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PASTEURELLACEAE FROM BIGHORN AND DOMESTIC SHEEP

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Abstract: Nasal, tonsil and lung samples from bighorn and domestic sheep (Ovis canadensis and Ovis aries) were cultured for members of the Pasteurellaceae family of bacteria. Pasteurella haemolytica was isolated from 38% of 120 bighorn sheep and 71% of 73 domestic sheep. Biotypes A, T and 3 were isolated from both bighorn and domestic sheep. Multiple biotypes were isolated from some animals. Biotype T organisms were isolated from 76% of the culture-positive bighorn sheep and 21% of the culture-positive domestic sheep. In contrast, biotype A was isolated from 30% and 75% of the culture positive bighorn and domestic sheep, respectively. Organisms identified as biotype 3 P. haemolytica were isolated from 13% and 11% of the 2 respective groups of animals. Only biotype T organisms were isolated from the lungs of bighorn sheep with respiratory disease. The outer membrane of each bighorn, domestic and American Type Culture Collection (ATCC) biotype T isolate was treated with sodium dodecyl sulfate and the proteins were separated by polyacrylamide gel electrophoresis (SDS-PAGE) to develop fingerprints for comparison. The bighorn isolates did not show patterns identical to those of either the domestic or ATCC strains.

The family Pasteurellaceae includes the closely related genera Pasteurella, Haemophilus and Actinobacillus. These organisms are Gram-negative, characteristically pleomorphic and non-motile. Most ferment glucose, produce oxidase and alkaline phosphatase and they vary in ability to produce catalase. Most grow aerobically although some are capnophilic and fail to grow without CO_2. All are obligate parasites of animals, with the majority being commensals of the upper respiratory tract (Mannheim 1984). Many species act as opportunistic pathogens in susceptible individuals. Susceptibility is increased by predisposing or concurrent infections with a variety of viruses or other bacteria and by stresses including fatigue, poor nutrition, temperature extremes and

transportation. Pasteurallaceae, most notably, Pasteurella haemolytica and P. multocida are associated with respiratory disease in domestic sheep and cattle (Timoney et al. 1988). Haemophilus somnus is a common pathogen of young calves and feedlot age cattle.

Pasteurellaceae are believed to occur in animals in all climates and can be detected in the nasal passages and tonsils of a high percentage of clinically normal animals. A typical disease due to a Pasteurellaceae organism is the "shipping fever" syndrome in cattle, a respiratory disease characterized by dyspnea, anorexia and pyrexia following a combination of stress factors including shipping. Inclement weather, weaning, castrating, dipping and shipping appear to be stresses most commonly associated with predisposition of domestic sheep to respiratory disease (Gilmour 1980).

Three biotypes of P. haemolytica, A, T and 3, are differentiated based on biochemical reactions. Biotypes A and T are further differentiated into serotypes by antisera which detect specific antigens. Biotype A contains serotypes 1, 2, 5, 6, 7, 8, 9, 11, 12, 13 and 14. Four serotypes, 3, 4, 10 and 15, are recognized within biotype T (Timoney et al. 1988). Biotype 3 organisms have not been separated into serotypes.

Pasteurella haemolytica A2 is most commonly associated with pneumonia in domestic sheep but can be cultured from nasal secretions of normal sheep. Biotype T organisms have a tropism for the tonsils of sheep and are rarely found elsewhere in clinically healthy animals. Biotype T serotypes are associated with septicemia and pneumonia in domestic sheep particularly in the late fall and early spring (Gilmour et al. 1974).

Pasteurella haemolytica strains have also been incriminated as the cause of disease and devastating losses of bighorn sheep populations (Post 1962). In some instances domestic sheep were believed to be sources of Pasteurella that caused illness and death of bighorn sheep (Foreyt 1989, Foreyt and Jessup 1982). It was speculated that clinically healthy bighorn sheep were free of strains of Pasteurella common in domestic sheep, and that strains present in healthy domestic sheep were highly virulent for the bighorn. Several authors have recommended that bighorn and domestic sheep be separated by buffer zones, assuming that even chance encounters would result in transmission of Pasteurella from domestic to bighorn sheep resulting in disease and death of the bighorn sheep (Foreyt 1989, Foreyt and Jessup 1982).

