The Pulmonary Clearance of *Pasteurella haemolytica* in Calves Infected with Bovine Virus Diarrhea or *Mycoplasma bovis*

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ABSTRACT

Based on current literature which commonly associates bovine virus diarrhea virus and *Mycoplasma bovis* with "pneumonic pasteurellosis," an investigation was conducted into the effect of these two pathogens on the capacity of bovine lung to clear inhaled *Pasteurella haemolytica*. There was no significant effect ($p > 0.05$) of either bovine virus diarrhea virus or *M. bovis* on the mean clearance rate of *P. haemolytica*, nor did the time interval of three, five or seven days between the first inoculation and exposure to *P. haemolytica* adversely affect the lung clearance rates. However, it was found that the left lungs had a higher bacterial retention ($p < 0.05$) than the right lungs.

RÉSUMÉ

En se basant sur la littérature courante qui associe communément le virus de la diarrhée à virus bovine et *Mycoplasma bovis* à la pasteurellose pulmonaire, les auteurs ont réalisé une expérience qui visait à déterminer l'effet de ces deux agents pathogènes sur la capacité de clairance des poumons de bovins, après l'inhalation de *Pasteurella haemolytica*. Ni l'un ni l'autre de ces deux agents pathogènes n'exerça d'influence appréciable ($p < 0,05$) sur le taux moyen d'élimination de *P. haemolytica*; des intervalles de trois, cinq ou sept jours entre la première inoculation et l'inhalation de *P. haemolytica* n'exercèrent pas non plus d'influence adverse sur le taux de clairance pulmonaire. Les auteurs constatèrent toutefois que le poumon gauche affichait une rétention bactérienne plus élevée ($p < 0,05$) que le droit.

INTRODUCTION

"Pneumonic pasteurellosis" is one of the most important disease complexes of cattle in North America (11,21). Despite large amounts of money, time and effort expended, the pathogenesis of the disease remains unclear (13,29,30). Many infectious agents, including various viruses, chlamydias, mycoplasmas and bacteria, have been postulated to interact with *Pasteurella haemolytica* in the genesis of the disease (7), however few investigators have succeeded in routinely reproducing the disease under laboratory conditions (4,12, 13).

Bovine virus diarrhea (BVD) virus is widely distributed in the cattle population (15,22). Isolation of the virus and seroconversion during outbreaks of pneumonia have led some investigators to believe that BVD virus is a respiratory pathogen (1,25), however to our knowledge there are no experimental reports supporting the role of BVD virus in bovine pneumonia. Similarly, *Mycoplasma bovis* has often been associated with bovine respiratory disease (16,24), but again the role of this organism in "pneumonic pasteurellosis" is still controversial (5,21,24). The objectives of this investigation were to test, by means of the pulmonary clearance technique, whether BVD virus or *M. bovis* could impair the normal clearance of *P. haemolytica* from calves' lungs.

MATERIALS AND METHODS

Twenty-six male Holstein calves seven to ten weeks of age, purchased from private owners, were selected from a group of 60 similar calves. The selection was based on general health status and low antibody titers to either BVD virus ($< 1:8$ serum neutralization test) (18) or to *M. bovis* (negative by the indirect hemagglutination test) (2). The calves were distributed at random in six groups of four calves

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TABLE I. Experimental Design

Experiment	Number of Calves	Prime Inoculation (day zero)	Time Interval Between Inoculations (days)	Second Inoculation[a]
BVD-3	4	BVD-virus[b]	3	*P. haemolytica*[c]
BVD-5	4	BVD-virus[b]	5	*P. haemolytica*[c]
BVD-7	4	BVD-virus[b]	7	*P. haemolytica*[c]
Myc-3	4	M. bovis[b]	3	*P. haemolytica*[c]
Myc-5	4	M. bovis[b]	5	*P. haemolytica*[c]
Myc-7	4	M. bovis[b]	7	*P. haemolytica*[c]
Past-control	2	—	—	*P. haemolytica*[c]

[a]Aerosol, all calves were euthanized four hours after *P. haemolytica* aerosolization
[b]Intratracheal
[c]Aerosol

