DEVELOPMENT OF A PROTECTIVE BACTERIN AGAINST PASTEURELLOSIS IN BIGHORN SHEEP

by

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ABSTRACT

The development of a protective bacterin for Rocky Mountain Bighorn Sheep was studied. A total of forty-five (45) *Pasteurella*-like organisms was isolated from nasal swabs and tissues of bighorn sheep from Wyoming, Colorado and Pennsylvania. Each isolate was examined for its cultural, morphological, staining and biochemical characteristics and compared to standard *Pasteurella multocida* and *Pasteurella hemolytica* cultures from domestic sheep. Two isolates showing characteristics most similar to *Pasteurella* sp. were chosen from each of the geographic locations: one from nasal swabs and the other from tissue isolates. These six isolates were tested both individually and combined in mice and combined in rabbits and domestic sheep for their virulence capacities. A living whole cell suspension was tested in domestic sheep using a two dose series given seven days apart. Hemagglutination titers were determined on blood samples from the sheep at given intervals to determine the ability of the suspension to be immunogenic as well as the numbers of organisms needed to yield highest antibody titer. Formalinized bacterins of individual organisms were tested in rabbits for antibody titers and in mice using both active and passive immunity studies. Formalinized whole cells and fractionated mixed bacterins were studied in rabbits and domestic sheep. These bacterins were tested for their ability to produce hemagglutination titers in the blood serum of the animals and the protective ability of the antibody against *Pasteurella* challenge.

The bighorn sheep population in the Rocky Mountain area has declined since the beginning of this century. One cause of these losses has been attributed at least in part to various species of *Pasteurella* bacteria when associated with a pneumonia-septicemia. The disease condition many times developes when the animals are under some specific stress factor. March (1927) reported losses of bighorn sheep on the Sun River Game Preserve, Montana. Necropsy reports showed typical pasteurellosis with lungs congested. Potts (1937) observed hemorrhagic septicemia among bighorns in Rocky Mountain National Park, Colorado in 1935 and 1936. *Pasteurella oviseptica*, along with *Corynebacterium pyogenes*, was isolated at necropsy. Packard (1946) reported additional losses at Rocky Mountain National Park due to *P. oviseptica*.

Post (1958) observed losses among bighorns which were held in captivity and attributed it to pasteurellosis. Even though commercial bacterins were used on the animals, death still occurred due to *Pasteurella* infections. A heat-killed bacterin was prepared from cultures of *Pasteurella* isolated from dead bighorns. Sheep losses were prevented when this was administered.

Post (1962) improved upon the bacterin and its protective ability by using formalinized cells in a septivalent bacterin. Rufi (1961) was able to show that this type of bacterin could give protection to bighorns and that low titers can give immunity to captive bighorns.

The purpose of the present study has been to isolate various *Pasteurella*-like bacteria from bighorn sheep and develop a protective bacterin against pasteurellosis in bighorn sheep so they may be held in captivity for further research. Since commercial bacterins do not seem to protect these animals, a bacterin using *Pasteurella* isolates with specific immunogenic properties from bighorns must be tested.

MATERIALS AND METHODS

Bacterial cultures were collected from nasal swabs from apparently healthy bighorn sheep and from tissue cultures of dead bighorns from Sybille Experimental Unit, Wyoming, Rachelwood Wildlife Research Preserve, Pennsylvania, Rocky Mountain National Park and Glenwood Park, Colorado. Isolated bacterial colonies grown on Brain-Heart-Infusion Agar (BHI) plates were picked, smeared and stained using gram stain. All gram negative rods or coccal bacillus were subcultured and subjected to differential examination. They were compared to results from ATCC *P. oviseptica* 9657 and *P. hemolytica* 9-2183 from domestic sheep. Those cultures yielding characteristics similar or identical to these *Pasteurella* sp. were held for further testing.

Six cultures were chosen to be most similar to the standard *Pasteurella* sp. and were tested both individually and in combination for toxicity in mice. Viable cultures were diluted in a peptone-saline buffer and injected into groups of mice in serial concentrations as well as used for challenges in rabbits and domestic sheep.