We report information from an ongoing study in which P. haemolytica isolated from bighorn and domestic sheep are being evaluated. Evaluation includes biotyping, serotyping and use of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to separate proteins from the outer membranes of P. haemolytica isolates. These procedures are being used to detect similarities and variances of organisms and to monitor the incidence and distribution of particular bacterial strains in sheep populations. Support for this study has been provided by the Idaho sheep Commission, the Idaho Department of Fish and Game, and USDA Formula Funds.

MATERIALS AND METHODS

Samples were collected from 120 bighorn and 73 domestic sheep. The bighorn sheep included 110 live-captured and released after sampling, 3 collected for disease investigation, 3 which died due to trauma which occurred during capture, 2 hunter kills and 2 animals found dead. Animals were captured by use of a net gun or a projectile dart containing a chemical tranquilizer (Dunbar et al. 1990). Samples taken from live-captured animals included nasal swabs from 52, tonsil swabs from 4, and both tonsil and nasal samples from 54. Tonsillar biopsies were taken from some of the latter group as described by Dunbar et al. (1990). All dead sheep, except 1 hunter-killed animal, were necropsied. Tissues including tonsils, lung and liver were collected from all necropsied animals. All samples were transported on ice to the University of Idaho, Caine Veterinary Teaching and Research Center (CVTRC) and were processed for bacterial cultures within 72 hr of collection.

Samples from domestic sheep included nasal swabs from 32 clinically normal 4-8 week-old lambs in a flock maintained at CVTRC, and lung samples from 18 adult sheep with respiratory disease that were brought to CVTRC by various owners. In addition nasal and tonsillar swab samples were collected from 23 adult sheep which had grazed on land adjacent to an area near Challis, Idaho, where bighorn sheep are known to range. Samples from the latter group were transported to the laboratory and cultured for bacteria within 48 hr of collection.

Pasteurella haemolytica type strains 1-12 were obtained (National Veterinary Services Laboratory, Ames, IA 50010) and used as controls to monitor agglutination reactions and for comparisons in evaluating outer membrane proteins of isolates from other sources.

All samples were cultured on Columbia Blood Agar (Becton Dickinson Microbiology Systems, Cockeysville, Maryland 21030, USA) with 5% citrated sheep blood (CBA). In addition, tonsillar and nasal samples were cultured on a selective CBA with added Vancomycin (6 ug/ml) and Nystatin (12.5 ug/ml, Sigma Chemical Co., St. Louis, Missouri 63178, USA) to reduce growth of other bacteria and thus enhance the detection of Pasteurellaceae. The inoculated media were incubated in an atmosphere of air with 5% added CO_2 at 37 C and bacterial growth was evaluated at 24 hr intervals for at least 3 days. Bacterial isolates with colonial morphology suggestive of Pasteurellaceae were speciated and identified to biotype by standard procedures (Carter 1984, Kilian and Frederiksen 1981). Serotyping was conducted by slide agglutination (Frank and Wessman 1978).

The outer membrane proteins of Pasteurella isolates were prepared by the procedure of Carlone et al. (1986) prior to separation by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Briefly, all bacteria were propagated on CBA for 16-24 hr at 37 C to standardize the growths of the organisms and reduce possible phenotypic variances. The protein isolation procedure used sonication for bacterial disruption, differential centrifugation for isolation of the membranous fractions and sodium N-lauroyl sarcosinate for dissolution of the cytoplasmic membranes. Outer membrane proteins were concentrated by centrifugation and the

protein concentration was determined by a modification of the Lowry procedure (Markwell et al. 1978). The outer membrane proteins (OMP) were solubilized in a buffer containing glycerol, 2-mercaptoethanol, SDS and 0.05% bromphenol blue. A 20 ul volume of the SDS treated OMP (2 ug/ul) was loaded per lane of the 12% polyacrylamide gels and electrophoresis was conducted using 35 MA/gel. The gels were fixed in a 7.5% Trichloroacetic acid aqueous solution for 2-8 hr, and stained in a 0.25% solution of Coomassie brilliant blue for 2-4 hr. Unbound stain was removed with an aqueous solution containing 25% methanol and 7.5% acetic acid.

