LABORATORY EVALUATION OF A POLYVALENT SHIPPING FEVER VACCINE

by

THOMAS EARL CLENDENNEN III, B.S.

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INTRODUCTION

Shipping fever is an acute pneumonic disease of cattle that is typically associated with stresses such as shipping or rough handling. This syndrome is believed to be the effect of combined infections of stressed cattle by bacteria of the genus Pasteurella and a number of viruses, especially parainfluenza-3 (PI-3), infectious bovine rhinotracheitis (IBR), and bovine viral diarrhea (BVD) (5, 26). The specific terminology, shipping fever, is misleading and frequently misapplied. Bovine pneumonic pasteurellosis is now suggested as preferred terminology since it is less likely to be misapplied and is more descriptive of the actual disease process. In the field, a wide variety of disease syndromes such as those produced by infection with Salmonella typhimurium, Arizona arizonae, and the enterotoxigenic strains of Escherichia coli may erroneously be labelled shipping fever (26).

Although there are vaccines available for PI-3, IBR, and BVD which are effective against the diseases specifically related to them, results with vaccines against bovine pneumonic pasteurellosis have been of questionable advantage to the livestock producer (4, 11, 12). Texas Vet Lab, Inc., San Angelo, Texas, has undertaken a project aimed at

producing an effective vaccine against the diseases associated with stressed cattle. Poly Bac B is a polyvalent bacterin prepared for use as an aid in immunizing cattle against bovine pneumonic pasteurellosis, salmonellosis, mixed bacterial enteritis, and bacterial pinkeye (bovine contagious keratoconjunctivitis). Organisms incorporated in Poly Bac B are: Pasteurella multocida, Pasteurella hemolytica, Salmonella typhimurium, Escherichia coli, Arizona arizonae, Proteus vulgaris, Pseudomonas aeruginosa, and Moraxella bovis. All these organisms were isolated from diseased cattle in Texas and may be considered primary or secondary pathogens for cattle.

A. Bovine pneumonic pasteurellosis.

Bovine pneumonic pasteurellosis can be distinguished from other types of bovine respiratory disease complexes by clinical signs and a distinctive fibrinous pneumonia evident by necropsy. Lesions of the lungs at necropsy frequently include swollen, firm anteroventral portions with red and purple discoloration of lobules separated by serofibrinous material in intralobular spaces. Pleuritis and serofibrinous adhesions are also characteristic of the disease. Clinical signs include depression, anorexia, serous nasal discharge, cough, fever, increased pulse rate, and rapid shallow breathing. Abnormal lung sounds are also characteristic of early stages with absence of lung sounds in later stages

when consolidation or pleural exudate accumulation are present (26).

Pasteurella multocida is closely associated with the characteristic clinical signs and lesions of bovine pneumonic pasteurellosis. This organism is sometimes isolated from the upper respiratory tract of healthy cattle but the frequency of isolation from stressed cattle is much greater (5). In the United States, P. multocida infection is most frequently encountered in livestock in association with PI-3, IBR, and BVD viruses (23). The host range of P. multocida is, as the name implies, virtually unlimited. Apparently all mammals and birds are suitable hosts. There is, however, some limited host range for each strain (13). Thus, many strains pathogenic for turkeys are not pathogenic for mice and vice versa (14). Carter designated types A, B, C, and D by hemagglutination (6). Roberts used serum protection tests to designate types II, I, III, and IV in the order that they correspond to Carter's types (5).

Type I, which is found only in Asian and African cattle, buffalo, and wild ruminants, is much more virulent than those types found in the United States and causes a different type of disease. For this reason, the term hemorrhagic septicemia is best reserved to denote the acute septicemic P. multocida type I infection seen in Asian and African ruminants. An LD_{50} of type I for buffalo calves has been estimated at as little as one (1) viable organism.

Necropsy of hemorrhagic septicemia victims revealed generalized hyperemic lesions of the gastrointestinal tract and lesions in the lungs (5).

Pasteurella hemolytica is morphologically similar to P. multocida and is also associated with bovine pneumonic pasteurellosis lesions. Pasteurella hemolytica is differentiated from P. multocida by its production of indole, growth on MacConkey agar, and complete hemolysis on blood agar, all of which are negative for P. multocida (29). Although P. hemolytica does not appear to be a primary pathogen in normal cattle, it is a primary pathogen in stressed cattle and is suspected as a contributing factor in morbidity and mortality associated with bovine pneumonic pasteurellosis (5).

B. Enteric diseases

The enteric diseases of shipped cattle present a much more complicated bacteriological picture than does bovine pneumonic pasteurellosis. This complication is partially due to the great diversity of normal gut flora as well as the possibility that many of the causative agents may be considered normal flora and are capable of causing disease only as secondary pathogens or in stressed cattle. The organisms included in Poly Bac B which are primarily associated with enteritis and diarrhea in cattle are: S. typhimurium, E. coli, A. arizonae, (Salmonella arizonae,

Arizona henshawii), P. vulgaris, and P. aeruginosa.

