Preliminary Studies with a Live Streptomycin-dependent *Pasteurella multocida* and *Pasteurella haemolytica* Vaccine for the Prevention of Bovine Pneumonic Pasteurellosis

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ABSTRACT

Twelve *Pasteurella*-free Holstein-Friesian calves were used in a study to test the efficacy of a live streptomycin-dependent *Pasteurella multocida* A:3 and streptomycin-dependent *Pasteurella haemolytica* A1 vaccine. The calves were inoculated intramuscularly twice at 14-day intervals with either the streptomycin-dependent vaccine, containing 1 x 10^6 colony forming units/mL *P. multocida* and 4 x 10^8 colony forming units/mL *P. haemolytica*, commercial bacterin, or phosphate buffered saline. Two weeks following the second vaccination, all calves were challenged by intranasal inoculation of 10^8 $TCID_{50}$/4.0 mL infectious bovine rhinotracheitis virus followed three days later by intratracheal injection with 2.3 x 10^7 colony forming units/mL of a 16 hour culture of *P. multocida* A:3 and 2.6 x 10^8 colony forming units/mL of an 8 hour culture of *P. haemolytica* A1. Seven days after challenge with *Pasteurella*, calves were killed for collection of tissues at necropsy. Each calf was given a score based on macroscopic and microscopic lesions. The scores for the calves receiving live vaccines were significantly lower ($p < 0.025$) than those for the controls. Also, the calves receiving live vaccines had a significant ($p < 0.05$) increase in the level of serum antibody to *P. haemolytica*. The results of this preliminary study showed that the streptomycin-dependent vaccine offered better protection than the commercial bacterin against a virulent homologous challenge.

Key words: Streptomycin-dependent, *Pasteurella multocida, Pasteurella haemolytica*, Bovine pneumonic pasteurellosis.

RÉSUMÉ

Cette expérience portait sur 12 veaux Holstein-Friesian, exempts de *Pasteurella*; elle consistait à vérifier l'efficacité d'un vaccin vivant et préparé avec les souches *Pasteurella multocida* A:3 et *Pasteurella haemolytica* A1, dépendantes de la streptomycine. À 14 jours d'intervalle, certains veaux reçurent deux injections intramusculaires du vaccin précité qui contenait 1 x 10^6 unités formatrices de colonies/mL de *P. multocida* et 4 x 10^8 unités formatrices de colonies/mL de *P. haemolytica*, tandis que d'autres veaux reçurent deux injections d'une bactérine commerciale ou d'une solution saline isotonique. Deux semaines après la deuxième injection, tous les veaux subirent une infection de défi qui consistait en l'inoculation intranasale de 10^8 TCID/4 mL du virus de la rhino-trachéite infectieuse bovine et, trois jours plus tard, en l'inoculation intratrachéale de 2,3 x 10^7 unités formatrices de colonies/mL d'une culture de *P. multocida*, âgée de 16 heures, et de 2,6 x 10^8 unités formatrices de colonies/mL d'une culture de *P. haemolytica*, âgée de huit heures. Six jours après l'inoculation de ces cultures de *Pasteurella*, on sacrifia les veaux pour en effectuer la nécropsie et prélever des tissus. On donna à chaque veau un pointage basé sur les lésions macroscopiques et microscopiques. Celui des veaux qui avaient reçu le vaccin vivant s'avéra significativement moins élévé ($p < 0,025$) que celui des témoins. Les veaux qui avaient reçu le vaccin vivant affichèrent aussi une élévation appréciable ($p < 0,05$) de leur taux d'anticorps sériques contre *P. haemolytica*. Les résultats de cette étude préliminaire démontrèrent que le vaccin dépendant de la streptomycine offrait une meilleure protection que la bactérine commerciale contre une infection de défi homologue virulente.

Mots clés: dépendant de la streptomycine, *Pasteurella multocida, Pasteurella haemolytica*, pasteurellose pulmonaire bovine.

INTRODUCTION

Bovine pneumonic pasteurellosis (BPP), also called shipping fever, is an important, widespread disease resulting in severe economic losses to the cattle industry. The disease is due to an interaction between environmental factors and infectious agents, such as viruses and bacteria (1). Although many viruses and bacteria may be associated with the bovine respiratory disease complex, there is very little doubt concerning the strong association of either *Pasteurella haemolytica* or *Pasteurella multocida* with fibrinous pneumonia of shipping fever (2). Recent scientific literature has emphasized the importance of *P. haemolytica* over *P. multocida* in BPP (3,4). A frequency of *P. haemolytica* as high as 90% has been reported from

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cases of fibrinous pneumonia (3). In North America, *P. haemolytica* biotype A, serotype 1, has been demonstrated to be the species most often recovered from the lungs of cattle suffering from BPP (5,6). In recent years, several methods have been developed to reproduce BPP in experimental calves with reasonable success (7-11). One such method of consistently reproducing BPP involves using aerosols of bovine herpesvirus-1 four days before the administration of *P. haemolytica* (7).