The stained gels were photographed with translucent and oblique lighting using Technical Pan (Eastman Kodak Company, Rochester, NY, 14650). Each SDS-PAGE lane was scanned with a densitometer (Hoefer Scientific Instruments, San Francisco, CA 94107) connected to a desktop computer (IBM-XT) with a GS-365 Data System software (Hoefer Scientific Instruments, San Francisco, CA 94107). The computer was used to monitor tracings and record data for determining molecular weight and comparing patterns ("fingerprints") produced from the evaluations of different organisms. Primary emphasis was given to evaluating biotype T organisms, since T biotypes predominated in the samples from bighorn sheep.

RESULTS

Pasteurella haemolytica was isolated from 46 of the 120 (38.3%) bighorn and 52 of the 73 (71.2%) domestic sheep. Although biotype T P. haemolytica isolates predominated in bighorn and biotype A were most common in domestic sheep samples, all three biotypes, A, T and 3, were isolated from both species of sheep (Figs. 1, 2). More than one biotype was isolated from some animals. Biotype combinations, A and T, and A and 3, were each isolated from 4 bighorn sheep (Fig. 1). Biotypes A and T, A and 3, and T and 3 P. haemolytica were isolated from 1, 1, and 2 domestic sheep respectively (Fig. 2).

Biotype A P. haemolytica was much more common in nasal than in tonsil samples of both sheep species. Ten of 11 A isolates from bighorn were from nasal samples; one was from a tonsil sample. Biotype A isolates were cultured from nasal samples of 11 and tonsil samples of 2 adult domestic sheep. Young lambs had a higher incidence of P. haemolytica in nasal samples (21 of 32) than was detected in samples from adult sheep.

Biotype T P. haemolytica was isolated from nearly equal numbers of nasal (12), of tonsillar (13), and in both nasal and tonsillar samples (12) of bighorn sheep. The 6 biotype T isolates from domestic sheep were from tonsil swabs of adult animals (3) and from nasal samples (3) of young lambs.

Only biotype T was isolated from the lungs of bighorn sheep including 4 which died with pneumonia, 1 which was killed in the terminal stage of pneumonia, and 2 which were live-captured and judged to be clinically normal prior to euthanasia of 1 and apparent death due to trauma of the other. Biotypes A and T were isolated in pure cultures from the lungs of 5 and 4 domestic sheep respectively, which died due to pneumonia. Biotype 3 organisms were isolated from equal numbers of

clinically normal bighorn and domestic sheep, (i.e. 6 [5%] and 6 [8%]) respectively.

The isolates of biotype T obtained from domestic sheep agglutinated rapidly in 1 of the antisera; 3, 4 or 10, with occasional weak reactions in 1 of the other 2 antisera. In contrast, most isolates from bighorn sheep agglutinated rapidly in more than 1 antisera, with the most frequent reaction being strong agglutination in all 3 antisera.

Biotype T and A isolates had marked differences in the number and placement of major OMP bands by which these biotypes could be readily differentiated (Fig. 3). All biotype T isolates from a single bighorn sheep gave the same agglutination pattern and identical SDS-PAGE profiles. Major differences were evident between biotype T isolates from different sheep (Fig. 4). These differences were most evident in comparison of the OMP bands in the 33-40 x 10^3 dalton range (Fig. 4). Minor bands of proteins with molecular weights less than 30 x 10^3 and more than 40 x 10^3 daltons were also present. However the concentrations of these proteins were too low for optimum detection.

