twofold serum dilutions. The microtiter plates were then centrifuged[4] at 10,400 × g for 25 minutes, washed in saline and recentrifuged. Then an indirect agglutination test using rabbit antibovine immunoglobulin as described by Wilkie and Markham (17) was performed and read after 18 hours.

EXPERIMENTAL DESIGN

The twenty selected calves were randomly assigned to five equal groups (Table I). In the PI-3-CONTROL group, calves were exposed to an aerosol of PI-3 virus and necropsy was performed seven days later. In the PI-3-PAST group, calves were exposed to an aerosol of PI-3 virus, followed seven days later by an aerosol of *P. haemolytica* and necropsy was performed four days after the bacterial aerosol. In the PAST-PI-3 group, calves were first exposed to an aerosol of *P. haemolytica*, followed four days later by an aerosol of PI-3 virus and necropsy was performed seven days after the viral aerosolization. In the PAST-CONTROL group, calves were exposed only to an aerosol of *P. haemolytica* and necropsy was performed four days later. Finally, in the UNTREATED CONTROL group, calves did not receive any aerosol treatment and necropsy was performed to assess the presence of preexisting pulmonary lesions.

AEROSOLIZATION

The aerosol apparatus and tech-

TABLE I. Experimental Protocol for Exposure of Calves to Aerosols of Parainfluenza-3 and *Pasteurella haemolytica*

	Group	Number of Calves	Prime aerosolization (day 0)	Time interval between aerosolizations (days)	Second aerosolization	Time interval between last aerosolization and necropsy (days)
I	PI-3-CONTROL	4	PI-3-Virus	—	—	7
II	PI-3-PAST	4	PI-3-Virus	7	*P. haemolytica*	4
III	PAST-PI-3	4	*P. haemolytica*	4	PI-3-Virus	7
IV	PAST-CONTROL	4	*P. haemolytica*	—	—	4
V	UNTREATED CONTROL	4	—	—	—	—[a]

[a]Killed without previous aerosolization

[1]Connaught Laboratories, Toronto, Ontario, Canada.
[2]Difco Laboratories, Detroit, Michigan, U.S.A.
[3]Cooke Microtiter plates. Dynatech Laboratories Inc., Alexandria, Virginia, U.S.A.
[4]Beckman Model TJ-6 centrifuge with microplate carriers, Beckman Instruments Inc., Spinco Div., Palo Alto, California, 94304, USA.

nique used for aerosolization of *P. haemolytica* were the same as previously described by Gilka *et al* (18) and Lopez *et al* (5). A modification of this apparatus was used when aerosolizing PI-3 virus. In order to quantify the amount of virus present in the air stream, all viral aerosols were sampled continuously during exposure by a large volume air sampler[5] situated downstream from the plexiglass chamber. Five hundred mL of Eagle's minimal essential medium (EMEM), to which were added 500 units of penicillin per mL, 200 μ g of streptomycin per mL, 500 units of nystatin per mL and 2% PI-3 virus antibody-free fetal calf serum, was used as the virus recovery medium in the large volume air sampler. An airflow of 4800 L/min was produced through the plexiglass chamber during aerosolization.

The virus used in this experiment was the 18th tissue culture passage on fetal bovine spleen cells of a field isolate (4644-9, O.V.C.) which was also used by Lopez *et al* (5, 19) and Al-Izzi *et al* (20). One hundred mL of viral suspension with a 50% cell culture infective dose (C.C.I.D. 50) of 10^3 to 10^4 per 0.2 mL was aerosolized for approximately 30 minutes.

A lyophilized strain of *P. haemolytica* biotype A, serotype 1, which has also been used by Wilkie *et al* (21) was cultured and prepared according to Lopez (5, 19). Briefly, two 6 L flasks containing 4 L of brain heart infusion broth were inoculated with five colonies of *P. haemolytica* and placed in a shaker bath at 37°C at 90 oscillations per minute for 12 to 14 hours and the culture was then centrifuged and bacterial pellets resuspended in potassium phosphate buffer (0.1 M; pH 7.5). During each bacterial aerosolization, three mice were placed into the plexiglass chamber and were used according to the technique described by Lopez (5) to determine by extrapolation the original deposition of *P. haemolytica* in lungs of calves immediately after aerosolization. The number of bacteria aerosolized ($\bar{x} = 2.11 \times 10^{12}$ bacteria/100 mL) and the extrapolated deposition of *P. haemolytica* in calf lung ($\bar{x} = 7.4 \times 10^5$ bacteria/g) was similar for each of the three experimental groups.

PI-3 VIRUS RECOVERY

Prior to each aerosolization of PI-3 virus a 30 minute preaerosol sampling of the ambient room air was performed using the large volume air sampler in order to detect any residual viral particles present in the room air or in the aerosol apparatus.

Swabs were collected from deep in the calves' nasal passages daily, starting on the day preceding aerosol exposure from both nostrils of each calf, and were pooled and rinsed in 3 mL of transport medium consisting of Hank's balanced salt solution with 0.5% lactalbumin (HLA) to which were added 500 units of penicillin, 200 μg of streptomycin and 500 units of nystatin.