Many bacterins consisting of *Pasteurella* spp. have been used to immunize calves against BPP, but there is some question as to their inefficacy (2,10,12-15). In addition, bacterin has been shown to cause adverse effect in vaccinated calves to challenge exposure with *P. haemolytica* (10,16). Because of the inadequate protection offered by bacterins, there is a need for a live or subunit vaccine for the prevention of BPP. Studies in recent years have indicated that immunization of calves with live *P. multocida* (17,18) or *P. haemolytica* (18-20) can protect them from experimental pneumonic pasteurellosis. A vaccine containing live bacteria, as opposed to killed, may offer better protection due to the presence of additional antigenic components which may not be present in the bacterin or may be chemically altered during its preparation. Candidates for these antigenic components include an exotoxin of *P. haemolytica* (21-23) and a capsular antigen of *P. multocida* (24). In addition, live vaccine may offer better cross-serotype protection than bacterin (25). A longer antigenic stimulus may also be seen in animals injected with a live product due to bacterial cell multiplication (26).

A live streptomycin-dependent (STR^D) *P. multocida* vaccine has been successfully used in mice and rabbits (26), in turkeys for the prevention of fowl cholera (27) and in rabbits for the prevention of snuffles (28). The advantage to using a STR^D vaccine is that the animal is given the benefits of a live vaccine without the danger of exposure to the virulent wild type organism. The purpose of the present study was to describe the testing of a live STR^D *P. multocida* and STR^D *P. haemolytica* vaccine for the prevention of BPP.

MATERIALS AND METHODS

VACCINAL STRAINS

Vaccinal STR^D *P. multocida* strain P1062, type A:3 and STR^D *P. haemolytica* strain P1148, type 1 (obtained from M.M.Chengappa, Kansas State University, Manhattan, Kansas) were grown for 18 hours on dextrose starch agar (Difco) plus 400 μg streptomycin (STR, Sigma)/mL. The mucoid colonies were then transferred to 25 mL tryptose broth (Difco), plus 0.3% yeast extract (Difco), plus 400 μg STR/mL and grown for 16 hours in a 37°C shaker water bath. Following incubation, the cells were harvested by centrifugation at 5000 x *g* for 30 minutes. The cells were washed twice in 0.15 M saline containing 400 μg STR^D/mL, resuspended in 10 mL of 10% skim milk (Difco) and lyophilized. The lyophilized cultures were resuspended in 10 mL phosphate buffered saline (PBS) just prior to intramuscular (IM) injection into the test animal. Viable cells were estimated by the standard pour-plate technique. The bacterins containing *P. multocida* A:3 and *P. haemolytica* A1 were obtained commercially (Pitman-Moore, Inc., Washington Crossing, New Jersey).

CALVES

Twelve Holstein-Friesian bull calves, weighing 131.7 ± 38.3 (mean ± SD) kg, were assigned to three equally sized groups (Table I). All calves had been clinically healthy since birth. Clinically evident infectious bovine rhinotracheitis (IBR) and BPP have not been encountered in this herd.

VACCINATION PROCEDURE

The calves in group A were vaccinated IM in the hip with two doses, two weeks apart, each containing 1 mL STR^D *P. multocida* and 1 mL STR^D *P. haemolytica*. The concentration of *P. multocida* and *P. haemolytica* were 1 x 10^8 colony forming units (CFU)/mL and 4 x 10^9 CFU/mL, respectively. Group B calves were vaccinated IM with 1 mL each of *P. multocida* and *P. haemolytica* bacterin, given twice, two weeks apart. Group C calves were injected IM with two doses of 2.0 mL PBS, two weeks apart. Two and three days after vaccination, nasal swabs of group A calves were checked for the presence of STR^D *Pasteurella* organisms.

