5-1989

Phenotypic Characterization of *Actinobacillus* and *Actinobacillus*-Like Species Isolated from Ram Lambs with Epididymitis

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PHENOTYPIC CHARACTERIZATION OF *ACTINOBACILLUS* AND *ACTINOBACILLUS*- LIKE SPECIES ISOLATED FROM RAM LAMBS WITH EPIDIDYMITIS

by

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A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

in

Bioveterinary Science

Approved:

UTAH STATE UNIVERSITY
Logan, Utah

1989

ACKNOWLEDGEMENTS

I would like to thank my committee, Dr. Mark C. Healey, Dr. Reed P. Warren, Dr. Bill B. Barnett, and Dr. Ross A. Smart, for their kindly suggestions and support during my graduate study. I would especially like to thank my major professor, Dr. Mark C. Healey, for his financial support and timely assistance. I would also like to thank the members in our laboratory (especially Dr. Hank H. Hwang and Mrs. Alice V. Johnston) for their technical help.

Finally, I would sincerely like to thank my dear wife, Chu-Tung, for her patient and moral encouragement and taking care of my family for the last three years.

Yen- Bou Liu

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ABSTRACT

Phenotypic Characterization of *Actinobacillus* and *Actinobacillus*-like Species Isolated from Ram Lambs with Epididymitis

by

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Utah State University, 1989

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The purpose of this study was to characterize phenotypically *Actinobacillus* and *Actinobacillus*-like species cultured from ram lambs with epididymitis (lamb epididymitis), so as to better understand the species and strains of *Actinobacillus* responsible for causing the disease. Two American Type Culture Collection (ATCC) type species of *Actinobacillus* (*A actinomycetemcomitans* and *A seminis*) and 24 field isolates were tested in this study. The field isolates were cultured from either lesions or semen of ram lambs with clinical epididymitis and had been identified as members of the genus *Actinobacillus*, based on cultural and morphological criteria. When analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), these field isolates could be divided into 4 groups, based on the similarities and differences in the protein profiles.

A panel of 13 MoAbs directed against 3 distinct proteins extracted from 1 field isolate (As8C) were also used in the enzyme-linked immunosorbent assay (ELISA), flow cytometry, and bacterial agglutination to screen these field isolates. Specificity of the MoAbs for the 3 proteins was confirmed by the enzyme-linked immunoelectrotransfer blot (EITB) technique.

Bacterial field isolates were also divided into 3 groups by the ELISA results, based on their different reaction intensities. Data generated by the flow cytometry, namely peak channel, mean value, and percent labeled cells, suggested that these field isolates could be divided into 2 groups. However, with the unique reaction pattern for each MoAb, the flow cytometry data suggested the possibility of preparing a bank of figures as a standardized reference to identify additional isolates belonging to the genus of *Actinobacillus*. Bacterial agglutination of some isolates was noted using hyperimmune mouse serum (HMS), but not using MoAbs. These data suggested that bacterial field isolates cultured from epididymal lesions of affected lambs were considered to be in the same group as the 2 ATCC-type species of *Actinobacillus*. Further studies will be required in determining whether *A seminis* or some other species of *Actinobacillus* is the primary pathogen responsible for lamb epididymitis.

(81 pages)

INTRODUCTION

Ram epididymitis in sheep-raising areas of the world, such as Australia, New Zealand and the western United States, represents a serious reproductive disorder resulting in significant economic losses to producers.[1-6] The pathological changes in the genital tract of clinically affected rams have been described in detail.[7] Briefly, when the epididymides become infected with pathogenic bacteria, masses of lymphocytes can be found among the epididymal epithelial cells. Polymorphonuclear neutrophils (PMNs) and plasma cells accumulate in the interstitial tissue. The PMNs also penetrate the epithelium. Intertubular areas show an increase of fibrous tissue in some rams. In acute cases of epididymitis, the interstitial tissues become infiltrated with lymphocytes and PMNs. These cells, together with amorphous material, cause swelling of the tubules and compression and degeneration of the epithelium.[7] Upon palpation, the scrotal contents are firm and usually more pronounced unilaterally. Semen smears from these rams contain fewer than normal spermatozoa, along with PMNs and bacteria. These pathologic changes result in reduced breeding potential for the ram.

Brucella ovis was first isolated from rams with clinical epididymitis in Australia.[5] For many years, this bacterium was thought to be the only pathogen responsible for causing ram epididymitis. However, the etiology of this disease has recently been associated with the sexual status of the ram.[8,9] In mature breeding rams on the range, *B ovis* is the primary bacterium that causes

epididymitis. However, in virgin ram lambs (lamb epididymitis), several fastidious gram-negative bacteria have been isolated from epididymitis lesions. These bacteria include *Actinobacillus*, *Haemophilus* and *Pasteurella*, as well as several other genera that are occasionally isolated.[3,8] Lamb epididymitis occurs almost exclusively in young, virgin ram lambs (6 to 15 months of age) from purebred ram-producing flocks. *Actinobacillus* and *Haemophilus* are the 2 genera of bacteria most often involved.[8] These bacteria appear to be transitory components of the ovine genital flora and are probably only opportunistic pathogens.[10] Although the exact species of *Actinobacillus* responsible for producing lamb epididymitis is still indefinite, all available information suggests that *A seminis* is most likely the principal pathogen.[11-15]

Commercial bacterins are presently available for only *B ovis* infection in mature range rams. Since lamb epididymitis is not associated with *B ovis* infection, the use of this bacterin is of no value in protecting against the disease in ram lambs. The most reliable method of preventing lamb epididymitis is the early detection of palpable lesions and the prolonged feeding of antibiotics to ram lambs at risk.[16,17]

Difficulties in diagnosing and preventing lamb epididymitis exist because the protective bacterial antigens have not been identified, isolated and purified.[18,19] For certain animal diseases, such as ovine pasteurellosis, improved bacterins prepared from outer membrane extracts of the causative bacteria have been developed.[20,21] These extract-derived bacterins contained more strains and demonstrated

greater efficacy than those prepared from formalin-inactivated whole-cell bacteria.[20,21] We are beginning to learn more about the species and strains of *Actinobacillus* responsible for causing lamb epididymitis. It appears that field isolates belonging to this genus constitute a heterologous group of organisms.[19] The purpose of this study was to characterize phenotypically *Actinobacillus* and *Actinobacillus*-like species isolated from ram lambs with epididymitis using SDS-PAGE, flow cytometry, ELISA and bacterial agglutination.

MATERIALS AND METHODS

Bacterial cultures -- Two American Type Culture Collection (ATCC) type species (*Actinobacillus seminis* and *A actinomycetemcomitans*)[a] and 24 bacterial field isolates cultured from either lesions or semen[b] of ram lambs with clinical epididymitis (Table 1) were grown on Columbia agar base[c] containing 5% fetal bovine serum (FBS).[d] Agar was poured into petri plates, streaked with bacteria and incubated in a GasPak jar[c] containing 15% CO_2 and 85% N_2 at 37°C for 48 hours. After incubation, bacterial colonies from the ATCC type species and field isolates were harvested in 3 different solutions: (1) 0.06*M* $NaHCO_3$ buffer (pH 9.6), (2) phosphate buffer saline solution (PBSS, pH 7.2) and (3) double-distilled water (DDH_2O). Each bacterial solution was McFarland standardized at an absorbance of 1 and read at 550 nm using a spectrophotometer,[e] which yields a stock solution containing approximately 2 X 10^9 cells/ml. The stock solutions were then aliquoted as needed in each experiment. The first 2 bacterial solutions were stored at -20°C, and the third solution was snap frozen and stored at -80°C for future use.