Salmonella typhimurium is a member of the family Enterobacteriaceae (18). Salmonella typhimurium has the Kauffmann-White antigenic formula 1,4,5,12:i:1,2 and is now properly known as Salmonella enteriditis serotype typhimurium (10). Salmonella typhimurium causes an acute contagious disease characterized by moderate fever and profuse hemorragic diarrhea. Transmission is generally fecal-oral, with contaminated feeds and waters apparently the greatest offenders. After ingestion, the bacteria rapidly invade the body from focal points in the intestine such as the Peyer's patches. Severe injury of the intestinal mucosa including necrosis of the epithelium and erosion of the blood vessels causes hemorrhaging into the intestinal lumen. Irritation of the intestinal tract provokes diarrhea early in the course of the disease. Bacteria soon penetrate the blood vessels and produce an active septicemia with foci of infection in the liver, spleen, and lymph nodes (28). In a few days, death may result from dehydration, hemorrhaging, intoxication, and septicemia. The salmonellae are rather indiscriminate pathogens and most species are capable of infecting almost any warm or cold blooded animal.

The economic impact of salmonellosis should not be overlooked. In the U. S. livestock and poultry industry, salmonellosis is the most economically destructive disease and the most frequent zoonotic infection of animals (19, 20).

It has been estimated that 1% of the human and livestock population is infected with Salmonella. At this rate of infection, livestock losses alone would approach $120 million annually (19). More recently a survey indicated that in cattle, horses, and dogs the infection rate may be nearer 10% (20).

Clinical manifestations of salmonellosis in livestock are increased by stress associated with overcrowding, calving, and shipping. Such stresses often result in fecal shedding by occult carriers. In feedlot situations this shedding by occult carriers may be particularly disastrous since newly arrived animals are unusually susceptible due to shipping stress. Dehydration, great loss of body weight, electrolyte imbalance, convulsions, and circulatory collapse characterize severe cases and usually precede death. The incubation period may be from 5 to 30 hours. Onset is sudden and the disease may follow an acute course of 1 to 2 weeks duration or evolve into a chronic form lasting 2 or 3 months (28).

Salmonellae survive in dust and soil and may multiply in river or lake bottom sediments. The bacteria have been shown to survive 47 days in a model manure oxidation ditch at $2^{o}C$ (19).

Arizona arizonae, now properly Arizona henshawii, is also a member of the family Enterobacteriaceae. Its biochemical properties are similar to those of the genus Salmonella with the primary exception of delayed lactose

fermentation. Both O and H antigens are defined serologically and given in a formula similar to those of Salmonella. Like the salmonellae, A. henshawii is an enteric pathogen both overtly and as an opportunist in previously traumatized animals (9).

Escherichia coli, the common colon bacillus, is the prototype coliform species. Although most strains of E. coli are of medical interest primarily as indicators of fecal contamination, there are some strains which produce enterotoxins and are thus important primary or secondary pathogens for almost all warm blooded animals. Enterotoxigenic strains produce both heat stable enterotoxins which closely resemble gram-negative endotoxin and heat labile enterotoxins (21). The heat labile enterotoxin produces cholera-like symptoms with profuse watery diarrhea and concomitant dehydration and electrolyte imbalance, both of which may be life threatening in affected animals. The heat labile enterotoxin is highly cross-reactive with cholera enterotoxin (3). Less is known about the heat stable enterotoxin.

Proteus vulgaris is also a member of Enterobacteriaceae and is related to but distinct from the other enteric bacteria (17). Proteus vulgaris is a common bacterium in both soil and water and is frequently part of the normal flora in animals. The numbers of P. vulgaris shed in feces is seen to increase during diarrheal diseases caused by other

agents (1). Proteus vulgaris is associated with a variety of pathological conditions. Infections of the eyes and ears, pleuritis, peritonitis, and suppurative abscesses in many parts of the body are among the instances in which P. vulgaris is highly suspected as the causative agent. In cystitis and pyelonephritis, P. vulgaris ranks second only to E. coli as a causative agent. Additionally, the urease of P. vulgaris seems to be nephrotoxic (1).

Pseudomonas aeruginosa is seen usually in cattle which are kept on relatively high levels of antibiotics incorporated in dietary rations. Those isolates examined from clinical cases were extremely resistant to antibiotics and produced prodigious amounts of pyocyanin. Pyocyanin, the soluble bluish pigment, is both a potent antibiotic and a powerful cytotoxin for mammalian cells (7). The organism frequently localized in the host's gall bladder and a characteristic leathery membrane of dead cells lined the bile duct and intestine below the junction with the bilary tract. In addition to pyocyanin, some strains seem to produce a heat labile enterotoxin which further contributes to their pathogenicity (2). Pseudomonas aeruginosa appears to be only an opportunistic pathogen in cattle, infections resulting from imbalance or eradication of normal flora by poor husbandry practices (W. Wohler, Texas Vet Lab, Inc., personal communication).