Ten to 20% suspensions of ground lung and trachea harvested at necropsy were produced in 6 mL of transport medium. The crude suspension was centrifuged and the supernatant was filtered through a 0.45 μm pore filter.[6]

VIRUS ISOLATION TECHNIQUE

Primary, secondary and tertiary fetal bovine lung cell cultures were used for PI-3 virus isolation. Eagle's MEM with 5% PI-3 virus antibody-free fetal calf serum and with added penicillin and streptomycin was used as maintenance medium. Four tissue culture tubes were used for each tested sample and 0.1 mL was inoculated into each tube. Tubes were examined every second day following inoculation for detection of cytopathic effects (CPE), and when positive CPE was noticed a hemadsorption assay (HAD) was performed. Hemadsorption consisted of gently pipetting 0.25 mL of 0.3% calf red blood cells into each tube which were left at room temperature for 20 minutes before pouring out and reading red blood cell adherence. Negative CPE tubes were incubated until the seventh day when HAD was performed on all remaining tubes. The supernatants of all negative tubes inoculated with lung or tracheal suspension were pooled for each sample, stored at -70°C and reinoculated and tested in the same manner for a second or third time if necessary. Lung and tracheal suspension samples were considered negative only after failure to produce CPE and HAD after three seven day passages.

CLINICAL EXAMINATIONS AND CLINICAL PATHOLOGY

Clinical examination, consisting of rectal temperatures, pulmonary auscultation, heart and respiratory rates, was conducted daily. Blood samples and nasal swabs were collected daily starting on the day preceding aerosolization. Blood was collected in E.D.T.A.[7] and sodium oxalate[7] anticoagulants from jugular venipuncture. The hematological variables measured were: total white blood cells,[8] differential leukocyte counts (100 cells counted per sample), red blood cell indices,[8] and platelet counts which were performed within four hours of blood sampling. Peripheral blood fibrinogen levels[9] were measured daily in plasma from blood collected in sodium oxalate[7] anticoagulant. Each calf was also examined six hours following aerosolization of *P. haemolytica*, and samples for hematology were drawn from calves in the PI-3-PAST and PAST-CONTROL groups.

NECROPSY

All calves were killed by intravenous injection of barbiturate.[10]

[5]Large Volume Air Sampler. Model LVS/10K Sci-Med Environmental Systems Inc., Minneapolis, Minnesota, U.S.A.
[6]Sterile Swinnex 0.45 μm, Millipore Inc., Mississauga, Ontario.
[7]E.D.T.A. (Ethylenediamine Tetracetic Acid, K Salt) and Sodium oxalate Vacutainers®, Becton Dickinson Co. Ltd., Mississauga, Ontario.
[8]Coulter Counter Model S, Coulter Electronics Inc., Hialeah, Florida, U.S.A.
[9]Fibrometer. Fibro System®, Becton Dickinson & Co., Clarkson, Ontario.
[10]Sodium Pentobarbital, MTC Pharm. Ltd., Mississauga, Ontario.

The right and left lung of every calf was weighed and the dorsal aspect was photographed. Four separate pieces of tissue were sampled for attempted virus isolation from each pair of lungs, taking suspected PI-3 virus lesions when present. The selected areas were from the right and left cranial or middle lobes and the right and left caudal lobes.

Samples of right and left cranial lobes were selected for bacterial isolation and one cranial lobe sample from each lung was submitted for *Mycoplasma* spp. and *Ureaplasma* spp. isolations (H.L. Ruhnke and S. Rosendal, personal communication). When preexisting lesions were suspected, samples were cultured for bacteria and mycoplasma.

HISTOPATHOLOGY

Tissues were fixed in Lillie's 10% neutral buffered formalin. Samples from right and left cranial, middle and caudal pulmonary lobes (six samples per calf) as well as mediastinal lymph node and midportion trachea were double embedded in paraffin, sectioned 6 μm thick and stained with hematoxylin-eosin.

STATISTICAL ANALYSIS

Changes in parameters postaerosolization were tested for the presence of statistically significant differences ($p < 0.05$) by use of the unpaired or paired Student's t test as was appropriate.

RESULTS

SERUM ANTIBODY TITRES

The calves used in this experiment had preinfection serum PI-3 antibody titres (HI) of 1:16 or 1:32 which did not increase at four to 11 days after exposure to aerosols containing PI-3 virus.

Preinfection direct and indirect serum antibody titres to *P. haemolytica* ranged from 1:2 to 1:16 and 1:16 to 1:512 respectively (Table II). An increase in titre of three serial dilutions after exposure to *P. haemolytica* and PI-3 virus occurred in one calf only (calf 9, day 11).

RESULTS OF CLINICAL EXAMINATION

Clinical signs were not recognized in calves exposed to PI-3 virus. Exposure to *P. haemolytica* produced a transient response which was similar in all inoculated groups, including calves previously infected with PI-3 virus. Six hours following aerosolization of *P. haemolytica*, most calves were in sternal recumbency, were reluctant to stand and exhibited a deep dry cough. Harsh lung sounds were present in every field on both sides of the thorax and there was marked tachypnea. Respiratory rates returned to normal (Fig. 1) and all clinical signs of disease had disappeared within 24 hours after exposure. None of the 20 calves in this experiment showed any other detectable signs of illness after exposure to *P. haemolytica*.

There was a significant increase ($p < 0.05$) in mean rectal temperatures from time zero to six hours after exposure to *P. haemolytica* in all three groups (Fig. 2). Rectal temperatures of all calves exposed

TABLE II. Serum Antibody Titres to *Pasteurella haemolytica* in Experimental Calves (Microagglutination Test)

Group	Calf no.	Preaerosol titre		Inteval (days)	Postaerosol titre	
		A	*B*		*A*	*B*
PI-3-CONTROL	1	(8)	128	7	(8)	128
	2	(4)	128	7	(4)	128
	3	(2)	128	7	(2)	128
	4	(4)	16	7	(4)	32
PI-3-PAST	5	(4)	64	4	(4)	256[a]
	6	(8)	128	4	(8)	256
	7	(8)	128	4	(16)	512[a]
	8	(8)	128	4	(4)	256
PAST-PI-3	9	(16)	256	11	(64)	2048[a]
	10	(8)	256	11	(8)	512
	11	(16)	256	11	(16)	1024[a]
	12	(8)	256	11	(16)	256
PAST-CONTROL	13	(4)	256	4	(2)	128
	14	(4)	128	4	(4)	128
	15	(2)	128	4	(2)	128
	16	(2)	128	4	(4)	256
UNTREATED CONTROL	17	(8)	512			
	18	(16)	512			
	19	(4)	512			
	20	(8)	256			