DISCUSSION

The percentage of bighorn sheep that was culture positive for Pasteurella was approximately half of that detected for domestic sheep. While the prevalence of Pasteurella may be less in bighorn sheep, this difference may also reflect variation related to geographic location, or due to sampling or handling procedures. Many samples from bighorn sheep captured early in the test period did not reach the laboratory for 48-72 hours. Samples collected later in the study included tonsillar crypt biopsies and most of the later samples reached the laboratory within 48 hr. Both the type of sample, i.e., tonsillar biopsies, and the shorter period from collection to processing, increased the probability of Pasteurella isolation. Since, Pasteurella are fastidious organisms which do not survive in dry material and do not survive out of the host for more than a few hours unless present with blood, mucus or tissue, detection is optimized by culturing samples within 4-6 hr (Mannheim 1984). Therefore, the lower incidence of isolations from bighorn than from domestic sheep may have occurred because samples collected in remote areas could not be processed within an optimum time. Lost viability of Pasteurella in samples and the fact that these organisms are inhibited in culture by other bacteria which are common in the environment (Corbeil et al., 1985) may have contributed to the low incidence of isolation of Pasteurella in another study (Onderka and Wishart 1988).

The relatively low numbers of biotype T isolates in samples from domestic sheep compared to bighorn sheep was an unexpected finding since biotype T P. haemolytica are common in domestic sheep, particularly in the tonsils of feeder-age lambs (Gilmour et al., 1974, Al-Sulton and Aitken 1985). Few herds and individual domestic sheep were tested and therefore the data from our limited study would probably include a limited number of P. haemolytica T strains and cannot be interpreted to represent all domestic sheep.

Similarly, the low incidence of biotype A isolated from bighorn sheep is in contrast to a high incidence detected in domestic sheep in our study and by Al-Sulton and Aitken (1985).

Although all bighorn Pasteurella isolates were serotyped, the fact that most of the isolates agglutinated in more than 1 antisera did not correlate well with tests results on domestic sheep isolates (Thompson et al. 1977). Therefore, greater emphasis was placed on evaluating SDS-PAGE profiles for detecting variation and shared components among the P. haemolytica isolates. The OMP profiles of these organisms are sufficiently discriminative to identify 3 distinct fingerprints (Fig. 4). In similar studies of Acinetobacter strains isolated from humans, major proteins were common and nondiscriminatory in a number of strains which were subsequently distinguishable by variation in molecular weights of minor proteins (Dijkshoorn et al. 1987).

We have evaluated a limited number of P. haemolytica isolates from animals in Idaho during 18 months. Additional samples will be collected from both domestic and bighorn sheep in Idaho and samples from other areas are being requested. As isolates are grouped based on similarity of major proteins, additional profiles will be produced using greater OMP concentrations to enhance evaluation of minor protein bands. Bacteria which appear to have identical fingerprints will also be tested by Western-blot assays to detect antigenic variances which may be present in organisms with common molecular weights of proteins. In addition, analyses of the DNA of these organisms will be conducted to detect variances in restriction endonuclease susceptibilities. These procedures will make it possible to determine if P. haemolytica isolates from different sources are identical. It may also be used to monitor transmission of particular bacterial strains within a herd or between different populations of sheep.

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Pasteurella haemolytica
Biotypes Isolated from Domestic Sheep