C. Bovine contagious keratoconjunctivitis.

Bacterial pinkeye, bovine contagious keratoconjunctivitis, is an acute or chronic contagious disease. Typical symptoms are lacrimation, swelling, corneal ulceration and opacity. After an incubation period of 3 to 5 days, the disease is initiated in either or both eyes. Swelling is prominent in the conjunctivae, eyelids, and nictitating membranes. Because of acute photophobia and pain, the affected eye or eyes are kept closed. Copious exudate and lacrimation escape from the eyelid and cause the eyelashes and facial hair to mat. Affected cattle seek cool, dark resting places and are inappetent. In early stages, there is acute inflammation of the conjunctival sac, eyelids, and nictitating membrane with petechial hemorrhages over the sclera. Three or four days after onset of the disease, an erosion approximately one (1) mm in diameter may develop in the center of the cornea. Corneal vascularization originating from the corneoscleral junction may be visible, and with the development of this sign most eyes begin to heal and recovery is uneventful. Visual impairment may be slight with only slight stellate scarring of the cornea. A few cases may progress from the initial erosion to an overt ulceration of the cornea with subsequent penetration and occasional eversion of the lens. Rarely, the disease process may continue into the

optic nerve and brain, causing death from bacterial meningitis. In most cases, recovery is apparent in 1 to 2 weeks following onset and visual recovery, even in cases with severe corneal perforation, is usually accomplished in 5 to 6 weeks (15, 27). Bovine contagious keratoconjunctivitis occurs in both sexes of all breeds and ages of cattle. Those breeds with non-pigmented eyes or faces are somewhat more susceptible both to the disease and to subsequent squamous cell carcinoma. Incidence of infection increases in spring, peaks in early summer, and is usually very rare during winter months. Such seasonal incidence suggests that allergic inflammation of the eyelids and conjunctivae from contact with grass and weed pollens may be an important predisposing factor. Other aggravating factors include wind, dust, concentrated sunlight, and foreign bodies (15, 27).

Direct contact with infected animals seems to be the primary mode of transmission in feedlots. In feedlots, the incidence of infection may be as high as 60 to 90% of the susceptible animals present. In the field, various flies and aerosols of bacteria from coughs and sneezes are implicated as modes of transmission.

Since only a very small percentage of infected cattle actually die, the major importance of bovine contagious keratoconjunctivitis is economic loss in the production of beef caused by weight loss (15, 27).

Moraxella bovis is the major causative agent of bovine

contagious keratoconjunctivitis in the United States. Moraxella bovis can be isolated from the conjunctival sacs and nasal secretions of affected cattle. Two toxic factors have been described, one is hemolytic, the other is dermonecrotic. Growth on blood agar results in rather large zones of complete hemolysis (16). Although viral agents such as infectious bovine keratoconjunctivitis virus and possibly mycoplasma may act as primary or secondary causative agents, it appears that M. bovis is the usual causative agent of pinkeye in United States cattle (23).

The aim of this research project was to attempt to duplicate in the laboratory the protection produced in the field by Poly Bac B. Since Boly Bac B was reportedly successful in field usage, a laboratory procedure for potency evaluation of Poly Bac B, as originally formulated, was desired. Studies were also included to evaluate the testing system and to determine an effective concentration of each desired organism. Two primary pathogens for mice in Poly Bac B, S. typhimurium and P. multocida, formed the basis of the potency evaluation of the vaccine.

The results presented in this thesis demonstrate that an effective test system has been developed. This system has also been compared with a modified version of the federal guidelines for testing monovalent vaccines containing either S. typhimurium or P. multocida which was published after the present system was developed.

CHAPTER II

MATERIALS AND METHODS

VACCINES: All vaccines used in this research were prepared as water in oil emulsions in Incomplete Freund's Adjuvant and were supplied by Texas Vet Lab, Inc., San Angelo, Texas. Bacterial concentrations were determined by viable cell counts which were performed prior to preparation of the vaccines. Unless otherwise noted, all bacteria were formalin killed.

Poly Bac B contains S. typhimurium, E. coli, A. arizonae, P. vulgaris, P. aeruginosa, and M. bovis at a final concentration of 5×10^8 cells per species per milliliter and P. multocida and P. hemolytica at a final concentration of 1×10^9 cells per species per milliliter.

Poly Bac B' contains S. typhimurium at a final concentration of 5×10^8 cells per milliliter and P. multocida and P. hemolytica at a final concentration of 2.5×10^9 cells each per milliliter. Dilutions of Poly Bac B' were prepared by diluting the aqueous phase prior to emulsification.

Monovalent Salmonella contains only S. typhimurium, the final concentration is 5×10^9 cells per milliliter.

Monovalent Pasteurella contains only P. multocida, the final concentration is 5×10^9 cells per milliliter. Two

batches of Monovalent Pasteurella vaccine were prepared, one formalin killed in the usual manner, the other heat killed by holding the aqueous phase at 80°C for 30 minutes in a water bath.

Divalent Pasteurella contains only P. multocida and P. hemolytica at a final concentration of 5×10^9 cells per species per milliliter.

ANIMALS: Male Swiss-Webster mice weighing approximately twenty grams were purchased from Laboratory Supply Company, Inc., Indianapolis, Indiana.