CHALLENGE INOCULUM AND PROCEDURE

Two weeks after receiving the second dose, all calves were challenged deep intranasally (IN) with a 4.0 mL inoculum (2.0 mL/nostril) of Cooper strain IBR virus (IBRV, provided by Donald Groghan, National Animal Disease Center, Ames, Iowa). The concentration of IBRV was 2.5 x 10^7 $TCID_{50}$/mL. The virus was deposited via a 12-inch cannula (Butler Company, Lexington, Kentucky) inserted deep into the nostril. Three days following IBRV challenge, all calves were anesthesized and a 12-inch cannula was inserted into the trachea of each. The challenge inoculum containing *P. multocida* and *P. haemolytica* was prepared in the following manner. *Pasteurella multocida* strain P1062, type A:3 and *P. haemolytica* strain P1148, type 1 were originally isolated from bovine pneumonic lungs. One-tenth of 1 mL suspension of each of *P. multocida* and *P. haemolytica* culture, turbidity corresponding to a number 8 McFarland nephelometer tube, was injected intraperitoneally into two adult Swiss Webster ICR albino mice and recovered from the heart blood. In the case of *P. haemolytica*, 0.1 mL of hemoglobin was injected along with the culture, as described by Chengappa *et al* (29). The mouse-passed organisms were then grown in 25 mL tryptose broth plus 0.3% yeast extract. The challenge dose, containing 2.3 x 10^7 CFU/mL of a 16 h culture of *P. multocida* and 2.6 x 10^8 CFU/mL of an 8 h culture of *P. haemolytica*, was suspended in 20 mL sterile PBS and deposited deep intratracheally through the cannula.

CLINICAL OBSERVATIONS

Rectal temperatures and lung sounds of the calves were recorded upon arrival and daily following IBRV challenge. Calves were observed for clinical signs daily following IBRV exposure.

SEROLOGICAL TESTS

Blood samples were collected from all calves upon arrival and prior to IBRV challenge. Serum was separated

and stored at -20°C until titrated for specific antibody activity. A serum neutralization test (30) was used to determine the presence of antibodies against IBRV. The indirect bacterial agglutination test as described by Wilkie and Markham (31) and the indirect hemagglutination test as described by Sawada *et al* (32) were used to detect specific antibodies against *P. haemolytica* and *P. multocida*, respectively.

NECROPSY PROCEDURES

All calves were euthanized by barbituate overdose six days following *Pasteurella* challenge. The lungs of each calf were examined and scored as follows: 0 = no visible lesions; 1 = fibrinous adhesions with adjacent lobes; 2 = firm, nodular, suppurative exudate in bronchioles, affecting < 50% of individual lobe; 3 = firm, dark pink to grey and necrotic with suppurative exudate filling bronchioles, affecting < 50% of two or more lobes or > 50% of one lobe; 4 = firm, nodular regions and suppurative exudate filling the bronchioles and affecting > 50% of two or more lobes.

MICROBIOLOGY

Nasal swabs were collected from all calves on day 0 and day 1 for microbiological evaluation. Sections of the lymph nodes, lung and bronchial lavage were submitted for bacterial isolation. Organisms recovered from these samples were identified as *P. multocida* and *P. haemolytica* using the characteristics described by Carter (33). Sections of trachea, lung and lymph nodes were collected for virus isolation (34).

HISTOPATHOLOGY

Lung tissues were fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. The stained sections were examined and scored as follows: 0 = no lesions; 1 = diffuse edema and slight thickening of alveolar septa; 2 = remarkable edema of interlobular connective tissues and diffuse mononuclear leukocytic infiltration; 3 = multiple large nodules containing centers of suppurative inflammation surrounded by connective tissues, fibroblasts, and aggregates of macrophages, and accumulation of neutrophils; 4 = similar to 3 but more severe in nature, multifocal large abscesses, large areas of necrosis and purulent inflammation of pulmonary lymph nodes.

STATISTICS

Scored data on microscopic and macroscopic lesions were analyzed for statistical significance using a Kruskal-Wallis test. Serum antibody titers were evaluated using Student's t test.

RESULTS

CLINICAL OBSERVATIONS

The injection of the STR^D vaccine did not cause obvious adverse effects, such as swelling at the injection site, lameness, or anorexia. The calves had normal lung sounds and rectal temperatures on the day of arrival at the research facility. Following IBRV exposure, the calves in all three groups had elevated rectal temperatures (Fig. 1). Temperatures peaked on the second and fourth day following IBRV exposure and then started declining toward normal base values. All calves had a mucopurulent nasal discharge by the fifth day following IBRV exposure. All calves from the control group, three from the bacterin group and two from the STR^D-vaccine group had raspy, shallow breathing and a cough by day 6 postchallenge. None of the calves died as a result of challenge.

NECROPSY AND HISTOPATHOLOGY FINDINGS

STR^D-vaccine group — Upon necropsy, lungs of calves 21, 77 and 106 appeared to have no visible lesions. However, in calf 25, 100% of the left apical lobe was firm and dark pink with suppurative exudate filling the bronchioles and the lymph nodes were two times the normal size. The microscopic examination of the lung of calf 25 showed aggregations of neutrophils within the bronchioles and the alveoli and multiple large abscesses. The other calves of the group were classified as normal since there were no microscopic changes.