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[a] American Type Culture Collection, Rockville, MD.

[b] Provided by Drs. Marie S. Bulgin, Veterinary Teaching and Research Center, Caldwell, Idaho, Leroy R. Maki, University of Wyoming, Laramie, and Clell V. Bagley, Utah State University, Logan, UT.

[c] Becton Dickinson & Co, Cockeysville, MD.

[d] Hyclone Laboratories, Logan, UT.

[e] Baush & Lomb Inc, Rochester, NY.

TABLE 1 -- Sources of 2 ATCC type cultures and 24 field isolates of *Actinobacillus* species

Source	Isolate
ATCC	29522* and 15768†
Lesions	As8C, 4101, 84-832(5), 84-832(9), 86-618, 86-722, 86-723, 86-724, 86-731A and 86-814A
Semen	R75, A11, A46, A22, 84-833, B30, B30-2, B111, Y136, S6, S15, D107, D56, and A2

*American Type Culture Collection (ATCC) of *Actinobacillus actinomycetemcomitans*.
†ATCC of *A seminis*.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) -- The procedure for bacterial protein extraction using a non-ionic detergent has been described by Healey et al.[18] Briefly, whole cell preparations of the ATCC type species and bacterial field isolates of *Actinobacillus* were individually extracted in a 2% solution of *n*-octyl-β-D-glucopyranoside (*n*-octyl-glucoside).[f] The extracted proteins were pretreated in 0.1% SDS and 0.5% 2-mercaptoethanol and boiled in water for 10 minutes. A 0.1% aqueous solution of bromphenol blue[g] was added as a tracking dye, and the extracts were evaluated by discontinuous-gradient SDS-PAGE. Using a casting cassette,[h] 2 gels (180 X 140 X 1.5 mm) consisting of 10% to 20% gradients were prepared, along with 5% stacking gels. Electrophoresis was done for approximately 6 to 7 hours at 60 mA and 250 V in 0.025*M* Tris-0.192*M* glycine buffer (TGB, pH 8.6) containing 1% SDS. Following electrophoresis, all gels were stained with Coomassie brilliant blue R250.[f]

Production of hyperimmune mouse serum (HMS) -- The HMS, provided by other researchers in Dr. Healey's laboratory, was prepared following the procedures described by Healey et al.[11]

Hybridoma production and cloning -- Monoclonal antibody (MoAb)-secreting hybridoma cell lines were prepared by other researchers in Dr. Healey's laboratory, with some modification in the procedure described by Healey et al.[11] An *n*-octyl-glucoside extract

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[f] Sigma Chemical Co, St. Louis, MO.
[g] Eastman-Kodak Co, Rochester, NY.
[h] Pharmacia Fine Chemicals, Piscataways, NJ.

of As8C was prepared as described previously, and the proteins (non-denatured and non-reduced) were separated by PAGE. After separation, 3 protein bands (74 kilodaltons (KD), 60 KD, and 45 KD), which appeared to be the most conserved bands between the 2 ATCC cultures and a majority of field isolates, were cut and eluded from the gel using a sample concentrator.[i] The protein concentrations of each band were determined by the Lowry method.[22] Adult female Robertsonian mice were twice inoculated IP with 100 µg of extracted protein at a 3-week interval. The extracted proteins were suspended in Freund's complete adjuvant[j] for the first inoculation and Freund's incomplete adjuvant[j] for the second inoculation. Four weeks after the second inoculation, the mice were intravenously (IV) administered 75 µg of extracted protein (suspended in PBSS). Three days after the IV inoculations, all mice were killed and their spleens were aseptically removed and placed in sterile PBSS. Immune mouse splenocytes were fused with FOX/NY myeloma cells.[d] The fused cells were collected by centrifugation and resuspended in 50 ml of Dulbecco's modified Eagle's minimum essential medium (DMEM)[k] containing 10% FBS, $10^{-5}M$ hypoxanthine, $10^{-6}M$ aminopterin and 1.5 X $10^{-5}M$ thymidine. Cells were pipetted at 100 µl/well into flat-bottom 96-well microtiter plates[l] and incubated in a 7% CO_2 incubator at 37°C and 100% relative humidity. Media from all wells

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[i] Isco Inc, Lincoln, NE.
[j] Difco Laboratory, Detroit, MI.
[k] Gibco, Grand Island, NY.
[l] Coning, Corning, NY.

with growth were screened by the enzyme-linked immunosorbent assay (ELISA) for the presence of As8C antigen-specific MoAbs.

Thirteen hybridoma cell lines were selected and cloned by limiting dilution as described by Healey et al.[11] Before cloning, each cell line was maintained in RPMI 1640[i] (RPMI) containing 10% FBS. The cells were counted and diluted to a concentration of 60 cells/ml. One milliliter of this diluted suspension was added to 39 ml of RPMI supplemented with 15% FBS, 7.5 X $10^{-5}M$ adenine, 8 X $10^{-7}M$ aminopterin and 1.6 X $10^{-5}M$ thymidine conditioned media, with the final concentration being 60 cells/40 ml. The FOX/NY myeloma cells were used as feeder cells. These myeloma cells were counted, centrifuged and resuspended in the conditioned media. A ratio of 100 µl of the conditioned media containing the hybridoma and the feeder cells was placed into each well of 2 flat-bottom 96-well microtiter plates. Plates were examined 7 and 14 days after cloning. Media from the wells with 1 colony were tested for MoAb secretion using the ELISA. Only 1 well was selected from each cell line on the basis of the strongest ELISA reaction. Selected cell lines were grown in RPMI supplemented with 10% FBS. Monoclonal antibody isotype and subisotype were then determined by testing each MoAb against goat anti-mouse IgA, IgM, IgG1, IgG2a, IgG2b and IgG3 sera, using a mouse MoAb subisotyping kit.[d]

Enzyme-linked immunoelectrotransfer blot (EITB) -- Procedures for analyzing the *n*-octyl-glucoside extract of As8C by the EITB technique were followed essentially as described by Healey et

al.[17] The polyacrylamide gels containing the separated As8C extract were transferred electrophorectically overnight to nitrocellulose membranes using a transblotting apparatus.[h] Transfer was done at 200 mA and 60 V in TGB containing 20% methanol (pH 8.3). After the transfer was complete, the membranes were removed from their respective gels. Strips of lanes containing the transferred extract were cut from the membrane and rinsed twice in DDH_2O for 15 minutes each. Individual lanes containing the molecular weight markers were stained with naphthol blue-black.[g] The remaining membranes were then incubated with 3% gelatin in 0.05*M* Tris-0.5*M* NaCl buffer (pH 7.5) at 37°C for 24 hours to block the unoccupied protein-binding sites.