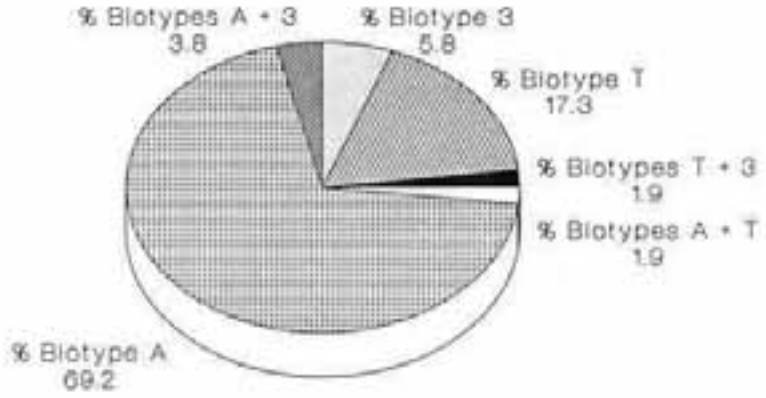

Fig. 1. Biotype distribution of Pasteurella haemolytica

Pasteurella haemolytica
Biotypes Isolated from Bighorn Sheep

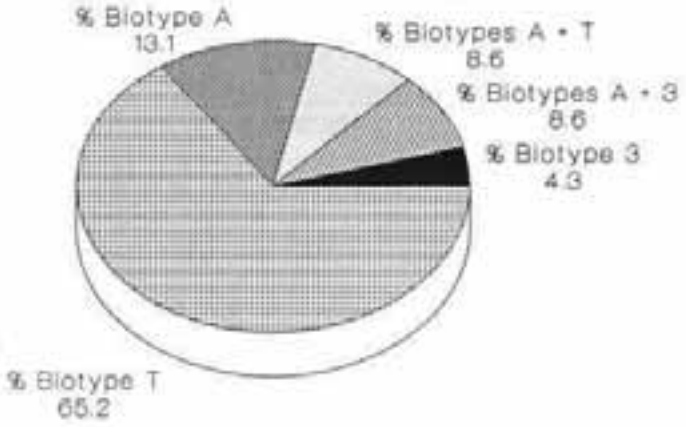

Fig. 2. Biotype distribution of Pasteurella haemolytica

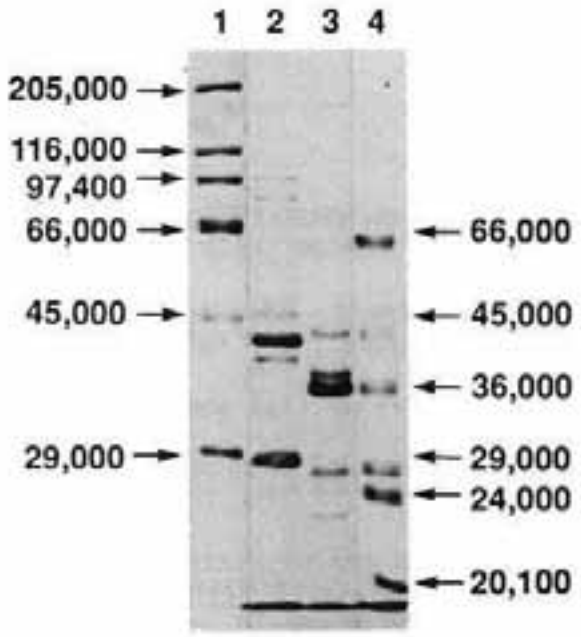

Fig. 3. Major outer membrane proteins present in Pasteurella haemolytica A1 (lane 2) and P. haemolytica T isolated from lung of bighorn sheep (lane 3). Proteins with indicated molecular weights are in lanes 1 and 4.

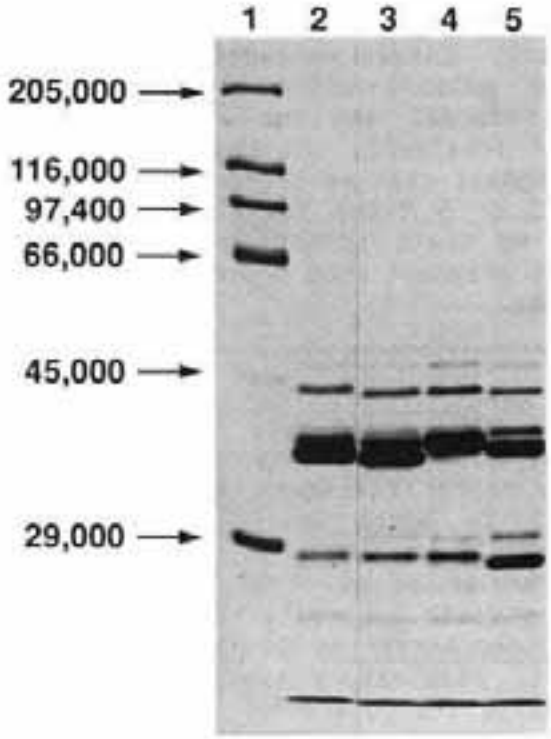

Fig. 4. Outer membrane protein SDS-PAGE profiles of 4 Pasteurella haemolytica isolates in lane 2, 4, and 5 (bighorn sheep isolates) and lane 3 (domestic sheep isolate). Proteins for molecular weight standards are separated in lane 1.