Bacterin group — Calf 19 had a few fibrinous adhesions between the right cardiac and apical lobes. In calf 20, 90% of the right apical lobe was firm with multifocal necrosis. The lymph nodes in this calf were twice the normal size. The apex of the left apical lobe of calf 41 was firm and red. The lower 50% of the left apical lobe of calf 51 was firm, dark red with suppurative exudate in the bronchioles. Microscopic examination of the lung of calf 20 revealed multiple scattered abscesses surrounded with diffuse infiltration of macrophages and fibroblasts that filled most of the alveoli. Calf 41 had diffuse edema and thickening of alveoli in lobules with linear aggregates of neutrophils present within the airways. The lung sections of calf 51 had a few scattered abscesses

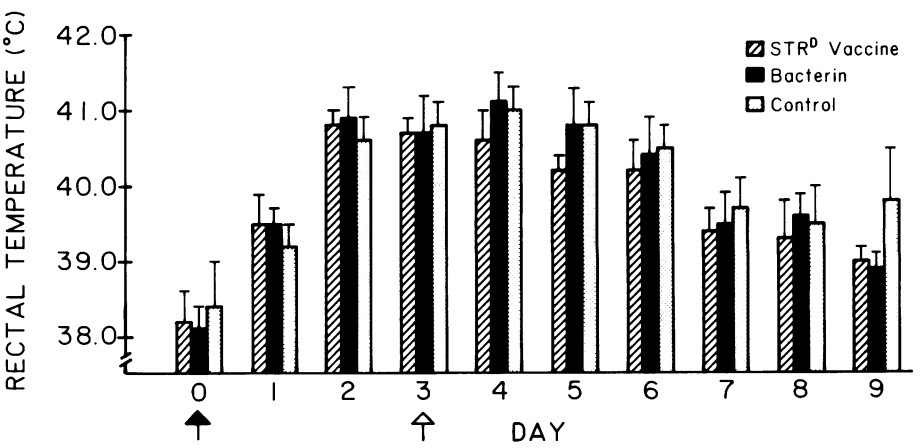

Fig. 1. Mean rectal temperatures of calves of STR^D vaccine, bacterin, and control groups after challenge exposure with IBRV (↑) and *P. haemolytica* and *P. multocida* (↑).

surrounded with macrophages and fibroblasts. Also, there was remarkable edema of the interlobular connective tissues and some bronchioles contained suppurative exudate.

Control group — Upon necropsy, calf 42 had dark red, firm areas containing fibrin in the interlobular septum covering 100% of the right apical and cardiac lobes and 60% of the left apical lobes. The affected lung areas of calf 44 was firm and dark red involving 100% of the right apical and cardiac lobes. The bronchioles were filled with suppurative exudate. Calf 200 had dark red, firm, necrotic areas in 20% of the right apical lobe and 40% of the left apical lobe and suppurative exudate filling the bronchioles. The lymph nodes were twice normal size in this calf. Microscopic examination of calves 42, 44 and 200 revealed multiple large abscesses and areas of necrosis surrounded with heavy clumps of neutrophils, fibroblasts and macrophages. Calf 79 had a few scattered small microabscesses present within the alveolar walls, which were infiltrated with suppurative exudate and surrounded with a few macrophages and fibroblasts.

The scores for the macroscopic and microscopic examination of lungs are listed in Table I. The total scores were: 6 for the STR^D vaccine group, 14 for the bacterin group and 25 for the control group. The difference between the average individual scores of live vaccine and control groups was significant ($p < 0.025$), but the difference between bacterin and control groups was not significant.

MICROBIOLOGY FINDINGS

The calves had negative isolation results for IBRV, *P. multocida* and *P. haemolytica* from two consecutive nasal swabs collected on day 0 and day 1. Following vaccination with STR^D vaccine, nasal swabs were negative for STR^D *P. multocida* and STR^D *P. haemolytica*. The presence or absence of IBRV, *P. multocida* and *P. haemolytica* in tissues of all the calves are presented in Table I.

SEROTEST RESULTS

Antibodies specific for IBRV and *P. multocida* were not detected in the calf serum collected before vaccination. However, low level serum antibodies specific for *P. haemolytica* were detected in the same serum samples (Table II). Analysis of the data using the Student's t test has shown a significant ($p < 0.05$) increase in serum antibody titer to *P. haemolytica* after vaccination only in the group that received STR^D vaccine. The results of the indirect hemagglutination test with *P. multocida* were inconsistent and not reproducible.

