Prevention of Experimental Bovine Pneumonic Pasteurellosis with an Extract of *Pasteurella haemolytica*

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ABSTRACT

A total of 36 calves were used in three experiments to test the efficacy of a potassium thiocyanate extract of *Pasteurella haemolytica* in protecting against experimental pneumonia. In each of experiments A and B, 12 calves were divided into three equal groups. The first group was vaccinated with an aerosol of a potassium thiocyanate extract twice, two weeks apart; the second group was vaccinated subcutaneously once only with the same extract. The third group of calves in both experiments remained as unvaccinated controls. In experiment C, six calves were vaccinated intramuscularly and six were left as controls. Approximately one month after vaccination all calves were challenged with an aerosol of bovine herpesvirus 1 (isolate 108) followed in 4 d by an aerosol of *P. haemolytica* type A1 (the same strain from which the potassium thiocyanate extract had been made).

Varying degrees of protection against subsequent development of experimental pneumonic pasteurellosis in cattle were seen in vaccinated calves as compared to control calves in these experiments. The results indicate that protection of cattle against pneumonic pasteurellosis may prove possible with a sub-cellular extract of *P. haemolytica*.

Key words: Pneumonia, cattle diseases, pasteurella infections, potassium thiocyanate extraction.

RÉSUMÉ

Cette étude consistait à déterminer l'efficacité d'un extrait au thiocyanate de potassium d'une souche de *Pasteurella haemolytica*, comme moyen de protection contre une pneumonie expérimentale. Les auteurs réalisèrent à cette fin trois expériences qui impliquaient en tout 36 veaux. Dans chacune des expériences A et B, ils utilisèrent trois groupes de quatre veaux. Le premier fut vacciné deux fois, à deux semaines d'intervalle, au moyen d'aérosols de l'extrait précité; le deuxième ne reçut qu'une seule injection sous-cutanée du même extrait. Les veaux du troisième groupe servirent de témoins, dans chacune des deux expériences. Dans l'expérience C, six veaux reçurent le vaccin, par la voie intramusculaire, et les six autres servirent de témoins. Environ un mois après la vaccination, tous les veaux furent soumis à une infection de défi qui impliquait l'utilisation d'aérosols de la souche #108 de l'herpèsvirus bovin #1 et de *P. haemolytica* A1, à quatre jours d'intervalle; c'est cette souche de *P. haemolytica* qui avait servi à préparer l'extrait précité.

Les auteurs constatèrent divers degrés de protection contre le développement ultérieur d'une pasteurellose pulmonaire expérimentale, chez les veaux vaccinés, comparativement aux témoins. Les résultats de ces expériences indiquent que la protection des bovins contre la pasteurellose pulmonaire peut s'avérer possible, lorsqu'on les vaccine avec un extrait subcellulaire de *P. haemolytica*.

Mots clefs: pneumonie, maladies bovines, infections à *Pasteurella* sp., extraction au thiocyanate de potassium.

INTRODUCTION

Shipping fever is a cause of substantial economic loss to the beef cattle industry of Canada and to feedlots in particular. It is usually associated with *Pasteurella haemolytica* or, less commonly, *Pasteurella multocida*, so that the term pneumonic pasteurellosis is also used. A similar condition can be produced experimentally by exposure to an aerosol of bovine herpesvirus 1 (BHV1) followed 4 d later by *P. haemolytica* (1). Development of this experimental disease can be prevented by vaccinating calves against the viral component (2,3,4). Corstvet *et al* (5) have commented on the paucity of data on the efficacy of pasteurella bacterins and on the importance of route of administration. The aim of the experiments described in this

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paper was to vaccinate cattle with a bacterially derived extract to determine if this would prevent subsequent development of fibrinous pneumonia after experimental challenge.