Blocked nitrocellulose membranes were washed in a buffer containing 0.025*M* Na_2HPO_4, 0.5*M* NaCl and 0.05% Tween 20[m] (ELISA diluent, pH 6.8) and gently stirred for 90 minutes at 37°C. Individual strips were then incubated with hybridoma culture fluids for 90 minutes at 37°C. The HMS and FOX/NY culture fluid were incubated as positive and negative controls, respectively. After incubation, strips were washed 3 times for 15 minutes each in ELISA diluent at room temperature with gentle stirring. A 1:1,000 dilution of horseradish peroxidase (HRPO)-conjugated goat anti-mouse IgG[d] in ELISA diluent was incubated with each strip for 90 minutes at 37°C. All strips were again washed in ELISA diluent as described. Individual strips were then placed in an enzyme substrate solution. The substrate solution was prepared from a mixture of 10 mg of 3.3'-

[m] Bio-Rad Laboratories, Richmond, CA.

diaminobenzidine tetrahydrochloride,[f] 0.1 ml of 8% aqueous $NiCl_2$ and 0.025 ml of 3% H_2O_2 in 20 ml of 0.05*M* Tris buffer (pH 7.6). To prevent excessive background staining, the reaction was stopped after 10 to 20 seconds by placing the strips in ELISA diluent and rinsing them in DDH_2O for 10 to 15 minutes. Nitrocellulose membrane strips were then photographed while they were wet.

Enzyme-linked immunosorbent assay (ELISA) -- Ninety-six-well, flat-bottom plates[l] were used for the ELISA. A plate was prepared by coating individual wells with 0.15 ml of whole cell bacteria (7.5×10^7 cells/ml) suspended in 0.06*M* $NaHCO_3$ buffer (pH 9.6) and was allowed to sit overnight in a 4°C cold cabinet. The plate was washed several times using ELISA diluent. A 0.1-ml volume of hybridoma culture fluid was placed in individual wells and incubated for 2 hours at 37°C. Hyperimmune mouse serum (1:2,000 dilution) and undiluted FOX/NY culture fluid were used as positive and negative controls, respectively. After incubation was complete, the plate was washed by immersing it in distilled water and rinsing it several times. One-tenth ml of HRPO-conjugated goat anti-mouse IgG diluted 1:3,000 with ELISA diluent was then placed into each well of the plate. The plate was again incubated for 2 hours and washed. Color was developed by adding 0.1 ml of o-phenylenediamine (OPD)[f] substrate to each well. The reaction was stopped by adding 0.05 ml of 1N HCl to each well 15 minutes after the addition of the OPD substrate. The optical density (OD) of each well was measured by analyzing the plate with a microplate reader (450 nm on mode 4).[n]

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[n] Biotek Instruments Inc, Burlington, VI.

Flow cytometry -- The fluorescence activated cells sorter (FACS) has many different uses in the application of MoAb technology.[23] Flow cytometry was done in an attempt to generate a graphic profile of each bacterial isolate as a function of MoAb specificity, using an EPICS-C FACS.[o] The 2 ATCC type species and 24 bacterial field isolates of *Actinobacillus* were prepared at a concentration of 1 X 10^8 cells/ml in DDH_2O. A 0.1 ml suspension of each bacterial isolate was mixed with culture fluids containing individual MoAbs. Hyperimmune mouse serum and FOX/NY culture fluid were used as positive and negative controls, respectively. Each bacterial suspension was placed in separate microcentrifuge tubes.[p] The tubes were incubated for 70 minutes at 37°C. After incubation, the tubes were washed by centrifugation at 12,000 x g in an Eppendorf bench-top centrifuge.[q] Following centrifugation, the supernatent was discarded and the bacterial pellet was resuspended in 0.8 ml PBSS (PH 7.2) on a vortex mixer.[r] The tubes were again washed and a 0.4 ml volume of a 1:20 dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG[f] diluted in PBSS was added to the pellet and incubated for an additional 70 minutes at 37°C. The tubes were washed again as described. The final pellet was resuspended in 0.4 ml PBSS and analyzed through the FACS. The FACS was equipped with an argon laser tuned to 400 mM at the 488 nm line. Cells were carried in Isoflow sheath fluid[o]

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[o] Coulter Co, Hialeah, FL.
[p] Fisher Scientific Co, Pittsburgh, PA.
[q] Brickman, Westbury, NY.
[r] Scienfitic Products, McGaw Park, Ill.

through a 76 micron flow tip. Fluorescence data for FITC were obtained using a bit-map format, gated on the forward light scatter versus right-angle scatter histogram. The bit map was used to exclude debris and doublets. Fluorescent signals were processed through a three-decade log amplifier and displayed on a 256-channel scale. Fluorescence intensity was standardized using 10 nm latex beads° and the laser power was adjusted to place the log-green histogram in channel 119. Five thousand cells were counted for each reaction, and the cursor line was set at a relative position of 45 on the X-axis (fluorescent intensity). The cursor was set based on the FOX/NY culture fluid's reaction in order to eliminate non-specific staining by fluorescein. If cells had a fluorescent intensity below (less than) the cursor, it was considered as background staining. Three parameters were evaluated in this experiment, namely peak channel, mean value and percent labeled cells. Values of the peak channel indicated the position on the X-axis where the highest cell count (indicated by the Y-axis) beyond the cursor occurred. The mean value indicated the position on the X-axis where the average fluorescent intensity beyond the cursor occurred, and the percent-labeled cells indicated the percentage of cells labeled by the fluorescent antibody that had intensities greater than the cursor.

Bacterial agglutination -- To determine the ability of the 13 MoAbs to agglutinate the ATCC type species and field isolates of *Actinobacillus*, spent hybridoma and FOX/NY culture fluids were

concentrated 10 times, using a Minicon macrosolute concentrator.[s] Bacteria were diluted to a concentration of 1 X 10^9 cells/ml in DDH_2O. Bacterial agglutination was performed with 4 concentrations (0.5X, undiluted, 5X and 10X) of each of the 13 MoAbs. Concentrated FOX/NY culture fluid was used as a negative control. Hyperimmune mouse serum was used as a positive control at 2 different concentrations (undiluted and 1:5). A 0.03 ml suspension of each bacterial isolate was mixed with an equal volume of culture fluid, and each suspension was placed in a separate ring of a 12-ring ceramic slide.[p] The slide was kept in a covered petri dish at room temperature and rotated at minimum speed on a rotator[o] every 5 minutes for 30 seconds. The slide was then observed for bacterial agglutination 3 times at 10-minute intervals, using a stereomicroscope[t] (6X magnification).

[s] Amicon Corp, Danvers, Mass.
[t] Wild Heerbrugg Ltd, CH-9435 Heerbrugg, Switzerland.

RESULTS

SDS-PAGE-- After separation by discontinuous-gradient SDS-PAGE and staining with Coomassie brilliant blue R250, the *n*-octyl-glucoside extracts of the 24 bacterial field isolates could be divided into 4 distinct groups according to similarities and differences in their protein profiles. The results are shown in Figs 1-4. The 2 ATCC type species of *Actinobacillus* (29522 and 15768) were also evaluated by SDS-PAGE to obtain data to be used as a standardized reference. Group 1 consisted of the 2 ATCC type cultures and 18 field isolates of *Actinobacillus* species. Field isolates in group 1 included As8C, 4101, 84-832(5), 84-832(9) and 86-618 (Fig 1); 86-722, 86-723, 86-724, 86-731A and 86-814A (Fig 2); R75, A11, A46, A22, 84-833, B30, B30-2 and B111 (Fig 3). Six major bands were shared by these 18 field isolates and the 2 ATCC type species, with molecular masses of 94 KD, 64 KD, 61 KD, 53 KD, 40 KD, and 27 KD. Group 2 consisted of field isolates Y136, S6 and S15. These 3 isolates shared 3 major protein bands at 45 KD, 30 KD and 16 KD (Fig 4). Group 3 consisted of isolates D107 and D56 (Fig 4). Four protein bands with molecular masses of 90 KD, 70 KD, 64 KD and 35 KD were shared by these 2 field isolates. Group 4 had only 1 isolate (A2, Fig 4). Individual field isolates in all 4 groups showed more than 25 distinct protein bands, ranging from 10 KD to 120 KD. Few protein bands were common between the 4 groups of isolates, but the protein profiles were similar among isolates within each group in the first 3 groups. Isolate A2 in group 4 had a unique protein profile.