TABLE I. Microbiology and Pathology Results of Vaccinated and Control Calves Following Challenge with IBRV, *P. multocida*, and *P. haemolytica*

Group[a]	Calf No.	Isolation at Necropsy[b]			Macroscopic Lung Lesion			Microscopic Lung Lesion Score[c]
		Pm	Ph	IBR	Lobe Affected	% Area Involved[d]	Score[c]	
A	21	-	-	-	None	0	0	0
	25	-	+	-	Left apical	100	3	3
	77	-	-	-	None	0	0	0
	106	-	-	-	None	0	0	0
B	19	-	-	-	Right cardiac	<10[e]	1	0
					Right apical	<10[e]		
	20	+	+	+	Right apical	90	3	4
	41	-	-	-	Left apical	<10	1	1
	51	-	+	+	Left apical	50	2	2
C	42	+	+	-	Right cardiac	100	4	4
					Right apical	100		
					Left apical	60		
	44	+	-	-	Right cardiac	100	4	4
					Right apical	100		
	79	+	+	+	None	0	0	2
	200	+	+	-	Right apical	20	3	4
					Left apical	40		

[a]A = STR^D vaccine; B = bacterin; C = control
[b]Pm = *P. multocida*; Ph = *P. haemolytica*; + = isolated; - = not isolated
[c]See text for description of scores
[d]Of affected lobe
[e]Fibrinous adhesions between the right cardiac and apical lobes

TABLE II. Serum Antibody Titer to *P. haemolytica* in Vaccinated and Control Calves

Group[a]	Treatment	Mean Antibody Titer[b]	
		Before Vaccination	After Vaccination
A	STR^D vaccine	<10 ± 0	67.3 ± 0.7
B	Bacterin	<10 ± 0	10 ± 0
C	Control	<10 ± 0	14.1 ± 0.7

[a]For each group n = 4
[b]Geometric mean titers ± SD

DISCUSSION

In the present study, a live STR^D *P. multocida* and STR^D *P. haemolytica* vaccine was shown to provide a safe and effective means of stimulating the calf immune system against BPP. The total group scores were 6 for the STR^D vaccine group, 14 for the bacterin group and 25 for the controls, with the lower score indicating reduced pulmonary tissue damage resulting from challenge with IBRV, *P. multocida* and *P. haemolytica*. The scores for the calves receiving STR^D vaccine were significantly lower ($p < 0.05$) than those for the controls.

The success of the live STR^D vaccine may be partially due to residual streptomycin being injected along with the live product, allowing some replication of the vaccinal strains within the animal and thus increasing the antigenic stimuli. A vaccine containing live organisms may offer better protection due to presence of intact, additional antigenic determinants. On the contrary, a vaccine containing killed bacterial cells may offer inadequate protection due to the lack of such antigenic determinants. These antigenic determinants may well be an exotoxin of *P. haemolytica* and a capsular antigen of *P. multocida*. The exotoxin produced by the growing *P. haemolytica* cells is cytotoxic to bovine pulmo-

nary alveolar macrophages and neutrophils (21). Studies have indicated that only the presence of specific immune antibodies directed against the capsular antigen of *P. multocida* appeared to offer substantial protection in challenged animals (24). The better protection observed in the present study with the live vaccine may be partly due to an enhanced stimulation of antibodies against the cytotoxin of *P. haemolytica* and the capsular antigen of *P. multocida*. Further study is needed to determine the presence of such antibodies in calves inoculated with the STR^D vaccine. Kucera *et al* (19) used a chemically altered strain of *P. haemolytica* to protect experimental calves against a homologous challenge. Smith (20) successfully used a live *P. haemolytica* vaccine to prevent the development of BPP in preweaned calves. Whether or not STR^D vaccine is superior to other live *Pasteurella* products needs further study with a greater number of calves.

The serological results indicate that injection of two doses of STR^D vaccine significantly ($p < 0.05$) increased the level of serum antibody to *P. haemolytica*. The results of the IHA test for *P. muultocida* antibody were inconsistent and not reproducible. This was possibly due to the lack of a suitable test procedure to detect low level antibodies in the serum. The observations that two injections of the commercial bacterin did not significantly increase the level of serum antibody to *P. haemolytica*, and that the scores of the calves receiving the commercial bcterin were not significantly lower than those of the controls support other reports of the ineffectiveness of these products (15). Although this is only a preliminary study with four calves per group, the results warrant extensive field trials and additional studies to investigate whether the STR^D vaccine provides cross-serotype protection. Studies may also be necessary to investigate the possible involvement of a cell-mediated immune system in calves vaccinated with STR^D vaccine.

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