MATERIALS AND METHODS

PRODUCTION OF ANTIGEN

The methods used were essentially those of Bain (6) and Mukkur (7), performed as follows. For experiment A, *P. haemolytica* type A1 was grown on blood agar plates at 37°C for 24 h. The organisms were harvested from the plates by washing them off with sterile normal saline. The washings were added to an equal volume of 1.0 M potassium thiocyanate (KSCN) and incubated without agitation for 6 h at 37°C. The extract was centrifuged at 16300 × g for 10 min and the supernatant was dialyzed against 0.01 M tris HCl-0.32 M NaCl buffer containing 0.1% sodium azide and the alkalinity adjusted to pH 8.0 with 0.1 M NaOH. The dialysis was continued for 24 h with three changes of buffer. The extract was divided into two equal volumes: the first was precipitated with alum (the potassium salt) for subcutaneous (SC) administration using the method outlined by Garvey *et al* (8), while the second was used with no further modification as the antigen for vaccination by aerosol.

The antigen used in experiment B was produced as for the first experiment with some modifications. The same organism was grown on blood agar under the same conditions and harvested and dialyzed similarly. However, on this second occasion the extract was found, using the same estimation procedure,[1] to have approximately 30% of the former amount of protein. Therefore the total volume of dialysate was reduced by freezing and sublimation (partial freeze drying). The final volume to which the sublimate was reconstituted contained approximately the same amount of protein per unit volume as the antigen prepared for the first experiment. The alum precipitated antigen in the first experiment contained 9.50 mg of protein/mL while that for the second experiment contained 8.70 mg of protein/mL. The intranasal antigen in the first experiment contained 4.63 mg of protein/mL while that for the second experiment contained 2.64 mg of protein/mL. Thus each calf of experiment A given the precipitated antigen received a total of 238 mg of protein in 25 mL and each calf in experiment B given the precipitated antigen received a total of 218 mg of protein in the same volume. Each calf of experiment A given the aerosolized antigen received a total of 324 mg protein and in experiment B a total of 185 mg of protein.

In experiment C a much smaller amount of antigen was used as an economical measure and to provide a preliminary test of the relationship between dose and efficacy. It was prepared as described above except that the bacteria were grown in brain heart infusion broth supplemented with 1% horse serum in 10 L fermentors. The bacteria from a 6 h culture were pelleted by centrifugation, washed once in saline, and resuspended to 200 mL in saline. They were extracted with 1 M KSCN and further processed as described above except that pH was adjusted to 7.6. Each of the six calves was vaccinated with 4 mg given intramuscularly (IM).

VACCINATION AND CHALLENGE PROCEDURES

Experiment A — An outline of the experimental design is given in Table I.

Twelve crossbred yearling bull calves, raised at ADRI, Lethbridge, and ranging in weight from 200-320 kg, were divided into three equal groups. Group A-I was vaccinated twice, on d 0 and d 14, with an aerosol of the unprecipitated extract for approximately 30 min using a DeVilbiss 65 apparatus[2] at a setting of 10 so that each calf received approximately 35 mL each time. Group A-II was vaccinated once (d 0) with 25 mL of the alum-precipitated antigen injected SC but divided equally between two sites. Group A-III served as untreated controls. All calves were challenged individually, by exposure to aerosols of BHV1 (isolate 108) on d 27 and *P. haemolytica* on d 31, using methods described previously (1). The same isolates of *P. haemolytica* were used to produce the vaccine antigen and the challenge culture.

The bovine herpesvirus 1 (BHV1) suspension used for the exposures contained 1.2×10^6 plaque forming units (PFU) before and 1.5×10^7 PFU after aerosol production. There were 3.6×10^8 *P. haemolytica*/mL in the suspension of organisms used to generate the aerosol when titrated before, and 2.4×10^8 organisms/mL after, bacterial aerosolization.

The following samples and tissues were taken. On d 0, 7, 14, 21, 27, 31 and 35, nasal swabs were collected for viral and bacterial

TABLE I. Experimental Design

Experiment	Group	No. of calves	Challenge
A	IN vaccinates	4	
	SC vaccinates	4	
	Controls	4	
B	IN vaccinates	4	
	SC vaccinates	4	BHV1 + *P. haemolytica*
	Controls	4	(all calves)
C	IM vaccinates	6	
	Controls	6	

[1]Bio-Rad Protein Assay, Bio-Rad Laboratories, Tech. Bull. 1051, Richmond, California.
[2]DeVilbiss Corp., Somerset, Pennsylvania.

isolation procedures and blood samples were taken. The rectal temperatures of the calves were taken on the day of bacterial aerosol and necropsy. Surviving calves were necropsied on d 35 at which time swabs were taken of nasal passages, tonsils, trachea and lung for viral and bacterial studies. Pulmonary tissues were fixed in 10% formalin, embedded in paraffin, and sectioned later for histological studies.