Immune sera for each of the individual six isolates and the ATCC 9657 culture were produced by injecting groups of rabbits intravenously with formalinized cells. The doses ranged from 1 mg to 11 mg of dry weight bacteria over a 21-day period. The rabbits were bled by heart puncture four weeks after the last dose, and the serum collected. Hemagglutination antibody titers were determined using polysaccharides from the individual bacteria coated to sheep red blood cells and the microtiter technique.

Passive immunity was determined in mice using either 0.3 ml, 0.2 ml, or 0.1 ml of immune sera from rabbits and sheep injected intraperitoneally. This was followed twenty-four hours later by a challenge dose of either individual bacteria or a combination of the isolates. The number of mice which survive challenge was compared to the number of uninoculated control mice to determine any immunity protection.

A living bacterin composed of a combination of the six chosen isolates was inoculated into a group of 5 domestic sheep in various doses. The doses were given as follows one week apart:

Sheep No.	1st Dose	2nd Dose
5	5×10^9 organisms	1.7×10^9 organisms
4	5×10^8 organisms	1.7×10^8 organisms
3	5×10^7 organisms	1.7×10^7 organisms
2	5×10^6 organisms	1.7×10^6 organisms
1	5×10^5 organisms	8.5×10^4 organisms

The doses were injected intrathoracically. Sheep sera were collected four weeks after the last dose for hemagglutination titer tests.

A combined formalinized cell bacterin was developed using equal amounts of formalinized cells of the six isolates. The dry weight of the bacterin was determined and diluted in normal saline to make a stock suspension containing 1 mg per ml. Various groups of laboratory animals were inoculated with different amounts of the material at one week intervals. The number of animals, kind and amounts received are as follows:

Mice - 0.33 mg dry weight bacteria per dose given I. P.

Group A - 3 doses - 20 mice
Group B - 2 doses - 20 mice
Group C - 1 dose - 20 mice
Group D - no doses - 20 mice controls

Rabbits - 3 mg dry weight bacteria per dose given S. Q.

Group A - 3 doses - 2 rabbits
Group B - 2 doses - 2 rabbits
Group C - 1 dose - 2 rabbits
Group D - no doses - 2 rabbits controls

Sheep - 5 mg dry weight bacteria per dose given S. Q.

Group A - 3 doses - 2 sheep
Group B - 2 doses - 2 sheep
Group C - 1 dose - 2 sheep
Group D - no doses - 2 sheep

The mice were challenged I. P. two weeks after the last doses with 5×10^9 organisms. The survivors were observed for 7 days after challenge.

H. A. titers were determined on the rabbits and sheep. The rabbits were challenged with 1.7×10^9 organisms five weeks after the last dose and the sheep were challenged with 8×10^{10} organisms. The body temperatures of the sheep were recorded before and after challenge for at least 72 hours.

A fractionated bacterin was developed using the six *Pasteurella* isolates grown on BHI agar. The cells were washed off the agar and heated in distilled water at 56°C for 1 hour to detach the capsular

material. Equal amounts of the individual cells were added to a suspension and fractionated under 20,000 psi pressure. The capsular material was twice precipitated out of the supernatant using 3 volumes of 95% alcohol and added back to the fractionated cell suspension. The dry weight was determined per ml and diluted in normal saline to a concentration of 1 mg per ml. Groups of rabbits and sheep were inoculated according to the same procedure as the formalinized bacterin. The sera was collected for antibody production testing and the animals were challenged. Body temperatures of the sheep were taken at given intervals after challenge.

RESULTS

A total of forty-five (45) *Pasteurella*-like organisms were isolated from cultures obtained from Wyoming, Colorado and Pennsylvania. They were all gram negative, small oval rods, bipolar stained and produced only acid in sugars if at all. From these isolates, six cultures were chosen with one from a nasal swab and one from tissues of dead bighorns from each area. Sources of each of the six organisms are given in Table 1. The bio-chemical differential of these isolates compared favorably to the standard cultures. Table 2 gives those specific biochemical activities which were used to estimate organism specificity. Table 3 shows the results obtained on individual virulence tests of the organisms as well as in combined suspensions in mice. Also, it shows protection given by serums from rabbits and sheep in passive immunity testing and the formalinized bacterin protective ability against challenge. These organisms as shown in Table 3 have a relatively low virulence in mice.