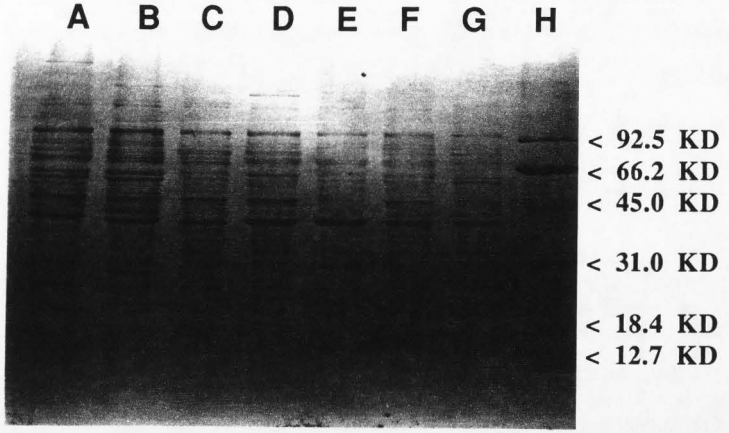

Fig 1 -- Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of n-octyl-β-D-glucopyranoside (*n*-octyl-glucoside) extracts of 2 ATCC type cultures and 5 field isolates of *Actinobacillus* species (group 1). Lanes A and B are extracts of ATCC *A actinomycetemcomitans* and *A seminis,* respectively. Lanes C through G are field isolates As8C, 4101, 84-832(5), 84-832(9), and 86-618, respectively. Molecular weight markers are shown in lane H, with relative molecular masses in kilodaltons (KD) indicated to the right.

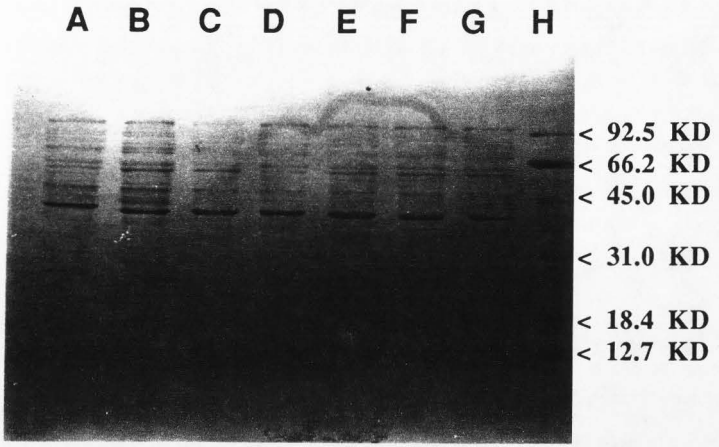

Fig 2 -- Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of *n*-octyl-glucoside extracts of 2 ATCC type cultures and 5 field isolates of *Actinobacillus* species (group 1). Lanes A and B are extracts of ATCC *A actinomycetemcomitans* and *A seminis*, respectively. Lanes C through G are field isolates 86-722, 86-723, 86-724, 86-731A, and 86-814A, respectively. Molecular weight markers are shown in lane H, with relative molecular masses in kilodaltons (KD) indicated to the right.

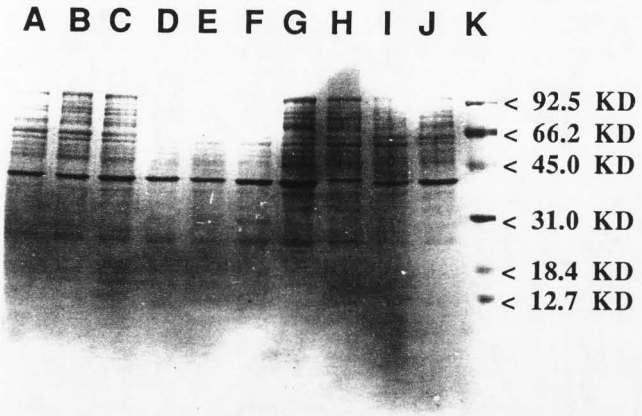

Fig 3 -- Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of *n*-octyl-glucoside extracts of 2 ATCC type cultures and 8 field isolates of *Actinobacillus* species (group 1). Lanes A and B are extracts of ATCC *A actinomycetemcomitans* and *A seminis*, respectively. Lanes C through J are field isolates R75, A11, A46, A22, 84-833, B30, B30-2, and B111, respectively. Molecular weight markers are shown in lane K, with relative molecular masses in kilodaltons (KD) indicated to the right.

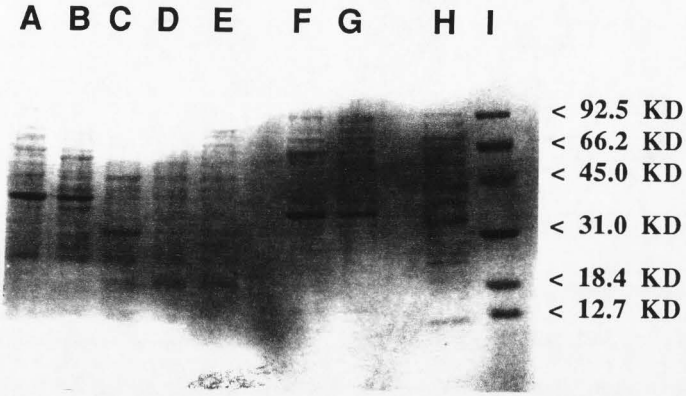

Fig 4 -- Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of *n*-octyl-glucoside extracts of 2 ATCC type cultures and 6 field isolates of *Actinobacillus* species (groups 2, 3, and 4). Lanes A and B are extracts of ATCC *A actinomycetemcomitans* and *A seminis*, respectively. Lanes C through H are field isolates Y136, S6, S15, D107, D56, and A2, respectively. Molecular weight markers are shown in lane I, with relative molecular masses in kilodaltons (KD) indicated to the right.

Hybridomas and monoclonal antibodies (MoAbs) -- Over 30 hybridoma cell lines produced MoAbs specific to As8C antigens, using the ELISA. Thirteen of these 30 hybridomas secreting MoAbs of the IgG isotype were cloned. The remaining hybridomas were suspended in DMEM supplemented with 20% FBS and 10% dimethyl sulfoxide (DMSO) and stored in liquid nitrogen.

Of the 13 hybridomas, 7 produced MoAbs of the IgG1 subisotype (LG51, LG57, LG60, LG61, LG63, LG65 and LG69), 2 produced MoAbs of the IgG2a subisotype (LG68 and LG75) and 4 produced MoAbs of the IgG2b subisotype (LG50, LG56, LG66 and LG70, Table 2). Culture fluid from each cloned hybridoma was checked twice using a mouse MoAb subisotyping kit to ensure MoAb purity.