Experiment B — The 12 calves used were of similar age and type to those in experiment A and were divided, treated and sampled in the same manner. However with Group B-I, because the antigen was more viscous, it was diluted 1:1 with sterile saline. Even so, after aerosol exposure for 1 h only half (approximately 35 mL) had been administered. The remainder of the vaccine dose (approximately 35 mL) was inoculated intranasally using a syringe and plastic cannula. Groups B-II and B-III were treated as in experiment A. The concentration of the BHV1 challenge suspension was $10^{7.8}$ $TCID_{50}$/mL before and after aerosolization. That of *P. haemolytica* was 8.4×10^8 before and 6.9×10^8/mL after the aerosol procedure.

Experiment C — Twelve 8-9 mo old Hereford calves were obtained from a ranch in southwest Saskatchewan. Calves from this herd have proven to be uniformly susceptible to BHV1-*P. haemolytica* experimental pneumonia in the past.

Each of the six vaccinates was given an IM injection consisting of 1 mL KSCN extract (containing 4 mg protein) and 1 mL adjuvant on d 0. The viral-bacterial challenge was given with the DeVilbiss nebulizer at setting 10 on d 36 by exposure to a suspension of BHV1 with a concentration of 1.6×10^7 PFU/mL and on d 40 by exposure to *P. haemolytica* with a concentration of 1.1×10^9 viable bacteria per mL. Approximately 4-5 mL of suspension was aerosolized for each calf at each exposure.

Nasal swabs for preexposure isolations were taken on d 36. At necropsy only the lung was cultured. Other procedures were similar to experiments A and B.

SEROLOGICAL STUDIES

Serum neutralization titers against BHV1 were determined by preparing serial dilutions of serum and reacting 0.5 mL with 0.5 mL of virus (10^2 plaque forming units). Serum virus mixtures were added to quadruplicate 7 mm wells in a microtiter plate containing confluent Georgia bovine kidney cells. The plaques were enumerated as described previously (9).

A number of tests were used to assess the titer of antibodies against *P. haemolytica* and the results will be reported elsewhere.

BACTERIOLOGICAL STUDIES

Routine aerobic methods were used for isolation of *Pasteurella* spp.

Specimens for *Mycoplasma* spp. were collected in noninhibitory horse serum broth composed of Hayflick's medium (10) with thallium acetate and penicillin omitted. *Mycoplasma* and *Acholeplasma* spp. were isolated by plating on Hayflick's medium with 1% purified agar under 10% CO_2, and identified by fluorescent antibody staining. *Ureaplasma* spp. were isolated using Ureaplasma broth (11) followed by A-7 agar (12) under H_2 and CO_2. Identification was by characteristic color and morphology of colonies. Isolation of *M. dispar* was done using the medium described by Gourlay and Leach (13) modified by substitution of ampicillin for penicillin (14) and replacement of 50% of the Hartley's digest broth by Viande Foie infusion (15). Mycoplasma studies were not done in experiment C.

RESULTS

CLINICAL SIGNS

Experiment A — There were no untoward effects of the vaccination procedures. All the cattle appeared normal for the first 2 d after BHV1 exposure (d 27 and 28). On the next day, two of the aerosol vaccinates and one control were standing apart from the group and had shallow, rapid respirations. The mean rectal temperature for all 12 calves on the morning of the fourth day after BHV1 exposure (d 31) was 40.8 ± 0.8°C.

Eight hours after aerosol challenge with *P. haemolytica* (d 31) two controls, two aerosol vaccinates and one SC vaccinate were depressed and had shallow, rapid respirations. On d 32 two control animals were in severe respiratory distress. Two of the calves vaccinated by aerosol were also severely dyspneic. Two calves of the SC vaccinated group had an increased rate of respiration but were bright and alert.

One control calf died on d 32 and the next day (d 33) two others were in severe respiratory distress; the fourth calf appeared normal. Of the aerosol vaccinates, three were bright while the fourth was dyspneic. The calves in the SC vaccinated group had no obvious signs of respiratory disease at this time.