A = Direct microagglutination titre
B = Indirect microagglutination titre
[a]Seroconversion

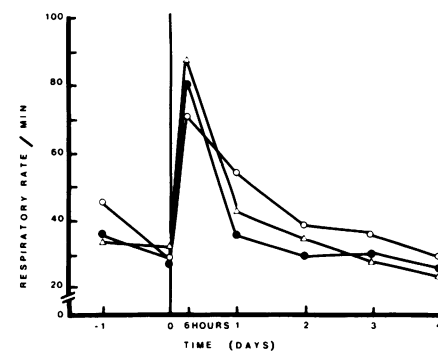

Fig. 1. Mean respiratory rates of groups of four calves given an aerosol of *Pasteurella haemolytica* on day 0. PI-3-PAST (-△-), PAST-PI-3 (-○-), PAST CONTROL (-●-)

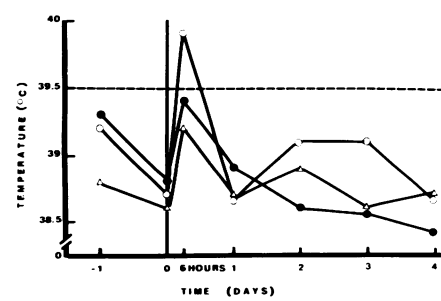

Fig. 2. Mean rectal temperatures of groups of four calves given an aerosol of *Pasteurella haemolytica* on day 0. PI-3-PAST (-△-), PAST-PI-3 (-○-), PAST CONTROL (-●-)

to *P. haemolytica* returned to normal (≤ 39.5°C) within 24 hours.

There was a significant increase ($p < 0.01$) in mean respiratory rates which approximately doubled from time zero to six hours after exposure to *P. haemolytica* in all three groups (Fig. 1). Respiratory rates of these calves returned to normal (≤ 50 respirations/minute) within 24 to 48 hours.

HEMATOLOGY AND FIBRINOGEN RESULTS

Hematological changes were not detected following exposure to PI-3 virus. Significant changes ($p < 0.05$) were noticed only at six hours following aerosolization of *P. haemolytica* in calves of the PI-3-PAST and PAST-CONTROL groups.

In the PI-3-PAST group, there were significant increases ($p<0.05$) in mean leukocyte counts (11.9×10^9/L) and segmented neutrophil counts (6.6×10^9/L) from time zero to six hours after exposure to *P. haemolytica*. However, this response was a mild transient neutrophilic leukocytosis[11] which persisted for only 24 to 48 hours.

There was a significant increase in the mean band neutrophil counts from time zero to six hours in both the PI-3-PAST ($p < 0.05$) and the PAST CONTROL ($p < 0.01$) groups (Fig. 3) with values returning to normal ($0\text{-}120 \times 10^8$/L) by 24 hours.

There were no significant changes ($p > 0.05$) in platelet counts or fibrinogen levels in any of the five experimental groups.

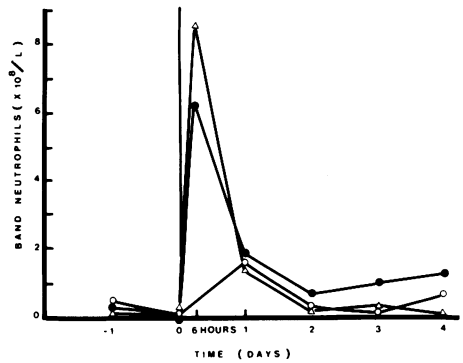

Fig. 3. Mean concentration of peripheral blood band neutrophils of groups of four calves given an aerosol of *Pasteurella haemolytica* on day 0. PI-3-PAST (-Δ-), PAST-PI-3 (-○-), PAST CONTROL (-●-)

PI-3 VIRUS RECOVERY

All preaerosol air samples were negative for PI-3 virus isolation and virus was recovered from the aerosol air sample at a concentration of 1CCID50 per 0.2 mL in the PI-3-CONTROL and PI-3-PAST groups.

Consistent PI-3 virus isolations were made for at least two consecutive days from nasal swabs of every calf exposed to PI-3 virus (Fig. 4). Positive PI-3 virus isolations from nasal swabs began on day 2, 3 and 4 respectively for the PI-3-PAST, PAST-PI-3 and PI-3-CONTROL groups.

Parainfluenza-3 virus was isolated from lung of three calves including one tracheal isolation from the PI-3-CONTROL group and from lung and trachea of one calf from the PAST-PI-3 group (Table III).

No other viruses were isolated from nasal swabs and tissues of the 20 calves of this experiment as demonstrated by the absence of CPE in the negative HAD cell cultures.