EITB -- Specificity of the 13 MoAbs to As8C target antigens was confirmed by the EITB. After the *n*-octyl-glucoside As8C extract was transblotted from the polyacrylamide gel to a nitrocellulose membrane and stained with naphthol blue-black, approximately 35 protein-containing bands were observed with molecular masses ranging from 10 KD to 120 KD (Fig 5). After incubation with HMS resulted in approximately 25 bands. The target antigen for MoAbs LG50, LG51, LG56, LG57, LG60, LG61, LG63, LG66 and LG70 appeared as a single band, having a molecular mass of about 60 KD (Fig 6). The MoAbs LG68, LG69 and LG75 also recognized a single band with molecular mass of 74, 45 and 74 KD, respectively (Fig 7). Target antigens for MoAbs LG65 showed 2 distinct bands, having molecular

TABLE 2 -- The IgG subisotype of 13 monoclonal antibodies (MoAbs)

MoAbs	Subisotype*			
	IgG1	IgG2a	IgG2b	IgG3
LG50	---	---	+	---
LG51	+	---	---	---
LG56	---	---	+	---
LG57	+	---	---	---
LG60	+	---	---	---
LG61	+	---	---	---
LG63	+	---	---	---
LG65	+	---	---	---
LG66	---	---	+	---
LG68	---	+	---	---
LG69	+	---	---	---
LG70	---	---	+	---
LG75	---	+	---	---

*Determined by the mouse MoAb subisotyping kit.[d]

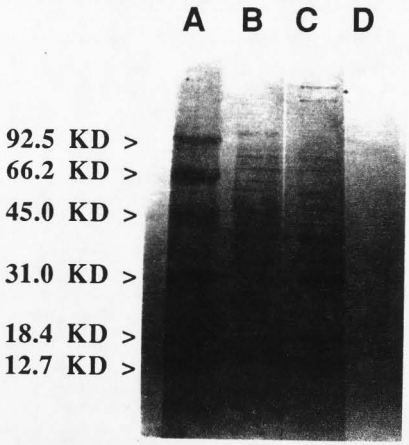

Fig 5 -- Enzyme-linked immunoelectrotransfer blot analysis of *Actinobacillus* species (As8C) antigens from an *n*-octyl-glucoside extract tested against hyperimmune mouse serum (HMS) and FOX/NY culture fluid. Lanes A and B are molecular weight markers and extracted As8C antigens after transfer from a polyacrylamide gel to a nitrocellulose membrane and stained with naphthol blue black, respectively. Lane C shows approximately 25 antigenic fractions of the extract after incubation with HMS. Lane D shows no antigenic fractions of the extract after incubation with FOX/NY culture fluid. Values to the left indicate molecular masses in kilodatons (KD).

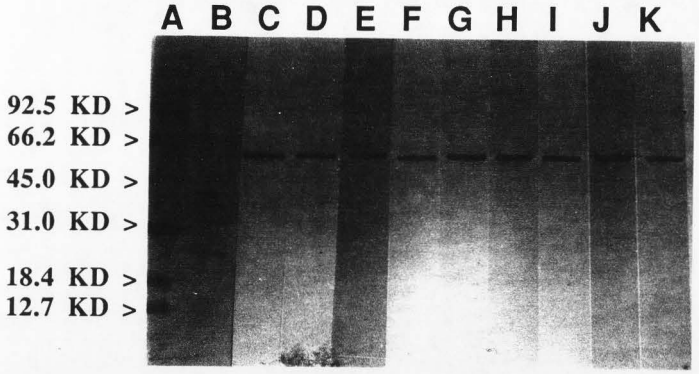

Fig 6 -- Enzyme-linked immunoelectrotransfer blot analysis of *Actinobacillus* species (As8C) antigens from an *n*-octyl-glucoside extract tested against 8 monoclonal antibodies (MoAbs). Lanes A and B are molecular weight markers and extracted As8C antigens after transfer from a polyacrylamide gel to a nitrocellulose membrane and stained with naphthol blue black, respectively. Lanes C through K show the target antigens for MoAbs LG50, LG51, LG56, LG60, LG61, LG63, LG66, and LG70, respectively. Values to the left indicate molecular masses in kilodatons (KD).

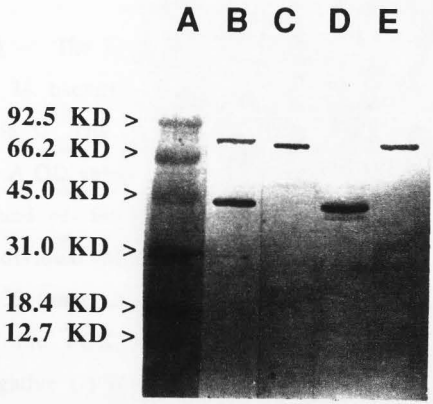

Fig 7 -- Enzyme-linked immunoelectrotransfer blot analysis of *Actinobacillus* species (As8C) antigens from an *n*-octyl-glucoside extract tested against 4 monoclonal antibodies (MoAbs). Lane A is molecular weight markers after transfer from a polyacrylamide gel to a nitrocellulose membrane and stained with naphthol blue black. Lanes B through E show the target antigens for MoAbs LG65, LG68, LG69, and LG75, respectively. Values to the left indicate molecular weight in kilodatons (KD).

masses of 74 KD and 45 KD, respectively (Fig 7). No bands were observed when the nitrocellulose membrane was incubated with FOX/NY culture fluid.

ELISA -- The ELISA results of 13 MoAbs against 2 ATCC type cultures and 24 bacterial field isolates of *Actinobacillus* species are given in Table 3. The scores were calculated from the Δ OD values of the MoAbs + Δ OD values of FOX/NY culture fluid. The symbols ++++, +++, ++, + and +/- indicate positive reactions (ranging from strong to weak) of individual MoAbs, which had intensities greater than 40 times, 30 to 39 times, 20 to 29 times, 10 to 19 times and 5 to 9 times greater than the FOX/NY value, respectively. The reaction was scored as negative (-) if the intensity was 5 times or less that of the FOX/NY culture fluid. Based on the ELISA results, the bacterial isolates could be divided into 3 groups. The first group contained field isolates As8C, 4101, 84-832(5), 84-832(9), 86-618, 86-722, 86-723, 86-724, 86-731A, 86-814A, R75, A11, A22, 84-833, B30, B30-2 and B111. The target antigens on these isolates appeared to be recognized by all 13 MoAbs, but with considerable variation in antigen frequency between isolates as indicated by differences in the scores of the reactions. The second group consisted of the isolates Y136, S6 and S15. Bacteria in this group showed essentially the same reactions when incubated with the 13 MoAbs. The isolates in group 3 consisted of A46, D107, D56 and A2, which showed variable or no recognition by most of the 13 MoAbs. Monoclonal antibody LG61 recognized all isolates of all 3 groups. The LG51 and LG60

TABLE 3 -- Results of the ELISA, using the 13 monoclonal antibodies (MoAbs) against 2 ATCC type cultures and 24 field isolates of *Actinobacillus* species