Table 4 shows that using serial concentrations of individual organisms in rabbits produces a variety of antigenic responses. It also shows the hemagglutination titers obtained in rabbits when inoculated with various doses of the two bacterins. All experimental control rabbit sera had negative titers. No rabbits died from the challenge.

Table 4 also contains the results of antibody titers from domestic sheep which received the various doses of the three bacterins. The living bacterin seemed to have low immunogenic response even when given in two doses. The sheep all had negative titers to these seven antigens before inoculations were begun. All experimental control sheep were negative for titers to these organisms before challenge.

Figure 1 shows the average body temperatures of sheep recorded after challenge at 0, 8, 24, 48, and 72 hours. Two pairs of experimental control animals were challenged and recorded as one group. All groups seem to have increased body temperature within 8 hours of challenge (Figure 1). Observations showed this was accompanied by increased respiration rate and coughing. Within 48 hours all animals except those receiving the fractionated bacterin (Group C) showed normal body temperature. Two sheep in Group C died within 60 hours after challenge. Necropsy showed heavy pneumonic lungs with *Pasteurella* sp. isolated from liver, lung, kidney, spleen and heart blood.

Table 1 - Original sources of the *Pasteurella* isolates

RM - 1	From tissue cultures of a dead bighorn ram in Rocky Mountain National Park. It is a gram negative, bipolar, encapsulated rod.
399-L	From nasal swabs taken from a young lamb ram at Glenwood Park Colorado. It is a gram negative, bipolar, encapsulated rod.
WYO-2	From tissue cultures of dead bighorns at Sybille Experimental Unit, Wyoming. It is a gram negative, bipolar, encapsulated small rod.
WYO-1	From nasal swabs taken from young bighorns at Sybille Experimental Unit, Wyoming. It is a gram negative, encapsulated rod.
70-326	From tissue cultures from a dead bighorn at Rachelwood Wildlife Research Preserve, Pennsylvania. It is a gram negative, bipolar, encapsulated, small rod.
2-29R	From nasal swabs of bighorn sheep at Rachelwood Wildlife Research Preserve, Pennsylvania. It is a gram negative, bipolar, encapsulated, small rod.

Table 2 - Comparison of specific biochemical tests of bighorn sheep isolates to standard cultures

	MEDIA			
ORGANISM	NITRITE	INDOLE	HEMOLYSIS	MAC CONKEY'S
RM-1	+	+	-	-
399-L	-	+	-	+
WYO-2	+	+	?	+
WYO-1	-	+	-	-
70-326	-	-	?	+
2-29R	+	+	?	-
9657	+	+	-	-
9-2183	+	-	+	+

Table 3 - Percent of survivors in active and passive immunity studies.

Challenge Organism	LD50	Active Immunity A	Active Immunity C**	Form. Bact. rabbit	Form. Bact. sheep	Fract. Bact. rabbit	Fract. Bact. sheep	Control Normal Sera	Control Exp. C**
Combined	1×10^8	75%*	0%	78%	72%	69%	56%	33%	25%

Challenge Organism	LD50	Passive Immunity A	Passive Immunity C
RM-1	5.4×10^6	86%	80%
399-L	5×10^9	86%	80%
WYO-2	1×10^6	53%	0%
WYO-1	1×10^9	N. D.	
70-326	7×10^7	46%	60%
2-29R	1×10^8	100%	80%
9657	5×10^9	N. D.	
9-2183	5×10^9	--	--

* This is the average for group A receiving 3 doses of formalinized bacterin.
** C is the averages for the experimental controls receiving only the challenge dose.
***N. D. means not done.
A is the average of all groups receiving serial doses of the specific immune sera.