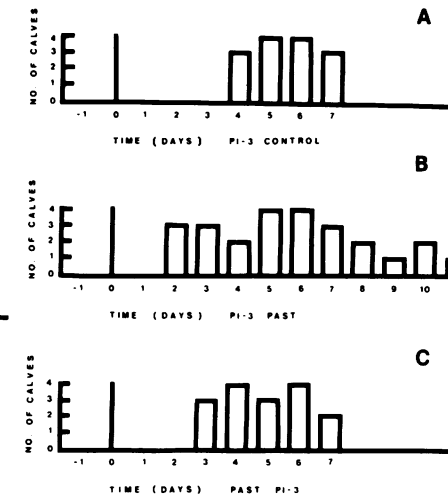

Fig. 4. (A) Number of calves with a positive PI-3 virus isolation* from nasal swabs after aerosolization of PI-3 virus on day 0. (PI-3 CONTROL group).
(B) Number of calves with a positive PI-3 virus isolation* from nasal swabs after aerosolization of PI-3 virus on day 0. (PI-3-PAST group).
(C) Number of calves with a positive PI-3 virus isolation* from nasal swabs after aerosolization of PI-3 virus on day 0. (PAST-PI-3 group).
***Positive isolation as determined by positive cytopathic effect (CPE) with positive hemadsorption (HAD).**

TABLE III. Parainfluenza-3 Virus Recovery in Calves from Tissues at Necropsy

			Lung Lobes			
			Right		Left	
Group[a]	Calf No.	Trachea	Cranial or Middle	Caudal	Cranial or Middle	Caudal
PI-3-CONTROL	1	—	—	+[b]	—	—
	2	—	—	—	+[c]	—
	3	—	—	—	—	—
	4	+[b]	—	—	+[b]	+[b]
PAST-PI-3	9	+[d]	+[c]	—	—	+[b]
	10	—	—	—	—	—
	11	—	—	—	—	—
	12	—	—	—	—	—

[a]PI-3-PAST, PAST-CONTROL and UNTREATED CONTROL groups: Tissues from all calves are negative
[b]Positive CPE/HAD at 1st passage
[c]Positive CPE/HAD at 2nd passage
[d]Positive CPE/HAD at 3rd passage

[11]Normal values established as being the total leukocyte and segmented neutrophil counts calculated from the mean + 2 SE of the UNTREATED CONTROL group. (WBC = 11×10^9/L, seg. neutrophil = 4.3×10^9/L).

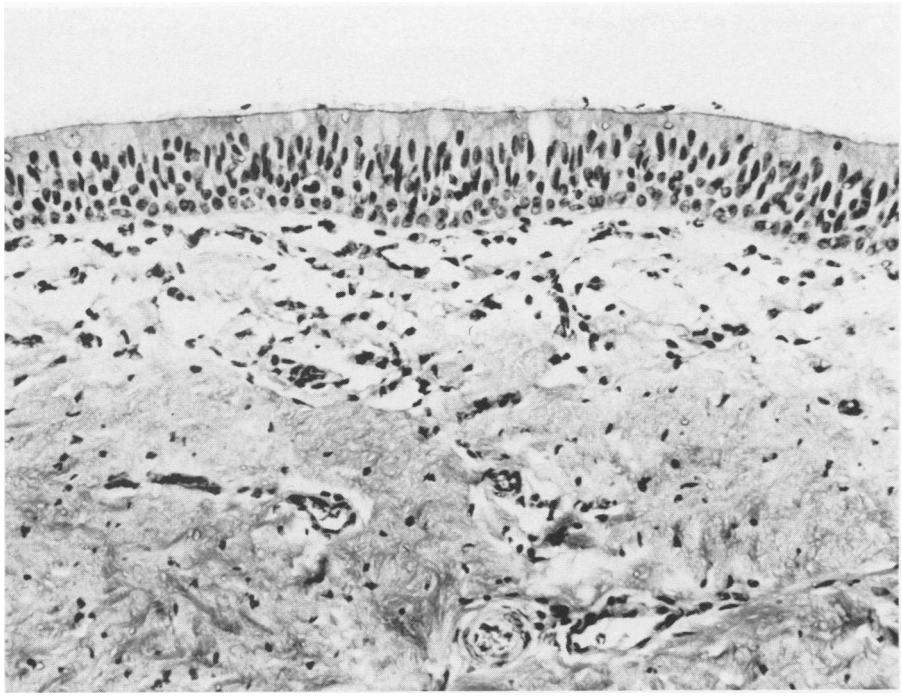

Fig. 5. Normal ciliated columnar pseudostratified epithelium from the midportion of the trachea. Note the paucity of mononuclear cells in the lamina propria. Section from the trachea of calf 18 of the UNTREATED CONTROL group. X130.

RECOVERY OF BACTERIA AND MYCOPLASMA FROM LUNG AT NECROPSY

Pasteurella haemolytica was isolated along with a *Mycoplasma* sp. only from the right cranial and middle lobes of calf 9 of the PAST-PI-3 group. In the PI-3-CONTROL group, *P. multocida* and a *Ureaplasma* sp. were isolated from the right and left cranial and middle lobes of calf 3. In the UNTREATED CONTROL group, a *Mycoplasma* sp. was isolated from the left cranial lobe of calf 18.

MACROSCOPIC AND MICROSCOPIC PATHOLOGY

Macroscopic and microscopic pulmonary lesions produced by experimental inoculation with PI-3 virus and/or *P. haemolytica* were mild and in some instances indistinguishable from mild lesions also encountered in the UNTREATED CONTROL group. Inclusion bodies were not present in any sections of trachea or lung of any of the 20 calves.

Tracheal lesions were present mostly in calves from the PI-3-CONTROL and PAST-PI-3 groups. These lesions consisted of moderate to marked exudation of mononuclear cells and neutrophils in the epithelium and lamina propria with degeneration of the superficial epithelial cells and loss of cilia (Figs. 5 and 6).