TABLE II. Concentration of Inoculum of BVD Virus and *Pasteurella haemolytica*

Experiment	*BVD Inoculum*[a] Titer/0.1 mL	*P. haemolytica Inoculum*[b] Titer/mL
BVD-3	1×10^4	6.5×10^{10}
BVD-5	1×10^4	1.3×10^8
BVD-7	1×10^4	7.0×10^{10}
Past-Control	Not exposed	4.0×10^8

[a]BVD virus aerosolized for 20 minutes
[b]Time of aerosolization = 30 minutes. Volume = approximately 150 mL

and one group of two calves. The groups were named BVD-3, BVD-5, BVD-7, Myc-3, Myc-5, Myc-7 and Past-control according to the type of inoculation (BVD virus or *M. bovis*) and the time interval between this inoculation and aerosolization of *P. haemolytica* (Table I). Included in the experiment were 35 male white mice[1] weighing about 30 g each.

PREPARATION OF INOCULA

The BVD virus inoculum was prepared from a 28th passage in embryonic bovine spleen cell culture of a cytopathic strain (Oregon) of BVD virus. The initial tissue culture infective dose (TCID) was 10^5 per 0.1 mL; it was diluted to an infective level of 10^4 per 0.1 mL and stored at -70°C until used. A volume of 80 mL of this culture was aerosolized to groups BVD-3, BVD-5 and BVD-7 during 20 minutes (Table II). (The variation in size of inoculum occurs under standardized conditions and is unexplained.)

A culture of *M. bovis* (stain 427 OVC) kept in liquid nitrogen was inoculated into 10 mL of mycoplasma medium (3) containing 15% fetal calf serum, and was incubated at 37°C for 24 hours. Two mL of this culture were then inoculated into 100 mL of broth and incubated at 37°C for 24 hours. The culture was centrifuged at 30,000 g for 30 minutes at 4 to 10°C. The sediment was resuspended in 2 mL of supernatant, titered and checked for bacterial contamination (Table III).

The preparation of *P. haemolytica* (biotype A, serotype 1) has been previously described (19) (Tables II and III).

INOCULATION METHODS

Bovine virus diarrhea virus and *P. haemolytica* were administered via aerosol. The aerosol apparatus and the method of aerosolization were the same as previously used in our laboratory and described elsewhere (19).

Two mL of *M. bovis* suspension were administered intratracheally to each calf by inserting a 16 gauge needle into the trachea and then passing a 15 cm long plastic tube[2] down to the bifurcation of the trachea. The syringe containing the mycoplasma inoculum was then connected to the tube. To facilitate the spreading of inoculum in airways, the tube was rotated gently during the slow injection. Once injection was completed, the syringe was disconnected and filled with 2 mL of phosphate buffer solution (0.1 M; pH - 7.5) and injected in order to wash the remaining *M. bovis* from syringe and tube. The tube was withdrawn first, followed by the needle, thus preventing accidental inoculation of the cervical subcutaneous tissue.

PULMONARY CLEARANCE

The four hour pulmonary clearance of *P. haemolytica* was calculated in all animals. The euthanasia, collection of samples and determination of bacteria per gram of lung in calves were the same as previously used in our laboratory and described elsewhere (19). The determination of initial deposition of *P. hemolytica* in calves lungs was calculated from the mices' lung deposition using the equation Y = -38222 + 0.419X. This equation was obtained from 14 different trials in which mice and calves were exposed to the same bacterial aerosol (20).

A total of 94 clearance values obtained from four samples of lung in each of the 26 calves were statistically analyzed[3] using logarith-

TABLE III. Concentration and Volume of *M. Bovis* Inoculum and Concentrations of *P. haemolytica*

Experiment	*M. bovis Inoculum*[a] Titer/mL	*Pasteurella haemolytica*[b] Titer/mL
Myc-3	4.4×10^9	4.0×10^9
Myc-5	9.0×10^{10}	5.0×10^8
Myc-7	6.7×10^{10}	1.2×10^9

[a]Volume of inoculum = 2 mL
[b]Time of aerosolization = 30 minutes. Volume = approximately 150 mL

[1]Connaught Laboratories, Toronto, Ontario.
[2]Becton Dickinson and Company, Rutherford, New Jersey.
[3]S.A.S. Institute Incorporated, Raleigh, North Carolina and Institute of Computer Science, University of Guelph, Guelph, Ontario.