VACCINATION PROTOCOL: Mice were vaccinated either subcutaneously (SQ) or intraperitoneally (IP). Revaccinations, when performed, were given on day 14 by the same route and in the same volume.

CHALLENGE DOSE: Each mouse was challenged with log phase bacteria resuspended in normal saline. Bacterial concentration was determined by viable cell count and correlated to absorption units in a Klett-Summerson photoelectric colorimeter, Klett Manufacturing Company, Inc., New York, using blue filter number 42. Since the route of challenge and number of organisms per challenge was varied considerably during the course of this research, the exact data were incorporated into the tables and table legends.

MOUSE LD_{50}: LD_{50} determinations were made by serial dilution of saline resuspended cultures at a Klett absorbance high enough to insure that at least one dilution was obtained

which killed more than fifty percent of the mice challenged and at least one dilution was obtained which killed less than fifty percent of the mice challenged. Actual calculation of the LD_{50} for each organism was by the method of Reed and Muench (24). Mice were observed daily for 7 days.

CHAPTER III

RESULTS

Preliminary testing of Poly Bac B used subcutaneous vaccinations with subcutaneous challenges. Challenges were carried out using a number of organisms near the previously determined subcutaneous LD_{50} for Swiss-Webster mice. Prior to conducting experiments requiring intraperitoneal challenges it was necessary to determine an LD_{50} by this route of inoculation. The results of replicate intraperitoneal LD_{50} determinations as calculated by the method of Reed and Muench are presented in Tables 1A and 1B and 2A and 2B (24).

TABLE 1A

LD_{50} DETERMINATION FOR INTRAPERITONEAL CHALLENGE WITH SALMONELLA TYPHIMURIUM*

				ACCUMULATED VALUES			
Bacterial Dilution A	Mortality Ratio B	Died C	Survived D	Total Dead E	Total Survived F	Mortality Ratio G	Mortality Percent H
1:4	4/5	4	1	15	1	15/16	93.75
1:8	5/5	5	0	11	1	11/12	91.67
1:16	4/5	4	1	6	2	6/8	75.00
1:32	1/5	1	4	2	6	2/8	25.00
1:64	1/5	1	4	1	10	1/11	9.09

*Bacterial suspension adjusted to 250 Klett absorbance units (1×10^{11} organisms per milliliter); 0.5 ml injections.

Calculations:

$$\text{Proportionate distance} = \frac{\text{\% mortality at dilution next above 50\%} - 50\%}{\text{\% mortality at dilution next above 50\%} - \text{\% mortality at dilution next below 50\%}}$$

$$= \frac{75 - 50}{75 - 25}$$

$$= \frac{23}{50}$$

Proportionate distance = 0.5

Correction factor = log dilution steps X proportionate distance

= 0.3 X 0.5

Correction factor = 0.15

Negative log of dilution next above 50% mortality = -1.20

Correction factor = -0.15

$\log LD_{50}$ titer = -1.35

Number of cells in caculated $LD_{50} = 0.5 \times 10^{11 - 1.35}$

$= 5 \times 10^{8.65}$

Calculated $LD_{50} = 2.24 \times 10^{9}$ cells

TABLE 1B

LD_{50} DETERMINATION FOR INTRAPERITONEAL CHALLENGE WITH SALMONELLA TYPHIMURIUM*

Bacterial Dilution A	Mortality Ratio B	Died C	Survived D	ACCUMULATED VALUES Total Dead E	Total Survived F	Mortality Ratio G	Mortality Percent H
1:4	5/6	5	1	11	1	11/12	91.67
1:8	3/6	3	3	6	4	6/10	60.00
1:16	2/6	2	4	3	8	3/11	27.27
1:32	1/6	1	5	1	13	1/14	7.14
1:64	0/6	0	6	0	19	0/19	0.00

*Bacterial suspension adjusted to 250 Klett absorbance units (1×10^{11} organisms per milliliter); 0.5 ml injections.

Calculations

$$\text{Proportionate distance} = \frac{\text{\% mortality at dilution next above 50\%} - 50\%}{\text{\% mortality at dilution next above 50\%} - \text{\% mortality at dilution next below 50\%}}$$

$$= \frac{60 - 50}{60 - 27.27}$$

$$= \frac{10}{32.73}$$

Correction factor = log dilution steps X proportionate distance

Correction factor = 0.3×0.336

Correction factor = 0.1008

Negative log of dilution next above 50%

mortality = - 0.9031

Correction factor = - 0.1008

$\log LD_{50}$ titer = - 1.0039

Number of cells in calculated $LD_{50} = 0.5 \times 10^{11} - 1.00$

Calculated $LD_{50} = 5 \times 10^{9}$ cells

TABLE 2A

LD_{50} DETERMINATION FOR INTRAPERITONEAL CHALLENGE WITH PASTEURELLA MULTOCIDA*

Bacterial Dilution A	Mortality Ratio B	Died C	Survived D	ACCUMULATED VALUES Total Dead E	Total Survived F	Mortality Ratio G	Mortality Percent H
10^{-6}	3/4	3	1	9	1	9/10	90
10^{-7}	3/4	3	1	6	2	6/8	75
10^{-8}	3/4	3	1	3	3	3/6	50
10^{-9}	0/4	0	4	0	7	0/7	0
10^{-10}	0/4	0	4	0	11	0/11	0

*Bacterial suspension adjusted to 170 Klett absorbance units (4.0×10^8 organisms per milliliter); 0.5 ml injections.