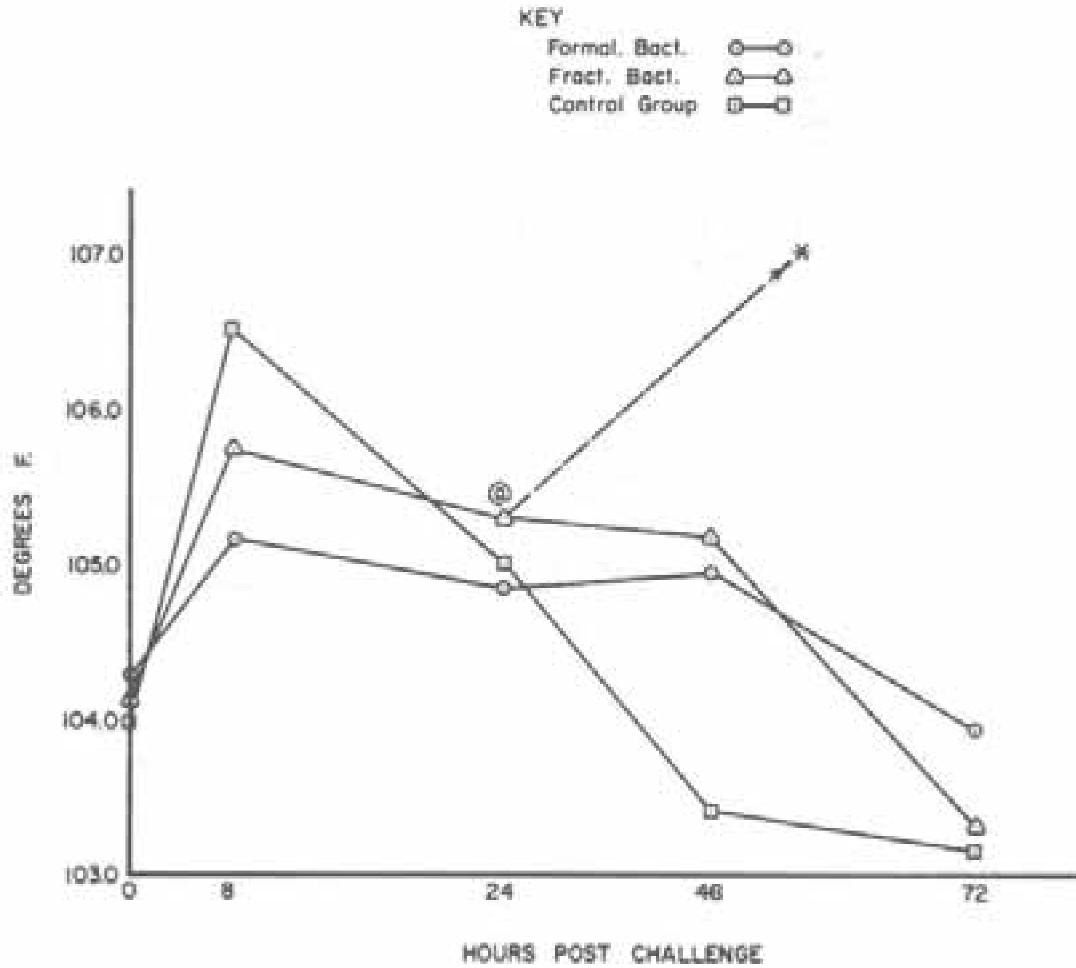
Table 4 - Average hemagglutination antibody titers from rabbit and sheep sera versus Polysaccharide *Pasteurella* antigens.

ANTIGEN	Individual Series RABBITS	Living Bacterin SHEEP	Formalinized Bacterin RABBIT	Formalinized Bacterin SHEEP	Fractionated Bacterin RABBIT	Fractionated Bacterin SHEEP
9657	1:8*	1:8	1:8	1:64	1:64	1:64
WYO-2	1:64	1:16	1:8	1:128	1:64	1:128
WYO-2	1:32	1:32	1:32	1:128	1:64	1:128
RM-1	1:256	1:32	1:8	1:128	1:128	1:128
399-L	1:256	1:16	1:32	1:128	1:128	1:128
70-326	1:64	1:8	1:32	1:128	1:128	1:128
2-29R	1:128	1:16	1:32	1:138	1:128	1:128
Controls						

*Average of all the animals in all groups given the specific bacterin and rounded off to the nearest dilution well. This is the highest dilution with a +2 reading in the microtiter well.