A second control died on d 33, leaving one dull and dyspneic calf and one apparently normal control. There was also one dyspneic aerosol vaccinate but all other calves appeared normal. The third clinically ill control calf died on d 34. On the day of elective necropsy (d 35), the affected aerosol vaccinate was moribund and was euthanized. The mean temperature of the surviving calves was 38.7 ± 0.7°C and they were sent for slaughter. The survival rates in all three experiments are given in Table II.

Experiment B — As in experiment A, all 12 calves were normal until the day of viral exposure (d 27). By the day of bacterial challenge, the mean rectal temperature for all calves was 40.5 ± 0.4°C. For the last 2 d of the experiment (d 33 and d 34), the control group's mean temperature was approximately one-half to one Celsius degree higher than the other two groups due to a fever in one control calf (41.7°C on d 33, 40.6°C on d 34).

There were no deaths in any group in experiment B.

Experiment C — Two of the control calves died on d 43, one on d 44 and one on d 45 whereas two survived until elective necropsy on d 49. One of the vaccinates died on d 46 whereas all the others lived until d 49.

PATHOLOGICAL FINDINGS

The following terms will be used in this paper for tabulated results and also individual descriptions. Gross pulmonary changes identified as "BHV1 (108)" lesions are those seen in the lungs of calves experimentally infected with BHV1, isolate 108 (1-4), and consist of areas of sublobular, lobular or multilobular pulmonary atelectasis scattered randomly throughout the lungs. Cross sections of such areas usually reveal white, viscous exudate in airways. These viral lesions are found on histological examination to consist of collapsed alveoli, diphtheritic bronchitis and bronchiolitis, accumulations of cellular debris in bronchioles and bronchi, and, on occasion, small focal areas of caseous pulmonary necrosis.

"Combined" lesions, as described in this paper, are the lesions of experimental fibrinous pneumonia produced by sequential infection with BHV1 (isolate 108) and *P. haemolytica* and indistinguishable from those associated with bovine pneumonic pasteurellosis. These include raised, reddish-black, hard areas of pulmonary consolidation, widespread parietal and visceral pleural deposits of fibrin, distended interlobular septa, and hydrothorax. The histological lesions associated with these gross morphological thoracic changes include septal distension with edema and fibrin, as well as lymphatic engorgement and thrombosis. Within alveolar parenchymal areas, there are waves of degenerate cells surrounding areas of necrosis, and alveoli containing streaming, oat-shaped cells, edema, fibrin and hemorrhage. In addition, there is pleural deposition of fibrin as well as occasional histological changes associated with BHV1 (108) infection.

The presence or absence of each type of lesion is tabulated in Table II.

Experiment A — Complete necropsies were done on all calves that died and the one that was moribund. The animals with no apparent illness on d 35 were sent to slaughter where only the lower respiratory tracts (i.e. tracheas and lungs) could be obtained.

All four calves of the aerosol vaccinated group had macroscopic and microscopic pulmonary lesions. In two animals there were BHV1 (108) lesions and in the remaining two there were combined lesions. Of these, the calf that would have died had widespread fibrinous pneumonia involving 80% of the right lung and 50% of the left. The other calf had an area of fibrinous pneumonia involving the lower 30% of the posterior part of the right cranial lobe.

All calves of the SC vaccinated group had BHV1 (108) lesions visible grossly in their lungs but no combined lesions.

There were severe combined lesions in all animals of the control group with the exception of the survivor that went for slaughter. In this latter calf there was fibrinous pneumonia involving the ventral 60% of the left middle lung lobe. This calf also had BHV1 (108) lesions throughout the rest of its lung (Table II).

Experiment B — A necropsy was done on the control calf that remained febrile to the end of the experiment; the others were sent to slaughter, thus only the lower respiratory tracts were available.

All 12 calves had BHV1 (108) lesions as described above. Their extent and distribution varied considerably between individual animals, with no apparent relation to type of vaccine exposure. In addition, two controls had fibrinous pneumonia, i.e. combined lesions. In the calf that had remained febrile, 80% of the right middle lobe was swollen, heavy and had distended interlobular septa, and 25% of the cranial part of the right cranial lobe was similarly affected. One other control had an area of fibrinous pneumonia involving 10% of the lobe (Table II).