Isolates	Monoclonal Antibody*												
	LG50	LG51	LG56	LG57	LG60	LG61	LG63	LG65	LG66	LG68	LG69	LG70	LG75
29522†	++++	++	++	+++	++	+++	++	+	+++	+	+++	++++	+/-
15768‡	+++	+++	++	+++	+++	+++	++	++	+++	+/-	++	++++	+/-
As8C§	+++	++	+	++	++	++	+	+	+++	+	++	+++	+
4101	+++	++	++	++	++	+++	++	+/-	+++	+/-	+	++++	-
84-832(5)	+++	+++	++	+++	+++	+++	++	+	+++	+/-	++	++++	+/-
84-832(9)	++++	+++	++	+++	++++	++++	++	+/-	++++	+/-	+	++++	-
86-618	+++	++	+++	++	++	++	+	+	+++	+	+	+++	+/-
86-722	+++	++	++	+++	+++	+++	++	+	+++	+	+	+++	+
86-723	+++	++	+	++	+++	++	++++	+	+++	+	++	+++	+
86-724	++++	+++	++	+++	+++	+++	++	+	+++	++	++	++++	+
86-731A	++++	++	++	+++	+++	+++	++	+	+++	+	++	+++	+/-
86-814A	++++	+++	++	+++	+++	+++	++	+	+++	+++	++++	+	+/-
R75	+++	++	++	++	++	++	+	+	++	+	++	+++	+
A11	++	+++	+	++	++	++	+	+	++	+	+	++	+
A22	++++	+++	++	+++	++++	+++	+	+	++++	+	+	+++	+
84-833	+++	++	+	++	++	+/-	+	+	+++	+/-	+	+++	+/-
B30	+++	+++	++	+++	+++	+++	++	+	+++	+/-	++	++++	-
B30-2	++	+++	++	+++	+++	+++	+++	+	+++	-	++	++	+/-
B111	+++	+++	++	+++	+++	+++	++	+	+++	+/-	++	+++	-
Y136	+/-	+	-	-	++	++	+/-	-	-	-	-	-	-
S6	+/-	+	-	-	++	++	+/-	-	+	+/-	-	-	-
S15	+/-	+	-	-	+	++	+/-	-	+/-	-	-	-	-
A46	+	-	-	+/-	-	+/-	-	-	+	-	-	+/-	-
D107	-	+++	+/-	+	-	+++	+	+	+/-	-	-	-	-
D56	++	-	+	-	++	++	-	-	-	-	-	-	-
A2	-	+++	-	-	+++	+++	-	-	-	-	-	-	-

*Hybridoma culture fluids were used as the sources of the MoAbs. †American type culture collection (ATCC) of *Actinobacillus actinomycetemcomitans*. ‡ATCC of *A seminis*. §The species of *Actinobacillus* from whih the 3 protein bands were extracted and the 13 MoAbs were prepared. ++++, +++, ++, +, and +/- = positive reactions ranging from strong to weak, - = negative.

MoAbs also recognized most of the isolates, but showed less reaction intensity with group 2 and group 3 isolates. Monoclonal antibody LG75 showed the weakest reaction intensity with isolates in group 1, and no recognition of group 2 and group 3 isolates.

Flow cytometry -- The flow cytometry results of 2 ATCC type cultures and 24 field isolates of *Actinobacillus* species, using the 13 MoAbs, HMS and FOX/NY culture fluid, are given in Figures 8 - 33. Three values were obtained from each graph. These included the peak-channel (Table 4a), the mean-value (Table 4b) and the percent-labeled cells (Table 4c). Quantitative results of fluorescent intensity were derived from the data presented in Table 4c and are shown in Table 4d. These results were generated by subtracting the percentage of cells labeled by the FOX/NY culture fluid from the percentage of cells labeled by the MoAbs for each bacterial isolate. The symbols ++++, +++, ++, + and +/- were used to indicate positive reactions, ranging from strong to weak, which had greater than 80%, 60 to 79%, 40 to 59%, 20 to 39% and 10 to 19% more cells labeled by the MoAbs than by the FOX/NY culture fluid. If 5% or less of the bacterial cells were labeled by the MoAbs as compared with the FOX/NY culture fluid, the reaction was scored as negative (-). Field isolates in Tables 4a-4d could be divided into 2 major groups. Isolates in group 1 were identical to the isolates assigned to group 1, except isolate A46, using the ELISA. Group 2 consisted of isolates Y136, S6, S15, D107, D56 and A2. Almost all of the 13 MoAbs showed specificity to group 1 isolates, but only weakly recognized

TABLE 4a -- Peak chanel* for flow cytometry, using the 13 monoclonal antibodies (MoAbs) against 2 ATCC type cultures and 24 field isolates of *Actinobacillus* species

Isolates	Monoclonal Antibody†												
	LG50	LG51	LG56	LG57	LG60	LG61	LG63	LG65	LG66	LG68	LG69	LG70	LG75
29522‡	135	106	56	57	65	105	68	122	126	64	125	122	67
15768§	91	112	47	47	112	105	50	48	77	48	47	55	50
As8C¶	98	74	47	45	68	57	51	94	45	119	119	70	121
4101	105	94	45	47	96	86	73	45	71	45	55	66	46
84-832(5)	109	134	46	64	123	112	50	70	55	57	140	53	55
84-832(9)	104	132	48	50	141	107	53	87	47	106	118	78	71
86-618	144	138	46	52	139	127	96	110	127	142	124	134	122
86-722	136	124	45	95	112	127	102	111	98	123	105	108	119
86-723	45	108	46	45	107	85	46	62	55	48	72	82	49
86-724	164	167	90	90	159	162	117	47	160	56	56	151	55
86-731A	151	129	58	85	148	134	89	113	50	45	124	119	46
86-814A	93	74	50	54	79	69	55	46	74	50	85	49	45
R75	102	109	52	48	88	123	55	48	80	51	61	117	48
A11	97	115	47	47	125	82	54	45	72	46	59	70	48
A46	129	93	49	45	109	93	59	95	85	57	122	64	49
A22	96	112	45	52	114	89	59	48	55	48	55	79	46
84-833	123	114	55	48	115	114	62	62	88	45	103	103	45
B30	140	119	60	69	129	134	107	70	131	46	78	46	49
B30-2	74	96	50	51	97	111	79	45	107	46	74	45	48
B111	118	56	58	45	55	137	55	47	126	47	122	45	57
Y136	46	106	46	45	54	72	53	53	48	45	48	49	74
S6	46	49	46	45	49	45	58	51	49	45	45	45	63
S15	46	55	49	45	67	70	59	46	50	54	53	45	46
D107	50	45	54	46	48	50	47	53	53	48	48	46	49
D56	45	45	45	46	45	48	52	48	46	46	48	52	45
A2	57	49	53	48	48	49	50	45	59	47	53	45	45

*Peak chanel indicates the position (X-axis) of the highest point (Y-axis) beyond the cursor line, located at position 45. †Hybridoma culture fluids were used as the source of monoclonal antibodies (MoAbs). ‡ATCC of *Actinobacillus actinomycetemcomitans*. §ATCC of *A seminis*. ¶The species of *Actinobacillus* from which the 3 protein bands were extracted and the 13 MoAbs were prepared.