Calculations:

$$\text{Proportionate distance} = \frac{\text{\% mortality at dilution next above 50\%} - 50\%}{\text{\% mortality at dilution next above 50\%} - \text{\% mortality at dilution next below 50\%}}$$

$$= \frac{75 - 50}{75 - 0}$$

$$= \frac{25}{75}$$

Proportionate distance = 0.33

Correction factor = log dilution steps X proportionate distance

= 1 X 0.33

Correction factor = 0.33

Negative log of dilution next above 50% mortality = - 7.00

Correction factor = - 0.33

log LD_{50} titer = - 7.33

Number of cells in calculated LD_{50} = $2.0 \times 10^{8-7.33}$

= $2.0 \times 10^{0.67}$

Calculated LD_{50} = 9.35×10^{0} cells

TABLE 2B

LD_{50} DETERMINATION FOR INTRAPERITONEAL CHALLENGE WITH PASTEURELLA MULTOCIDA*

Bacterial Dilution A	Mortality Ratio B	Died C	Survived D	ACCUMULATED VALUES Total Dead E	Total Survived F	Mortality Ratio G	Mortality Percent H
10^{-5}	5/5	5	0	22	0	22/22	100
10^{-6}	5/5	5	0	17	0	17/17	100
10^{-7}	5/5	5	0	12	0	12/12	100
10^{-8}	5/5	5	0	7	0	7/7	100
10^{-9}	2/5	2	3	2	3	2/5	40

* Bacterial suspension adjusted to 170 Klett absorbance units (4.0×10^{8} organisms per milliliter); 0.5 ml injections.

Calculations:

$$\text{Proportionate distance} = \frac{\text{\% mortality at dilution next above 50\%} - 50\%}{\text{\% mortality at dilution next above 50\%} - \text{\% mortality at dilution next below 50\%}}$$

$$= \frac{100 - 50}{100 - 40}$$

$$= \frac{50}{60}$$

Proportionate distance - 0.83

Correction factor = log dilution step X proportionate distance
= 1 X 0.83
Correction factor = 0.83

Negative log of dilution next above 50% mortality = -8.00

Correction factor = -0.83

log LD_{50} titer = -8.83

Number of cells in calculated LD_{50} = $2.0 \times 10^{8-8.83}$

= $2.0 \times 10^{-0.83}$

Calculated LD_{50} = 2.9×10^{-1} cells

The results shown in Table 3 indicate that with S. typhimurium as the challenge organism, Poly Bac B injected subcutaneously produced protection from subcutaneous challenge at 30 hours. This protection was shown to dissipate rapidly because further challenge failed to show protection under the conditions of our experiment. Subcutaneous vaccination with Poly Bac B when challenged intraperitoneally did not produce the 30 hour protection shown with subcutaneous challenge; however, some erratic protection was observed at later challenge dates.

When Poly Bac B was administered intraperitoneally and challenged intraperitoneally significant protection was observed at both 30 hours and 30 days (Table 4).

A series of experiments was conducted using Poly Bac B vaccinations administered subcutaneously and challenged with P. multocida (Table 5). When the challenge was presented

subcutaneously, protection was observed at 30 hours. At later challenge times no protection was observed under the conditions of our experiments. When vaccinated mice which received Poly Bac B subcutaneously were challenged intraperitoneally no protection was demonstrated at 30 hours or 14 days; only limited protection was observed in one of three trials conducted at 30 days (Table 5).

In results not shown, mice vaccinated intraperitoneally and challenged intraperitoneally were completely unprotected. Because of the complete lack of protection afforded by a single injection of Poly Bac B by this route, no repeat of the experiment was attempted.

The results shown in Table 6 were obtained using Poly Bac B' vaccinations in a modified version of the federal guidelines for animal vaccine potency testing (22). The mice were revaccinated at 14 days and were challenged at 24 days as proposed in the guidelines (Table 6 and Table 7). Excellent protection against S. typhimurium challenge was produced by revaccinating mice with Poly Bac B'. Dilutions of Poly Bac B', both 1:5 and 1:25, also provided excellent protection against challenge with this organism (Table 6).