TABLE IV. Deposition and Retention of *P. haemolytica*

Group	*P. haemolytica* in calves lungs at zero hour (X10/g)	*P. haemolytica* in calves lungs at four hours (X10/g)	Percent of *P. hemolytica* retained in lung
BVD-3	9.7	7.1 ± 16[a]	76.9 ± 106.1[a]
BVD-5	11.0	2.8 ± 2.2	25.7 ± 21.9
BVD-7	4.3	0.83 ± 0.77	19.3 ± 17.0
Myc-3	13.0	2.9 ± 3.6	22.0 ± 21.1
Myc-5	3.8	25.7 ± 68	602.9 ± 1186.4
Myc-7	2.8	0.43 ± 0.34	15.5 ± 5.4
Past-Control	8.7	3.3 ± 3.4	37.9 ± 25.6

[a]Mean ± one standard deviation

mic transformation. The following null hypotheses were tested:

Effect of treatment
H_0:BVD = *M. bovis* = Control
Effect of time
H_0:day 0 = 3 = 5 = 7
Effect of topography
H_0:Right lung = left lung; Apical lobe = diaphragmatic lobe

Interaction between treatment and days, and between different anatomical parts of the lungs were also investigated.

RESULTS

The deposition of *P. haemolytica* in the lungs of calves at zero hours was in the range of 10^5 to 10^6 organisms per gram of lung in all groups. The mean bacterial retention in the lungs of calves four hours after aerosolization was in the range of 10^4 to 10^6 organisms per gram of lung (Table IV). The percentage of *P. haemolytica* retained in the lungs was markedly higher in the Myc-5 group and to a lesser extent in the BVD-3 group; however the variance among these two groups of calves was also high as reflected in the standard deviation (Table IV). The statistical analysis did not reveal any significant differences ($p > 0.05$) among groups of calves treated with BVD virus or *M. bovis* compared to the Past-control group (Table V); nor did the time interval between inoculations have any significant effect (Table VI).

The interaction of treatment and day did not have any significance either ($p > 0.05$) (Table VII). A highly significant difference ($p < 0.01$) was detected in the bacterial retention between the left and right lobes of the lungs. The right lungs showed higher bacterial clearance rates than the left lungs (Table VIII). Apical and diaphragmatic lobes failed to show any significant difference ($p > 0.05$) in their ability to clear inhaled bacteria (Table IX). In the interaction (right-apical, right-diaphragmatic, left-apical, left-diaphragmatic) of topography, although the differences are not statistically significant ($p = 0.06$), the right apical lobes of the lung had much less retention of bacteria than the other three lobes (Table X). Bovine virus diarrhea virus was isolated from the lung in all but two of the inoculated calves. *Mycoplasma bovis* was isolated from the lungs of four out of 12 inoculated calves.

TABLE V. Mean Bacterial Retention (Log) of *P. haemolytica* in Calves Treated with BVD Virus or *M. bovis*

Treatment	N	$\bar{x}$ retention	P
BVD	48	0.86156	
M. bovis	48	0.84919	0.75
Past-control	8	0.90711	

N = Number of lung samples
P = Probability (F calculated < F tabulated)

TABLE VI. Mean Bacterial Retention (Log) of *P. haemolytica* for Days Zero, Three, Five and Seven

Day	N	$\bar{x}$ retention	P
0	8	0.90711	
3	32	0.85093	0.58
5	32	0.88282	
7	32	0.83237	

N = Number of lung samples
P = Probability (F calculated < F tabulated)

TABLE VIII. Mean Bacterial Retention (Log) of *P. haemolytica* for the Right and the Left Lungs

Lung	N	$\bar{x}$ retention	P
Right	52	0.83622	0.013
Left	52	0.88249	

N = Number of lung samples
P = Probability (F calculated < F tabulated)

DISCUSSION

The null hypothesis stating that neither BVD virus nor *M. bovis* have any effect on the bacterial clearance of *P. haemolytica* in bovine lung could not be rejected at the 0.05 level of significance. That is, the null hypothesis is accepted. This lack of effect of treatment was largely due to great variation within and between animals of the same experimental group. Logarithmic or square root transformation (8) did not greatly reduce the large dispersion of values and therefore had no meaningful effect on the statistical analysis.