FIGURE 1

BODY TEMPERATURE AVERAGES FOR DOMESTIC SHEEP AT GIVEN TIME INTERVALS AFTER I.V. CHALLENGE



1. Ⓢ Time when first animal of I dose Fract. bact. died so temperature not included.

2. * Time when second animal died.

DISCUSSION

Any one working with *Pasteurella* organisms soon finds that they are a difficult group of bacteria to understand. These organisms may be isolated from both living and dead bighorns, as seen by the number of isolates made in this study. The *Pasteurella* sp. from bighorns are difficult to differentiate.

These organisms seem to be virulent to mice both individually and in combination but in high doses. The organisms isolated from dead bighorns appeared to be more virulent that those from nasal swabs. The antigenic response tests show that they vary in immunogenic response to rabbits which may indicate different antigens or that some organisms may have a larger quantity of one antigen than another. This factor seems to be further substantiated in the amount of capsular material that was observed around each specific culture of organisms.

The problem of experimental pasteurellosis becomes greater when attempts are made to reproduce the disease in larger laboratory animals. A variety of factors must be combined to produce the disease in rabbits and domestic sheep. An example of this is the Wyo-2 organism which was isolated from a number of dead bighorns and seemed to kill the animals quite rapidly. It killed mice at a low dose, yet would not cause death in inoculated rabbits or experimental control sheep. It did produce the disease and death in Group C of the fractionated bacterin group. This lack of reproductability of the disease makes for difficulty in estimating the protective ability of a given bacterin against active challenge. The results do indicate that a variety of organisms should be used in a bacterin to get a heterogeneous antigenic make-up even if all of the organisms do not stimulate high titers. Both the inoculations in rabbits and mice seemed to indicate that this variation was needed due to the variability in immune response in the various animals.

The formalinized bacterin showed good protection (75%) for those mice receiving 3 doses of the bacterin. All the rabbits showed low titers (Table 4) when immunized with this bacterin yet were protected against challenge. The formalinized bacterin showed about equal ability in protecting sheep whether given in one, two, or three doses. These results seem to indicate that the amount or number of doses needed depends upon the animal used.

The fractionated bacterin seemed to give high titers in both rabbits and sheep and was best among all animals as far as total antibody production was determined. This was especially true for those animals receiving three doses. The results seem to indicate a need for multiple inoculations of this bacterin as indicated by the results of Group C deaths.

Both bacterins appeared to have good points and should be tested further. As mentioned above, one problem with the one dose of fractionated bacterin could mean enhanced infection with only one dose of this bacterin. This may also indicate that titers of anti-body may not mean that the animals are protected from disease since

the animals had relatively high H.A. titers. The passive immunity tests in mice (Table 3) seemed to support the idea. All immune serums, no matter what titer, were protective to the mice passively to some degree.

Possibly both bacterins can be used effectively, as Rufi (1961) pointed out, the protective ability of a bacterin may not be in how high the titers are, but in the ability of it to protect the animal from disease.

Further studies must be done on bighorn sheep. New parameters may be introduced which may help choose the best bacterin for protective ability to bighorn sheep.

LITERATURE CITED

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DISCUSSION

QUESTION BY MILT FRAN, FISH AND WILDLIFE SERVICE: I'd be curious to know if the isolates you made from living animals were characterized as opposed to the ones from the dead animals in terms of the form of bacteria. Were they rough or smooth colonies?

REPLY BY NASH: They were characterized. The bacteria from the living animals were the rough form and the bacteria from the dead animals were the smooth form.

REPLY BY FRAN: Would you care to comment on the possibility of stress? You have two populations of bacteria in the sheep, the rough form and the smooth form, and stress making environmental conditions in the host more preferable for multiplication of the smooth or more virulent form to cause the outbreak of disease. Did you have any comment or ideas on this?

REPLY BY NASH: I have not done work in this, but the literature indicates that with *Pasteurella pestis* that this is the case. You have an avirulent form going to a virulent form when psychological, biological and chemical changes occur in the body of various laboratory animals. I can grow this organism on a defined media and get a capsule material greater than when I isolated the organism. All this is tested from the capsule material, not from the cell wall.