Macroscopic pulmonary lesions related to experimentation were multifocal small dark red areas measuring less than 0.5 cm in diameter which were randomly distributed on the surface of the lungs of two calves from the PI-3-CONTROL group and one calf from the PAST-PI-3 group (Fig. 7). Microscopically these areas consisted of foci of acute alveolitis and bronchiolitis with exudation of alveolar macrophages and neutrophils (Fig. 8). This microscopic lesion was seen in the lung of only one calf from the two groups which did not receive an aerosol of PI-3 virus and was present in most lung sections of all four calves from the PI-3-CONTROL group and in the lungs of three out of four calves from each of the PI-3-PAST and PAST-PI-3 groups.

Dark red focal areas of atelectasis measuring between 0.5 and 2 cm in diameter (Fig. 9) were encountered in every group except the PAST-CONTROL group and consisted microscopically of areas of atelectasis, sometimes associated with bronchiolar plugging by neutrophils.

Diffuse areas of consolidation,

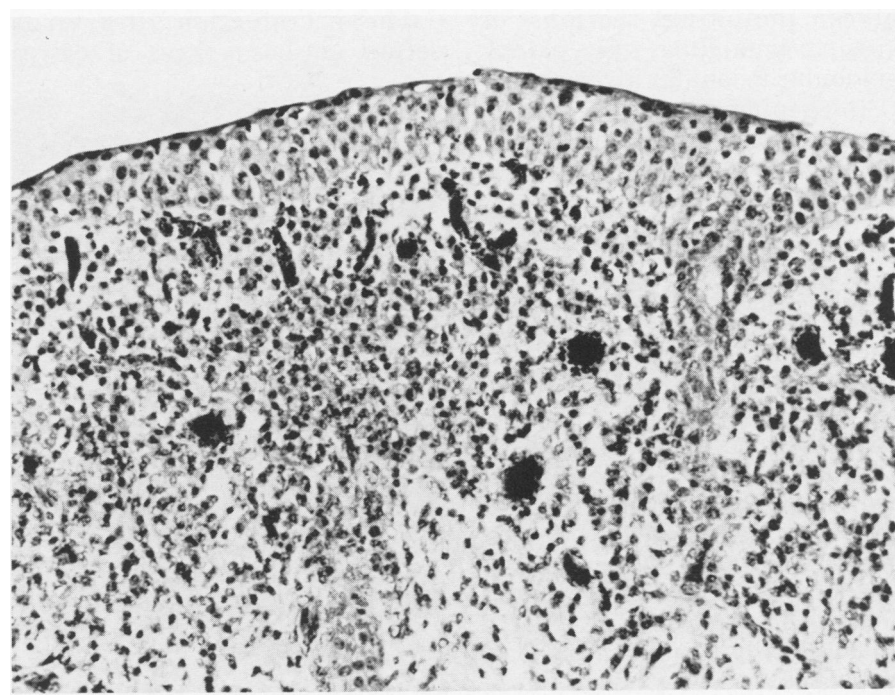

Fig. 6. Midportion tracheal mucosa from a PI-3 virus infected calf. Marked inflammatory mononuclear cell infiltration in the lamina propria and also through the tracheal epithelium. Note the absence of cilia and the flattening of the superficial layer. Section from the trachea of calf 3 of the PI-3-CONTROL group. X130.

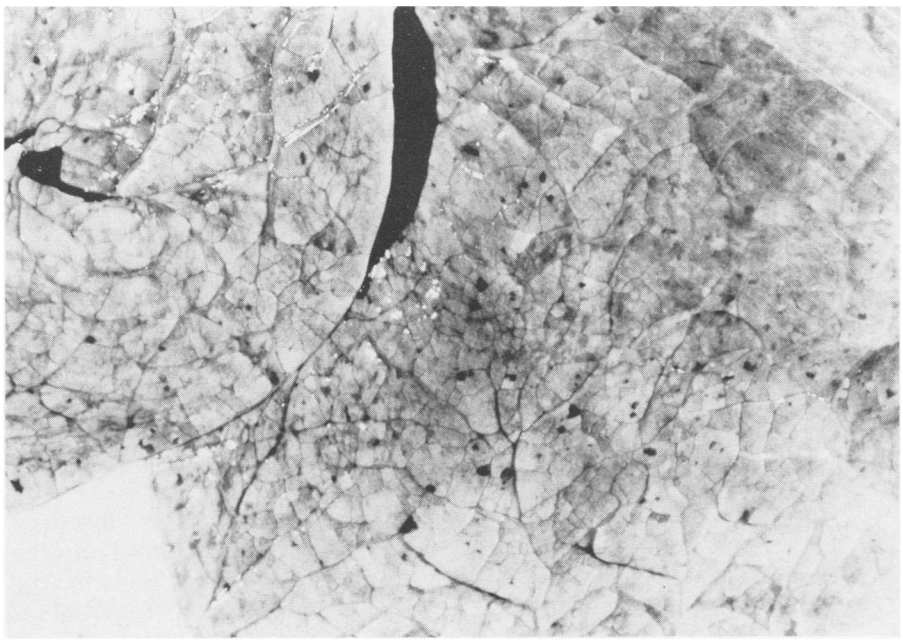

Fig. 7. Dark red focal areas randomly distributed on the surface of the lung and measuring less than 0.5 cm in diameter. Left lung of calf 2 from the PI-3-CONTROL group.

which usually involved one pulmonary lobe, were present in some calves from every group and were considered to be preexisting lesions of enzootic pneumonia (Fig. 10).

A microscopic lesion which was present in most lung sections of all calves, including sections of grossly normal lung, was a patchy sublobular to lobular alveolar septal thickening with dense hypercellular interlobular septa (Fig. 11).