The marked dispersion of values among different animals of a group receiving the same treatment, either BVD virus or *M. bovis*, is similar to that already

TABLE VII. Mean Bacterial Retention (Log) of *P. haemolytica* for BVD-3, 5, 7 and MYC-3, 5, 7 and Past-control

Treatment	Day	N	$\bar{x}$ retention	P
BVD	3	16	0.88041	
BVD	5	16	0.87038	
BVD	7	16	0.83388	
M. bovis	3	16	0.82144	
M. bovis	5	16	0.89525	0.68
M. bovis	7	16	0.83086	
Past-Control	0	8	0.90711	

N = Number of lung samples
P = Probability (F calculated < F tabulated)

TABLE IX. Mean Bacterial Retention (Log) of *P. haemolytica* for the Apical and Diaphragmatic Lobes of the Lung

Lung	N	$\bar{x}$ retention	P
Apical	52	0.85213	0.475
Diaphragmatic	52	0.86658	

N = Number of lung samples
P = Probability (F calculated < F tabulated)

TABLE X. Mean Bacterial Retention (Log) of *P. haemolytica* for the Apical and Diaphragmatic Lobes with the Right and Left Side of Lungs

Lung	N	$\bar{x}$ retention	P
Right-apical	26	0.81078	0.06
Right-diaphragmatic	26	0.86166	
Left-apical	26	0.89347	
Left-diaphragmatic	26	0.87150	

N = Number of lung samples
P = Probability (F calculated < F tabulated)

described in the infected lungs of laboratory animals (6,9,14) and in calves infected with parainfluenza-3 (PI-3) virus (19). The reason why similar animals respond so differently in lung clearance when they have been previously infected with a virus remains to be solved.

The null hypothesis of day 0 = 3 = 5 = 7, indicating that the time interval between treatment with BVD virus or *M. bovis* and *P. haemolytica* does not affect bacterial clearance, could not be rejected at the 0.05 significance level. That is, these experiments indicate that there is not significant inhibition of *P. haemolytica* clearance at any of three, five or seven days postinoculation with either BVD virus or with *M. bovis*. These results are in contrast to previous experiments with PI-3 virus in which significant differences were observed among clearance rates at the intervals of three, seven and eleven days between virus and bacteria, with a severe detrimental effect on bacterial clearance occurring only on day 7 (19). Similarly, a critical time interval between bovine herpesvirus 1 (IBR) and *P. haemolytica* inoculations has also been demonstrated in the experimental induction of "pneumonic pasteurellosis" (12).

The null hypothesis indicating that all four topographical areas of the lung will have the same bacterial clearance was rejected at the 0.05 level for the right versus left lung but could not be rejected for the apical versus diaphragmatic lobe. In other words, it was demonstrated statistically that the way in which the left lung handled bacterial clearance significantly differed from the right lung. According to these results, the right lung had less retention than the left, therefore the right lung had better clearance than the left one. It is possible to accept this only if we assume that the bacterial deposition at the time of aerosolization will be the same for the right and left lungs. It has been reported that the deposition of inhaled bacteria is equal in all parts of bovine lung with the exception of the tips of the diaphragmatic lobes (17). However, slight differences have been reported in clearance of *Serratia marcescens* for dorsal or ventral, and cranial or caudal lung but not between right and left lung (31). To our knowledge, no scientific evidence is available to correlate these findings of different clearance activity between left and right lung with pneumonia under field conditions (26,27).

Some of the animals used in this investigation reacted atypically in the pulmonary clearance studies, and it remains obscure why some calves receiving the same experimental treatment reacted so differently. Jericho clearly pointed out this phenomenon in experimental "pneumonic pasteurellosis" stating, "What determines susceptibility? Not all calves in a group of four would succumb to the experimental model of infection . . ." (13). The same phenomenon is also well known in field conditions (26,27).

Our results indicate that neither BVD virus, nor *M. bovis* had the capacity to significantly impair the pulmonary clearance of *P. haemolytica*. It is known in laboratory animals that decreased clearance correlates with susceptibility to pneumonia (9,10), however this has not been proven or disproven in cattle, therefore our results do not rule out the possibility that either BVD virus or *M. bovis* may predispose to pasteurella pneumonia by mechanisms other than depressed clearance of bacteria from bovine lung. Based on our results and on the lack of other supporting experimental evidence, these two agents cannot unequivocally be included in the long and already confusing list of etiological agents involved in pneumonic pasteurellosis.

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