Experiment C — Complete necropsies were done on all calves. Five of six controls had combined lesions; in all but one of these, entire lung lobes were affected. One vaccinated calf had combined lesions involving 70% of the right lung field and one other had a similar lesion of the accessory lobe. The remaining four calves had no fibrinous pneumonia (Table II).

BACTERIOLOGICAL FINDINGS

In experiment A there were no isolations of *P. haemolytica* from the experimental calves until d 35 of the experiment, i.e. after the challenge exposures.

In experiment B both *P. haemolytica* and *P. multocida* were isolated from nasal swabs from some calves at various times prior to aerosol exposure. *Pasteurella haemolytica* was present in one control, two SC vaccinates and two

TABLE II. Survival Rates and Pathological Changes in the Lungs of Experimental Calves

Experiment	Group and route of vaccination	Survival rate	Macroscopic Lesions	
			BHV1 (108)	Combined
A	I (aerosol)	3/4	2/4	2/4
	II (SC)	4/4	4/4	0/4
	III (control)	1/4	1/4	4/4
B	I (aerosol)	4/4	4/4	0/4
	II (SC)	4/4	4/4	0/4
	III (control)	4/4	4/4	2/4
C	IM	5/6	5/6	2/6
	Control	2/6	5/6	5/6

aerosol vaccinates, whereas *P. multocida* was cultured from three controls and one SC vaccinate. Only one calf harbored both bacteria and this was the control calf in which the most severe fibrinous penumonia later occurred.

In experiment C, four of the six controls had *P. haemolytica* in their nasal passages on d 36, prior to viral aerosol. Of the four vaccinates that were sampled on that day, each had *P. haemolytica* and two also had *P. multocida* in the nasal passages.

The isolations of *P. haemolytica* at necropsy or slaughter are given in Table III.

Samples of one lung lobe taken from each calf of experiments A and B at the time of necropsy were negative for *Ureaplasma* and *Mycoplasma* spp., including *M. dispar*.

VIROLOGICAL FINDINGS

In experiment A no BHV1 isolations were made prior to viral aerosol exposure. Virus was recovered from nasal swabs of one control animal and two aerosol vaccinates on d 31 (the day of bacterial aerosol) and from two control calves on the day of necropsy. Bovine herpesvirus 1 was isolated at necropsy from tonsil, trachea and lung of two control calves and from one lung lobe of one other control calf. Virus was also isolated from the trachea and lung of one calf vaccinated by aerosol and from the lung of one calf vaccinated SC.

Similarly in experiment B, there were no isolations of BHV1 prior to experimental exposure. Bovine herpesvirus 1 was isolated from nasal swabs of one control, two SC vaccinates and two aerosol vaccinates on the day of bacterial exposure and from two controls, all four SC vaccinates and two aerosol vaccinates immediately prior to necropsy. At postmortem the virus was isolated from the trachea and lung of the control calf that had remained febrile and from one other control, and from the lung only of a third. It was isolated from the lungs but not the tracheas of all four SC vaccinates and from the lung and trachea of one aerosol vaccinate and the lung only of a second.

Antemortem nasal swabs for BHV1 were not taken in experiment C. At postmortem, BHV1 was isolated from the lungs of two of five controls (one not done) and none of four vaccinates (two not done).

SEROLOGICAL FINDINGS

In experiment A, no calves of the aerosol vaccinated group had serum neutralizing antibodies to BHV1 on either the day of vaccination or on the day of viral aerosol. In contrast, two calves of the SC vaccinated group had antibodies, one with a titer of 1:8, the other with a titer of 1:16 at these times. One calf in the control group, the sole survivor of that group, had a titer of 1:16 on the same two days.

In experiments B and C none of the calves had detectable antibodies to BHV1.