TABLE 4b -- Mean value* for flow cytometry, using the 13 monoclonal antibodies (MoAbs) against 2 ATCC type cultures and 24 field isolates of *Actinobacillus* species

Isolates	Monoclonal Antibody†												
	LG50	LG51	LG56	LG57	LG60	LG61	LG63	LG65	LG66	LG68	LG69	LG70	LG75
29522‡	117.4	109.2	83.9	84.5	92.4	112.2	95.1	110.5	116.0	91.7	118.6	116.4	90.1
15768§	110.9	115.3	81.2	87.3	116.8	108.2	90.4	72.7	104.0	97.2	78.9	106.8	98.2
As8C¶	97.6	87.1	76.6	75.1	86.6	86.9	86.4	105.4	88.9	114.9	126.8	85.2	114.6
4101	105.4	91.9	67.0	66.4	92.8	93.0	81.4	70.1	87.9	84.0	81.0	93.8	75.7
84-832(5)	110.6	115.4	89.5	85.2	121.4	106.4	88.4	101.2	107.4	95.6	127.0	108.5	96.6
84-832(9)	109.2	120.0	76.4	74.3	121.2	114.7	81.2	88.2	89.6	114.2	99.7	103.2	93.0
86-618	129.8	131.7	75.6	77.6	133.7	125.6	95.6	103.0	113.7	122.1	122.2	115.2	117.8
86-722	132.4	127.6	83.7	89.6	113.0	131.2	103.2	94.8	110.1	113.2	113.5	120.4	111.7
86-723	77.4	109.2	75.6	70.7	106.0	89.6	77.6	85.8	93.8	73.4	94.7	85.3	77.6
86-724	142.9	144.1	96.4	97.5	139.0	133.8	103.4	83.3	129.2	85.1	94.5	133.7	91.2
86-731A	116.4	132.3	81.2	86.4	145.4	124.7	92.6	112.6	100.6	72.5	119.2	118.4	70.7
86-814A	105.8	101.0	82.3	92.0	103.4	101.7	91.8	98.7	97.2	81.5	111.6	98.2	89.9
R75	106.3	100.9	77.2	79.0	95.6	101.2	94.8	82.3	95.8	78.6	91.6	99.6	77.2
A11	101.8	106.8	65.5	68.1	118.8	96.7	71.6	60.5	88.4	70.7	69.5	84.5	72.0
A46	110.3	102.6	75.9	82.3	104.2	104.9	87.3	102.2	97.9	93.2	110.1	97.7	92.7
A22	104.3	108.6	74.4	81.2	102.9	100.6	83.7	78.6	85.6	87.1	82.6	90.2	86.5
84-833	120.1	112.2	71.2	78.4	109.8	116.8	91.6	87.0	107.0	62.2	104.4	106.3	62.0
B30	117.8	117.8	82.2	86.8	114.6	118.9	99.5	88.6	118.9	92.2	96.2	85.4	92.3
B30-2	103.8	109.2	84.2	83.6	107.6	100.7	91.5	83.9	112.0	77.8	96.2	86.3	79.0
B111	108.5	116.0	100.8	99.8	112.7	116.9	88.7	99.8	113.7	92.3	106.2	80.7	91.3
Y136	81.6	101.2	83.9	80.3	102.4	108.4	84.1	79.8	86.6	88.4	87.7	82.2	87.9
S6	69.2	81.4	81.0	77.0	79.2	68.0	80.9	70.9	81.3	83.0	79.4	83.1	83.2
S15	81.7	93.6	78.4	74.2	103.1	109.8	85.3	71.9	85.3	99.0	71.1	86.4	94.0
D107	78.6	72.6	76.5	73.2	74.8	74.6	81.6	75.8	82.1	79.9	73.6	75.3	85.8
D56	91.1	77.6	94.5	92.7	81.5	82.1	81.5	86.3	92.8	94.5	78.1	87.8	93.2
A2	87.0	92.8	81.4	84.7	88.6	87.6	85.5	78.0	88.5	91.8	78.1	73.1	88.7

*Figures of these values indicate the position (X-axis) of the average height (Y-axis) beyond the cursor line, located at position 45. †Hybridoma culture fluids were used as the source of monoclonal antibodies (MoAbs). ‡ ATCC of *Actinobacillus actinomycetemcomitans*. §ATCC of *A seminis*. ¶The species of *Actinobacillus* from which the 3 protein bands were extracted, and the 13 MoAbs were prepared.

TABLE 4c -- Percent labeled cells* for flow cytometry, using the 13 monoclonal antibodies (MoAbs) against 2 ATCC type cultures and 24 field isolates of *Actinobacillus* species

Isolates	Monoclonal Antibody†												
	LG50	LG51	LG56	LG57	LG60	LG61	LG63	LG65	LG66	LG68	LG69	LG70	LG75
29522‡	62.2	50.8	53.8	33.7	48.1	65.6	65.5	60.2	68.3	44.9	65.4	67.9	52.8
15768§	63.9	76.9	39.0	46.9	73.1	79.3	65.5	31.3	71.2	19.0	46.5	66.4	12.4
As8C¶	58.2	62.6	20.6	22.1	64.5	57.7	53.2	48.8	38.0	69.7	72.3	73.8	68.5
4101	72.2	58.5	33.6	35.1	77.3	57.7	70.8	33.1	62.3	16.8	70.9	66.6	21.3
84-832(5)	63.0	73.7	21.9	37.3	73.5	71.5	56.6	37.6	33.4	64.0	68.0	58.0	48.6
84-832(9)	54.1	77.5	42.0	46.3	69.2	72.1	61.8	47.2	39.9	75.1	72.8	37.8	63.4
86-618	76.0	82.0	33.3	57.7	82.9	78.9	78.4	73.4	77.9	81.3	80.0	71.9	77.0
86-722	50.8	77.6	22.1	66.3	55.2	78.0	74.0	57.9	54.1	71.7	74.3	75.0	67.0
86-723	37.4	58.9	23.0	28.8	65.9	53.5	46.4	45.5	45.9	16.5	44.8	52.4	18.5
86-724	45.8	58.5	36.5	20.8	61.6	60.3	52.8	38.0	55.7	33.6	26.6	44.5	21.2
86-731A	40.0	87.9	67.0	70.4	86.5	86.1	79.4	78.3	38.8	49.8	58.8	84.0	9.3
86-814A	53.0	63.9	29.1	28.5	58.7	56.6	58.1	55.7	63.6	42.8	60.8	50.5	27.2
R75	55.9	59.6	34.8	32.0	55.3	58.5	57.7	46.1	50.7	40.1	43.0	57.9	41.8
A11	81.8	90.3	26.3	42.4	90.8	83.9	59.8	23.2	77.8	51.4	61.6	75.8	37.8
A46	70.5	75.0	39.4	26.4	75.3	64.2	61.8	65.3	63.6	54.5	66.7	58.2	54.1
A22	66.4	63.6	28.1	29.0	65.5	69.6	54.2	24.3	36.8	36.5	44.9	59.3	23.5
84-833	85.6	88.3	42.0	53.5	87.0	89.2	75.2	75.2	81.0	28.6	83.6	84.3	22.9
B30	61.6	75.8	43.0	64.8	74.7	75.2	70.3	42.2	77.3	23.9	49.1	38.2	18.0
B30-2	49.1	55.6	26.5	43.8	57.4	34.7	49.7	34.7	58.8	19.2	48.9	22.0	15.4
B111	52.9	52.2	39.9	48.2	58.0	55.6	46.6	40.5	63.3	54.1	52.0	18.8	25.7
Y136	26.6	46.6	6.0	11.8	41.3	46.6	37.0	12.8	18.8	19.1	11.8	1.4	6.4
S6	11.7	22.7	7.3	7.8	15.5	29.3	25.3	25.6	24.4	21.3	13.4	9.8	18.8
S15	23.2	35.7	4.6	11.4	54.2	53.4	21.3	9.8	32.4	18.4	6.0	11.0	12.1
D107	18.6	15.9	6.5	11.9	20.1	25.9	17.3	9.7	11.6	14.7	11.5	3.0	19.3
D56	12.8	20.2	6.3	8.4	22.7	18.8	36.1	9.2	19.2	16.2	16.0	6.5	11.8
A2	14.0	33.9	3.2	6.4	36.1	30.4	30.4	8.1	17.0	17.8	9.2	3.7	13.3

*Values indicate the percentage of cells with a fluorescent intensity beyond that of the cursor line. †Hybridoma culture fluids were used as the source of monoclonal antibodies (MoAbs). ‡ATCC of *Actinobacillus actinomycetemcomitans*. §ATCC of *A seminis*. ¶The species of *Actinobacillus* from which the 3 protein bands were extracted and the 13 MoAbs were prepared.