DISCUSSION

Calves in the present study did not exhibit clinical signs of disease following aerosolization of PI-3 virus even though viral infection of the upper respiratory tract was established as demonstrated by repeated isolations of PI-3 virus from nasal secretions. Several other investigators have failed to observe any detectable clinical signs of disease following experimental PI-3 virus inoculation of conventionally-raised calves despite virus recovery from nasal secretion and/or lung (8, 22, 23, 24). It is well accepted that clinical signs of disease in calves experimentally infected with PI-3 virus are at best considered to be mild and that colostrum-free calves are known to be more susceptible (9, 25, 26). For these reasons, several researchers have relied upon virus recovery from nasal secretions and upon serology to confirm infection (27, 28).

The preinfection PI-3 virus serum antibody titres of calves used in the present study were similar to those obtained by several authors interested in studying pulmonary clearance in conventionally-raised calves of similar source (5, 18, 19, 20). The preinfection PI-3 as well as *P. haemolytica* serum antibody titres were most likely passive antibody derived from colostrum. During the course of selection of calves, monthly serological tests for both pathogens demonstrated stable or decreasing antibody titres in calves only a few days old until they were used for experimentation.

Virus-neutralizing, colostrally-acquired, maternal antibody to PI-3 virus may also appear in the nasal secretion (29). In conventionally-raised calves experimentally infected with PI-3 virus, a possible explanation for the variability of clinical response as well as virus recovery from the respiratory tract, may be related to the level of colostrally-acquired PI-3 virus neutralizing antibody, namely IgA, in the nasal and bronchoalveolar secretions.

Seroconversion following experimental PI-3 virus infection is variable (5, 8, 9, 19, 20, 23), usually occurring later than 14 days after inoculation. In the present study,

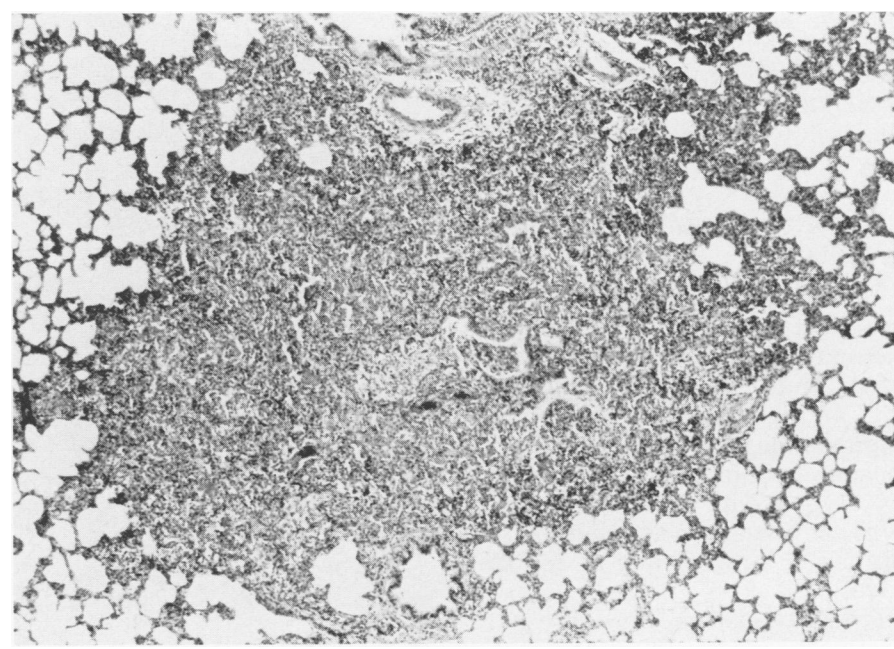

Fig. 8. Focal exudative alveolitis and bronchiolitis. Note that the focal exudative area is centered around the affected bronchiole. Section from the left middle lobe of calf 2 of the PI-3-CONTROL group. PI-3 virus was isolated from this lobe. X30.

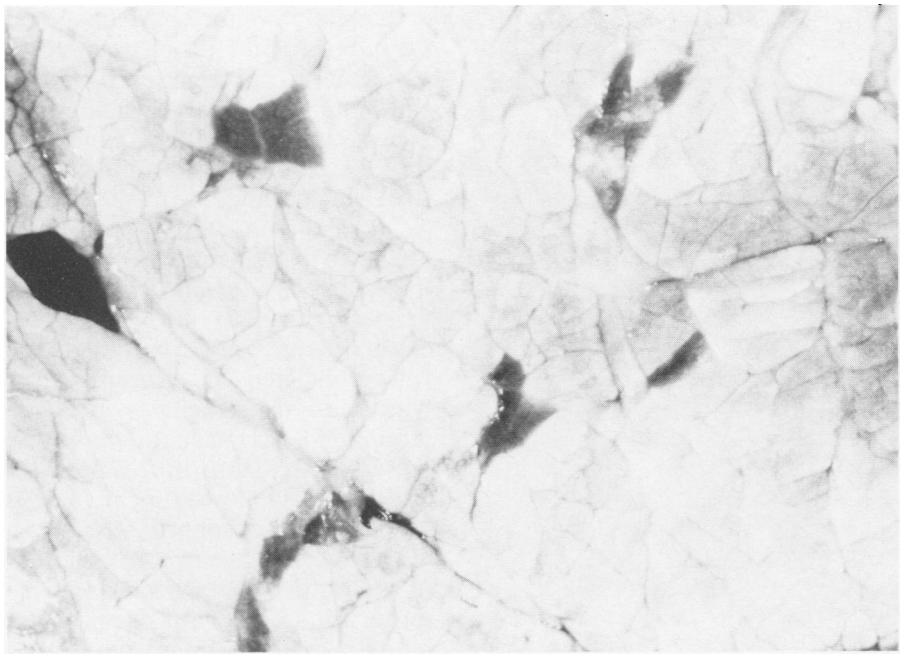

Fig. 9. Dark red focal areas of atelectasis measuring between 0.5 and 2 cm in diameter. Right lung of calf 17 from the UNTREATED CONTROL group.

time intervals between exposure to PI-3 virus and necropsy (seven and 11 days) were probably insufficient to allow calves to mount a detectable humoral immune response.