Resistance to P. multocida challenge with revaccinated Poly Bac B' and with revaccinated Poly Bac B' diluted 1:5 showed complete protection in one experiment, but in a repeat experiment only 1 of 5 mice from each group survived. No protection from challenge was obtained with Poly Bac B'

TABLE 3

THE EFFECT OF SUBCUTANEOUS VACCINATION ON PROTECTION

AGAINST SALMONELLA TYPHIMURIUM CHALLENGE

Vaccination Protocol		Challenge		Survival Ratios Time of Challenge (Days)			
Volume	Route	Number	Route	1.25	7	12-14	26-30
REPLICATE 1							
0.20 ml	SQ	1.12×10^8	SQ	5/6*	0/6	0/6	0/6
Unvaccinated	--	1.12×10^8	SQ	3/6	0/6	0/6	0/6
0.25 ml	SQ	2.3×10^{10}	IP	0/6	---	0/6	0/6
Unvaccinated	--	2.3×10^{10}	IP	0/3	---	0/3	0/6
REPLICATE 2							
0.20 ml	SQ	1.12×10^8	SQ	6/6	0/6	0/6	0/6
Unvaccinated	--	1.12×10^8	SQ	0/6	0/6	0/6	0/6
0.25 ml	SQ	2.3×10^{10}	IP	0/6	---	1/6	1/6**
Unvaccinated	--	2.3×10^{10}	IP	0/3	---	0/6	1/3**
REPLICATE 3							
0.20 ml	SQ	5×10^9	IP	---	---	---	4/4
Unvaccinated	--	5×10^9	IP	---	---	---	1/4

*Survivors/Challenged after 7-10 days observation.
**Challenge dose modified, 5×10^9 viable cells.

TABLE 4

THE EFFECT OF INTRAPERITONEAL VACCINATION ON PROTECTION

AGAINST SALMONELLA TYPHIMURIUM CHALLENGE

Vaccination Protocol		Challenge		Survival Ratios Time of Challenge (Days)	
Volume	Route	Number	Route	1.25	30
REPLICATE 1					
0.20 ml	IP	5×10^{9}*	IP	6/6**	4/4
Unvaccinated	--	5×10^{9}	IP	0/4	1/4
REPLICATE 2					
0.20 ml	IP	5×10^{9}	IP	2/4	4/4
Unvaccinated	--	5×10^{9}	IP	0/4	2/4

*Viable Cells
**Survivors/Challenged after 7 days observation.

TABLE 5

THE EFFECT OF SUBCUTANEOUS VACCINATION ON PROTECTION

AGAINST PASTEURELLA MULTOCIDA CHALLENGE

Vaccination Protocol		Challenge		Survival Ratios Time of Challenge (days)			
Volume	Route	Number	Route	1.25	7	12-14	26-30
REPLICATE 1							
0.20 ml	SQ	1.5×10^8	SQ	6/6*	0/6	0/6	0/6
Unvaccinated	--	1.5×10^8	SQ	0/6	0/6	0/6	0/6
0.25 ml	SQ	2.0×10^3	IP	0/6	---	0/6	0/6
Unvaccinated	--	2.0×10^3	IP	0/3	---	0/3	0/6
REPLICATE 2							
0.20 ml	SQ	1.5×10^8	SQ	6/6	0/6	0/6	0/6
Unvaccinated	--	1.5×10^8	SQ	0/6	0/6	0/6	0/6
0.25 ml	SQ	2.0×10^3	IP	0/6	---	0/6	0/5**
Unvaccinated	--	2.0×10^3	IP	0/3	---	0/6	0/3**
REPLICATE 3							
0.20 ml	SQ	2.0×10^2	IP	---	---	---	2/4**
Unvaccinated	--	2.0×10^2	IP	---	---	---	0/6**

*Survivors/Challenged after 7 days of observation.
**Challenge modified, 2.0×10^2 viable bacteria.

TABLE 6

THE EFFECT OF REVACCINATION ON PROTECTION AGAINST

SALMONELLA TYPHIMURIUM CHALLENGE

Vaccination Protocol		Challenge		Survival Ratio
Vaccine Dilution	Route	Number	Route	24 days
REPLICATE 1				
Revaccinated Poly Bac B'*	IP	2.5×10^{10}	IP	5/5**
Revaccinated Poly Bac B' (1:5)	IP	2.5×10^{10}	IP	4/5
Revaccinated Poly Bac B' (1:25)	IP	2.5×10^{10}	IP	5/5
	--	2.5×10^{10}	IP	1/5
REPLICATE 2				
Revaccinated Poly Bac B'	IP	2.5×10^{10}	IP	4/5
Revaccinated Poly Bac B' (1:5)	IP	2.5×10^{10}	IP	5/5
Revaccinated Poly Bac B' (1:25)	IP	2.5×10^{10}	IP	5/5
Unvaccinated	--	2.5×10^{10}	IP	0/5

*All vaccinations were 0.10 ml. Revaccinations were at 14 days.
**Survivors/Challenged after 7 days observation.