TABLE III. Isolations of *P. haemolytica* from the Respiratory Tracts at Necropsy

Experiment	Group and route of vaccination	Tissue			
		Nasal swab	Tonsil	Trachea	Lung
A	I (aerosol)	3/4[a]	4/4	3/4	3/4
	II (SC)	1/4	2/3	1/4	0/4
	III (control)	2/3	4/4	4/4	3/4
B	I (aerosol)	3/4	4/4	1/4	0/4
	II (SC)	1/4	1/4	0/4	0/4
	III (control)	3/4	4/4	3/4	1/4
C	IM	ND[b]	ND	ND	1/5
	Control	ND	ND	ND	3/6

[a]Number of calves from which *P. haemolytica* was isolated/number of calves sampled
[b]Not done

DISCUSSION

From the clinical, pathological and bacteriological results it is concluded that SC and IM vaccination with the KSCN extract of *P. haemolytica* conferred a certain degree of protection against subsequent experimental challenqe. A much lower degree of protection occurred in the calves in which the KSCN extract was used as an aerosol. Protection was demonstrated by reduced mortality, illness, and fibrinous pneumonia in all calves of the vaccinated groups, even with the small dose used in experiment C. Wilkie and Markham (16) did not find important quantitative differences in serum and lung antibodies to *P. haemolytica* in calves exposed by the SC versus the intrabronchial route. These facts may indicate that other defense mechanisms, such as cell-mediated immunity, are more important in protecting calves from *P. haemolytica* infection. No evidence for the possible deleterious effects associated with other *P. haemolytica* preparations given SC (17,18) were found in this study. This may indicate that there are numerous antigenic determinants associated with *P. haemolytica* and its products, some of which stimulate harmful responses, such as hypersensitivity, whereas others may stimulate immunity.

Pulmonary lesions similar to those described here as "BHV1 (108)" have been observed by workers dealing with other or unspecified strains of BHV1. Markson and Darbyshire (19), Pavlov (20), Braca *et al* (21) all recorded areas of atelectasis and these authors as well as Shroyer and Easterday (22) described necrotizing effects on the mucosa of pulmonary airways. These observations would correspond to the bronchitis, bronchiolitis and atelectasis referred to here as BHV1 (108) lesions.

In earlier work, development of experimental pneumonic pasteurellosis of cattle was prevented by vaccinating against the viral component of the disease (2,3,4). In those experiments challenge with

the homologous virus and *P. haemolytica*, i.e. vaccination with BHV1 (isolate 108) and challenge with BHV1 (isolate 108), produced complete immunity with no lesions either viral or combined being seen. In animals vaccinated with a commercial vaccine containing BHV1 and challenged with BHV1 (isolate 108) and *P. haemolytica*, i.e. heterologous viral challenge, small areas of viral and combined lesions were seen. In the present study viral [i.e. BHV1(108)] lesions were found in all groups both vaccinated with *P. haemolytica* and unvaccinated, but no combined lesions, i.e. fibrinous pneumonias, were found in any of the SC vaccinates.

The bacteriological data were consistent with those of the clinical and pathological findings. In the SC vaccinated group of experiment A the lungs were cleared of *P. haemolytica* by the day of necropsy whereas it was still present in the control and aerosol vaccinated groups, which had fibrinous pneumonia. In experiment B the only animal with *P. haemolytica* remaining in its lungs at the time of necropsy was the control calf with extensive fibrinous pneumonia of one lobe. In experiment C more controls than vaccinates were positive for *P. haemolytica* at necropsy. The presence of various pasteurellae in the nostrils and of antibodies to BHV1 in some calves may have affected individual susceptibility to the challenge system, but the number of animals used, with and without these complications in separate experiments and from two different sources, suggests that the bacterin was protective.

Prevention of the viral-bacterial synergism that produces pulmonary lesions has been reported in at least two other mammalian species. Vaccination against either the viral component of the combined Sendai virus-*P. pneumotropica* infection or the bacterial component of Sendai virus-*Proteus mirabilis* infection in mice will prevent the subsequent development of pneumonia in that species (23,24). Similar results have been reported for the combined parainfluenza type 3 virus-*P. haemolytica* infection in sheep where vaccination has been directed against the two agents separately (25,26).

The results presented here indicate that immunological methods of control may become important management tools in the prevention of pneumonic pasteurellosis, but much more information is needed about which antigenic determinants must be included in a preparation in order to ensure a beneficial immunological response.

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