There are very few reports in the literature of experimental PI-3 virus induced lesions in the respiratory tract of conventionally-raised calves. Tsai and Thomson (23) infected colostrum-deprived (CD) and conventionally-raised (CR) calves with aerosolized PI-3 virus and observed microscopic tracheal lesions as well as gross and microscopic pulmonary lesions similar to those encountered in the present study in CD calves killed on days 5 and 6 and to a lesser extent in CR calves killed on days 7 and 12. These authors also reported the presence of intranuclear and intracytoplasmic inclusion bodies in the trachea and lungs of CD calves but not from CR calves. The presence of intranuclear and intracytoplasmic inclusion bodies in the respiratory tract of calves experimentally infected with PI-3 virus is transient and is more likely to occur during the first few days of infection (24, 30).

Variable results have been reported regarding PI-3 virus recovery from lung following experimental viral infection (10, 18, 19, 23). Betts *et al* (31) and Omar *et al* (26) recovered PI-3 virus from trachea and lung of specific-pathogen-free (SPF) calves infected with the virus five days previously but failed to recover virus from these tissues by the seventh day. Similarly, PI-3 virus was not recovered and macroscopic lesions were not present in the lungs of the calves from the PI-3-PAST group which were euthanized 11 days after viral aerosolization. However, the absence of macroscopic pulmonary lesions and the absence of PI-3 virus recovery from the lungs of three out of four calves from the PAST-PI-3 group were unexpected since these calves were killed seven days after viral exposure. An explanation for this could be that previous exposure to *P. haemolytica* may have enhanced rather than depressed the viricidal defense mechanisms of the lung and therefore prevented the production of PI-3 virus induced macroscopic pulmonary lesions. A possible mechanism for this could be that during the four day interval between bacterial and viral aerosol exposures the bone marrow mononuclear cell reserve may have been stimulated to release more circulating monocytes, therefore increasing the alveolar macrophage pool.

Serum antibody titres to *P. haemolytica* in the present study could not be directly compared with those from previous studies since different serological tests have been used (5, 17-21). The indirect

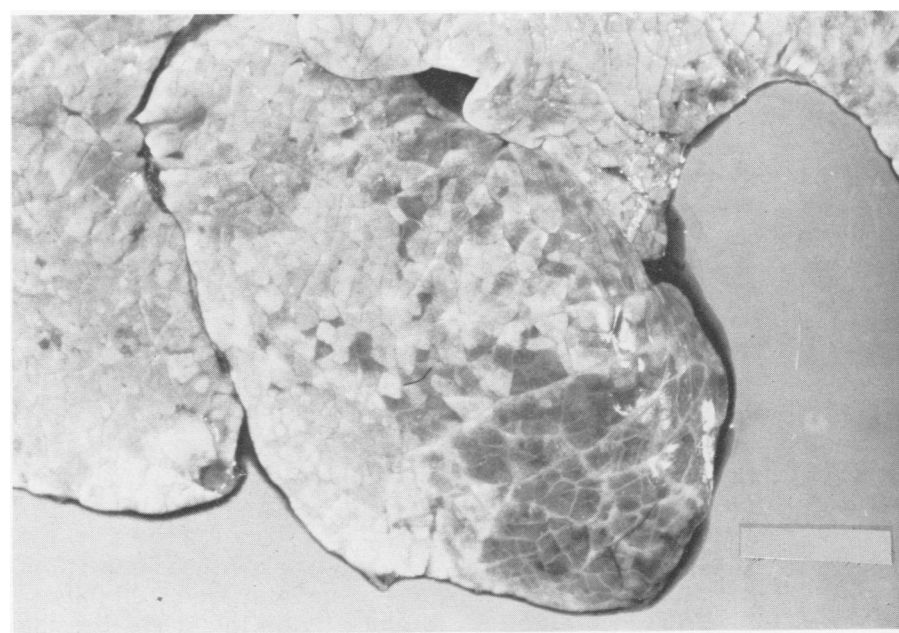

Fig. 10. Confluent dark red areas of consolidation measuring more than 0.5 cm in diameter. Right lung of calf 20 from the UNTREATED CONTROL group (enzootic pneumonia).