TABLE 7

THE EFFECT OF REVACCINATION ON PROTECTION AGAINST

PASTUERELLA MULTOCIDA CHALLENGE

Vaccination Protocol		Challenge		Survival Ratio
Vaccine Dilution	Route	Number	Route	24 days
REPLICATE 1				
Revaccinated Poly Bac B'*	IP	2.0×10^2	IP	5/5**
Revaccinated Poly Bac B' (1:5)	IP	2.0×10^2	IP	5/5
Revaccinated Poly Bac B' (1:25)	IP	2.0×10^2	IP	0/5
Unvaccinated	--	2.0×10^2	IP	1/5
REPLICATE 2				
Revaccinated Poly Bac B'	IP	2.0×10^2	IP	1/5
Revaccinated Poly Bac B' (1:5)	IP	2.0×10^2	IP	1/5
Revaccinated Poly Bac B' (1:25)	IP	2.0×10^2	IP	0/5
Unvaccinated	--	2.0×10^2	IP	0/5

*All vaccinations were 0.10 ml. Revaccinations were at 14 days.
**Survivors/Challenged after 7 days observation.

TABLE 8

TRANSIENT PROTECTION AGAINST SALMONELLA TYPHIMURIUM CHALLENGE

PRODUCED BY VACCINATION WITH DIVALENT PASTEURELLA VACCINE

Vaccination Protocol*	Challenge		Survival Ratio	
	Day	Dose	Replicate 1	Replicate 2
Divalent Pasteurella	1.25	5×10^{10}	5/6	4/6
Unvaccinated	1.25	5×10^{10}	2/6	1/6
Revaccinated Divalent Pasteurella	24	2.5×10^{10}	3/6	0/5
Unvaccinated	24	2.5×10^{10}	0/6	0/6

*Vaccinations and revaccinations were 0.1 ml IP.

TABLE 9

THE EFFECT OF INTRAPERITONEAL VACCINATION WITH MONOVALENT SALMONELLA VACCINE ON PROTECTION AGAINST INTRAPERITONEAL CHALLENGE WITH PASTEURELLA MULTOCIDA AT 30 HOURS

Vaccination Protocol	Challenge Dose	Survival Ratio
REPLICATE 1		
Monovalent Salmonella	2.0×10^3_3	0/6
Unvaccinated	2.0×10^3	0/6
REPLICATE 2		
Monovalent Salmonella	2.0×10^2_2	1/6
Unvaccinated	2.0×10^2	1/6

*All vaccinations were 0.1 ml.

TABLE 10

THE EFFECT OF REVACCINATION WITH VARIOUS VACCINES ON PROTECTION AGAINST 24 DAY CHALLENGE WITH PASTEURELLA MULTOCIDA

Vaccination Protocol	Challenge Dose	Survival Ratio
REPLICATE 1		
Monovalent Pasteurella*	8.0×10^{1}	5/6
Monovalent Pasteurella	8.0×10^{1}	5/6
Divalent Pasteurella	8.0×10^{1}	5/6
Poly Bac B	8.0×10^{1}	0/5
Unvaccinated	8.0×10^{1}	0/6
REPLICATE 2		
Monovalent Pasteurella	8.0×10^{1}	11/12
Monovalent Pasteurella**	8.0×10^{1}	5/6
Divalent Pasteurella	8.0×10^{1}	6/6
Poly Bac B	8.0×10^{1}	1/5
Unvaccinated	8.0×10^{1}	1/6

*All vaccinations, except as noted, were 0.1 ml at 14 day interval, IP.
**Monovalent Pasteurella administered at 0.2 ml.

diluted 1:25 (Table 7).

Mice vaccinated with a single dose of Divalent Pasteurella given IP were challenged at 30 hours with S. typhimurium. The data in Table 8 shows that some non-specific protection was afforded those mice vaccinated. Some irregular protection against S. typhimurium challenge was also evident at 24 days in revaccinated mice.

Mice vaccinated with Monovalent Salmonella and challenged 30 hours later with P. multocida were not protected, as shown in Table 9.

Revaccination of mice with Monovalent Pasteurella or Divalent Pasteurella gave excellent protection against P. multocida challenge. No difference in protection was observed between the formalin killed and heat killed Monovalent Pasteurella vaccines. Poly Bac B was not shown to be protective against P. multocida challenge under similar conditions (Table 10).

CHAPTER IV

DISCUSSION

Results shown in these studies indicate that vaccination with Poly Bac B produces excellent protection at 30 hours against both S. typhimurium and P. multocida if challenged by homologous route. The exception to the pattern of 30 hour protection was that no protection to IP challenge with P. multocida was shown. These studies have shown that the observed 30 hour protection is at least partially of a non-specific nature and has been shown to be transient. A possible cause of this transient response could be increased local infiltration by phagocytic cells, or it may be related to the protection produced experimentally by "Protodyne" (8). The nature of this early protection is worthy of further investigation.

Although animals vaccinated SQ were never revaccinated during the course of this study, it appears that a single SQ vaccination in mice may not be sufficiently protective to be useful in a potency evaluation system which specifies a challenge date greater than 30 hours.

In contrast to the transient nature of protection produced by SQ vaccination against S. typhimurium, the

protection produced by a single IP vaccination was more durable as shown by the survival patterns of mice challenged at 30 days. No protection was shown to P. multocida challenge with a single IP injection of Poly Bac B as originally formulated.