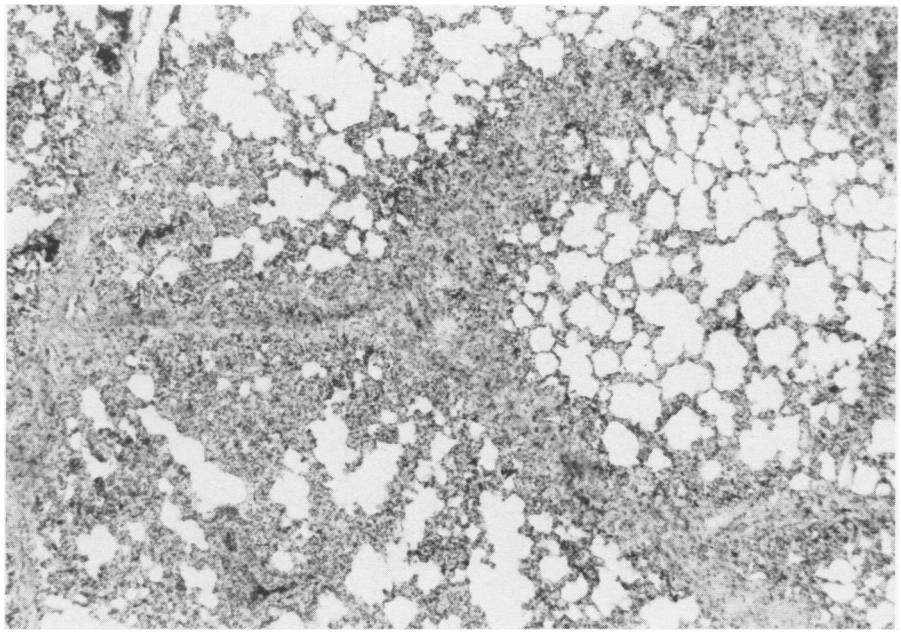

Fig. 11. Sublobular to lobular alveolar septal thickening with dense hypercellular interlobular septa. Section from the right middle lobe of calf 18 of the UNTREATED CONTROL group. X30.

microagglutination test used in the present study greatly increased the *P. haemolytica* serum antibody titre readings compared to the direct microagglutination titre. The assumption that the preinfection *P. haemolytica* serum antibody titres in calves of the present study may have been protective in preventing the establishment of *P. haemolytica* infection is speculative. Even though there is evidence (32, 33) that *P. haemolytica* antibody is positively correlated with protection, there is no indication in the literature of what constitutes protective levels of serum antibody. Although seroconversion to *P. haemolytica* infection has been shown to occur in calves after one week (34), only two out of four calves seroconverted after four days in the PI-3-PAST group and after 11 days in the PAST-PI-3 group (Table II).

In the present study, all 12 calves which received an aerosol of *P. haemolytica* demonstrated similar transient clinical signs of respiratory illness. Hematological changes observed in the present study are similar to those reported by Lopez (19) who also observed a significant increase in band neutrophils four hours after exposure to *P. haemolytica*. The variation in leukocyte response is likely the result of interaction between pulmonary injury and chemotaxis, and marrow granulocyte reserves.

The strain of *P. haemolytica* used in this study has been repeatedly used by others for experimental induction of fibrinous pneumonia by intrabronchial inoculation (21, 35). Distinctive pulmonary pathology was not produced by the exposure to aerosols of *P. haemolytica* in any of our groups. Very few researchers have reported the effect of experimental exposure to aerosols of *P. haemolytica* alone (36) and respiratory disease in calves has not been reported following exposure to single aerosols of *P. haemolytica*. Although, based on experimental work, *P. haemolytica* can be considered a primary pathogen without the help of a virus (21, 35), experimental induction of fibrinous pneumonia by aerosol inoculation appears to require a preceding pulmonary injury such as viral infection. This suggests that virulence factors, which may be important in the initial stages of development of pneumonia, may be lost in part or completely in the aerosolization procedure.

In the present study, it was not possible to demonstrate that reduced pulmonary clearance of *P. haemolytica* in PI-3 virus infected calves led to production of severe pulmonary lesions as was demonstrated in bacterial clearance studies in laboratory animals using different pathogens (37, 38, 39).

Jericho *et al* (10) exposed 22 calves to aerosols of PI-3 virus followed by *P. haemolytica* at intervals of three to ten days. Two experiments were conducted, the first consisted of exposing conventionally-raised calves with variable PI-3 HI serum antibody titres (0 to 1:128) to diluted aerosols of PI-3 virus followed six or 14 days later by an aerosol of *P. haemolytica*. The viral treatment resulted in sporadic rectal temperature elevations, necropsy revealed lobular-sized pulmonary lesions and these organisms did not appear to have any synergistic effect. The second experiment consisted of exposing conventionally-raised calves without any PI-3 HI serum antibody titre to undiluted aerosols of PI-3 virus followed three, four, six, seven and ten days later by an aerosol of *P. haemolytica*. The viral treatment produced consistent rectal temperature elevations and a synergistic effect in the form of purulent pneumonia at all time intervals in ten out of 14 calves. Although synergism of PI-3 virus and *P. haemolytica* infections was demonstrated in the second experiment, it is difficult to compare those results with the first experiment as well as with the present study. As stated by the authors, it is not clear if the failure to produce viral-bacterial synergism in the first experiment is related to the 14 day interval between aerosols, the preexposure HI serum antibody titres or the use of diluted PI-3 aerosols. The negative PI-3 virus serum antibody status of the conventionally-raised calves of the second experiment is unusual and could be important in determining susceptibility in this dual infection experimental model. In their study, Jericho *et al* (10) postulated that compromised clearance of bacteria may not be the most important effect of virus in the pathogenesis of viral-

bacterial synergistic pneumonia.

It should be stressed that the disparity between the results of the present study and those obtained by Jericho *et al* (10) are difficult to analyse. Aerosol apparatus, dose of virus, breed of calf, infectious agent antibody status of calves were different in both studies. Furthermore, due to the high prevalence of lesions of enzootic pneumonia in clinically healthy conventionally-raised calves, the use of untreated control calves in similar studies should be emphasized. There is increasing evidence that the effect of viral dose in dual infection experimental pneumonia in calves (10, 40) as well as in mice (41) is a critical factor. Although the concentrations of PI-3 virus inocula in the present study was similar to that used by researchers studying pulmonary clearance of *P. haemolytica* (5, 18, 19, 20), this dose may have been insufficient in compromising the pulmonary defense mechanisms and therefore unable to predispose to secondary *P. haemolytica* infection.

Finally, in the present *in vivo* study it was not possible to demonstrate that *P. haemolytica* could markedly impair the pulmonary defense mechanisms as has been suggested in *in vitro* studies with alveolar macrophages (12, 14, 42).

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