During the course of this study, amendments to the former federal requirements for potency testing of proposed animal vaccines containing either S. typhimurium or P. multocida became law (22). Previously, each laboratory had been allowed to establish its own potency standards and evaluation system (25). These amendments were published in the Federal Register during the latter stage of this study. The federal guidelines specified revaccination at 14 days and that dilutions of each bacterin had to be tested. Vaccination volumes were specified and under the conditions of this experiment only 0.1 ml was allowed per mouse dose.

Previous results with S. typhimurium indicated that the concentration of this organism in Poly Bac B was sufficient. When Poly Bac B', which contained the standard concentration of S. typhimurium, was tested on a revaccination protocol, excellent protection was obtained against S. typhimurium challenge at day 24.

Since the original Poly Bac B formulation was not effective in protecting mice against IP challange with P. multocida, Poly Bac B' and appropriate dilutions were specially prepared with a 2.5-fold increase in the

concentration of P. multocida. Mice vaccinated with Poly Bac B' according to this protocol were challenged with P. multocida in two separate studies. One set of results indicated that Poly Bac B' afforded excellent protection even at a 1:5 dilution. The second set of results obtained in this study indicated that only limited protection was afforded by Poly Bac B' against P. multocida challenge.

These results may be related to the variability shown in the LD_{50} determinations and reported in the literature by other investigators (14). Another possible explanation of this inconsistency could be related to the uncertainty of working with extremely high concentrations of organisms in vaccine preparation and the extremely small mouse protection dose. Even though protection against P. multocida challenge was highly variable, a certain amount of protection was provided by Poly Bac B'. Additional results obtained in a study employing vaccines with further increased P. multocida concentrations designed to compensate for the loss in volume showed that revaccination with Monovalent Pasteurella which contained five times the standard concentration of P. multocida gave good protection against challenge, indicating a threshold effect for protection against P. multocida. The possibility of antigenic interference by P. hemolytica was eliminated by demonstrating that Divalent Pasteurella which contained equal numbers of each Pasteurella species afforded excellent protection against P. multocida challenge.

One source of difficulty inherent in a mouse system employing very small doses of vaccine is that significant reduction in the quantity of vaccine actually delivered may be caused by apparently insignificant capillary loss from the syringe between injections. Another source of error in vaccine delivery is encountered when viscous materials such as the emulsified vaccines used in this study are drawn into the syringe through a small diameter needle. Such a procedure allows air to enter by leaking around the plunger as the material is withdrawn and if this air is not eliminated prior to delivery then compression of the contents of the syringe occurs during delivery which could cause the actual volume delivered to be significantly less than indicated. A third complication is that such air trapped at the base of the plunger is not visible through the opaque liquid contained in the syringe. A fourth possible source of error is also related to the nature of the emulsified vaccines. That is, air is trapped in the liquid as the aqueous and oil phases are emulsified. Under vacuum produced while filling the syringe, trapped air bubbles expand and coalesce to form larger and easily compressible defects in the liquid column. Like air trapped at the base of the plunger, these defects in the liquid column are masked by the opacity of the emulsion and lead to overestimation of the amount of vaccine delivered at each injection sequence. The data supplied with the federal guidelines showed that a 1:5 dilution of an

aqueous bacterin decreased the protection from 100% to 65% in an experiment using P. multocida challenge.

To minimize the effects of these purely mechanical sources of error, which may only be significant when dealing with very small dose volumes, it is suggested that vaccine preparations to be used in mouse testing systems be held in vacuo for a period to remove as much incorporated air as possible, and, whenever possible, small volume syringes filled from the back by larger syringes should be employed. In the filling of the filler syringes, a long, large bore needle should be attached so that the emulsion can be picked up from an open container and the use syringe can be filled from a point near the point of delivery so that any air in the liquid can be voided prior to injecting any animals. In larger animals, where larger doses and equipment are used, the relative importance of these considerations is greatly reduced and is probably less significant. Large animal studies might also show that because of the difference in species susceptibility to P. multocida, the threshold concentration of this organism in Poly Bac B may be quite different in cattle and mice. Additional studies should be conducted on the basic research level to more clearly define the immune response to P. multocida and the antigenic properties of the organism.

The federal standards committee was aware of the

inconsistency of any testing system involving mouse protection against P. multocida when extrapolated to large animals. For this reason, a relative potency standard based upon sheep studies rather than an absolute potency standard based on bovine studies was established, since the latter appear to be less susceptible.

CHAPTER V

SUMMARY

Poly Bac B, a polyvalent bacterin prepared in Incomplete Freund's Adjuvant, was designed for use as an aid in preventing those bacterial diseases commonly encountered in stressed cattle in Texas. Results obtained with a mouse protection test system showed that Poly Bac B was effective in protecting mice against S. typhimurium or P. multocida challenge administered 30 hours after vaccination. Thirty days after a single vaccination with Poly Bac B, mice were still protected against S. typhimurium challenge but not against P. multocida challenge. A revaccination protocol showed that a five-fold increase in the standard concentration of P. multocida when given as a monovalent or divalent preparation in 0.1 ml doses did protect mice against P. multocida challenge at 24 days.

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