TABLE 4d -- Quantitative results* for flow cytometry, using the 13 monoclonal antibodies (MoAbs) against 2 ATCC type cultures and 24 field isolates of *Actinobacillus* species

Isolates	Monoclonal Antibody†												
	LG50	LG51	LG56	LG57	LG60	LG61	LG63	LG65	LG66	LG68	LG69	LG70	LG75
29522‡	++	++	++	+	++	+++	+++	++	+++	++	+++	+++	++
15768§	++	+++	+	+	+++	+++	++	+	+++	-	+	++	-
As8C¶	++	++	-	+/-	++	++	++	+	+	++	+++	++	++
4101	+++	++	+	+	+++	++	++	+	++	-	+	++	+/-
84-832(5)	++	+++	+/-	+	+++	+++	++	+	+	++	+++	++	++
84-832(9)	++	+++	+	+	+++	+++	++	+	+	+++	+++	+	++
86-618	+++	+++	+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++
86-722	++	+++	+/-	++	++	+++	+++	++	++	+++	+++	+++	++
86-723	+	++	+/-	+	++	++	+	+	+	-	+	++	+/-
86-724	++	++	+	+/-	++	++	++	+	++	+	+	+	+/-
86-731A	+	+++	++	++	+++	+++	+++	+++	+	+	++	+++	-
86-814A	++	++	+/-	+/-	++	++	++	++	++	+	++	+	+/-
R75	++	++	+	+	++	++	++	+	++	+	+	++	+
A11	++	+++	+	+	++++	++++	++	+/-	+++	++	++	+++	+
A46	+++	+++	+	+/-	+++	++	++	++	++	++	++	++	++
A22	++	++	+/-	+/-	++	++	++	+/-	+	+	+	++	+/-
84-833	++++	++++	++	++	++++	++++	+++	+++	+++	+	++++	++++	+
B30	++	++	+	+++	+++	+++	+++	+	+++	+	++	+	+/-
B30-2	+	++	+/-	+	++	+	+	+	++	-	+	+/-	+
B111	+	+	+	+	++	+	+	+	++	+	+	-	-
Y136	+/-	+	-	-	+	+	+	-	-	-	-	-	-
S6	-	+/-	-	-	-	+/-	+/-	+/-	+/-	+/-	-	-	-
S15	+/-	+	-	-	++	++	+/-	-	+	+/-	-	-	-
D107	+/-	-	-	-	+/-	+/-	-	-	-	-	-	-	+/-
D56	-	+/-	-	-	+/-	+/-	+	-	+/-	+/-	+/-	-	-
A2	-	+	-	-	+	+	+	-	+/-	+/-	-	-	-

*Determined by values of percent cells labled by fluorescent antibody. †Hybridoma culture fluids were used as the source of monoclonal antibodies (MoAbs). ‡ ATCC of *Actinobacillus actinomycetemcomitans*. §ATCC of *A seminis*. ¶The species of *Actinobacillus* from which the 3 protein bands were extracted and the 13 MoAbs were prepared.
++++, +++, ++, +, and +/- = positive reactions ranging from strong to weak, - = negative.

isolates in group 2. Data shown in Table 4d indicated that MoAb LG61 recognized a public epitope shared by all of the isolates in both groups. The LG51, LG60 and LG66 MoAbs also recognized group 1 isolates, but showed less reaction intensity with group 2 isolates. From the graphic data (Fig 8-33), it was noted that each MoAb showed a unique reaction pattern when incubated against different bacterial isolates. This suggested that it may be possible to prepare a bank of graphs as references in the identification of other bacterial isolates belonging to the genus of *Actinobacillus* using a standardized panel of MoAbs.

Bacterial agglutination -- Agglutination of the 2 ATCC type cultures and 24 field isolates of *Actinobacillus* species was not observed with any of the 13 MoAbs or the FOX/NY culture fluid. Undiluted HMS agglutinated all ATCC type species and field isolates except Y136, S6, D107, D56 and A2. Diluted HMS (1:5 dilution) also agglutinated all of the isolates except 86-731A, R75, Y136, S6, S15, D107, D56 and A2 (Table 5). The symbols ++, + and +/- indicate positive bacterial agglutination, ranging from moderately strong to weak, and a negative (-) symbol was used if no bacterial agglutination was observed.

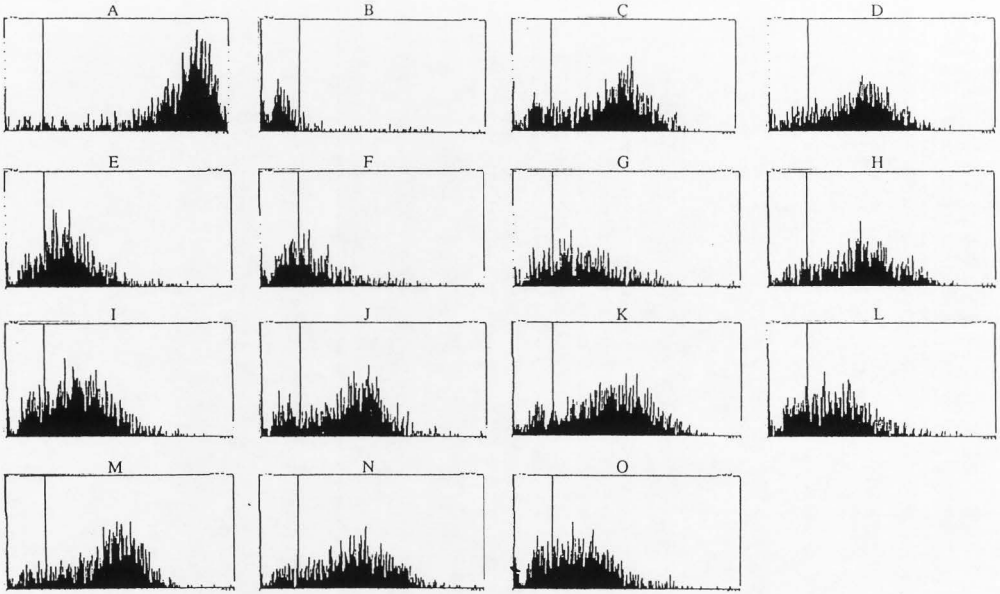

Fig 8 -- Flow cytometry graphic results of hyperimmune mouse serum (A), FOX/NY culture fluid (B), and monoclonal antibodies LG50, LG51, LG56, LG57, LG60, LG61, LG63, LG65, LG66, LG68, LG69, LG70, and LG75 (C-O, respectively) following reaction with ATCC type species *Actinobacillus actinomycetemcomitans* (29522).

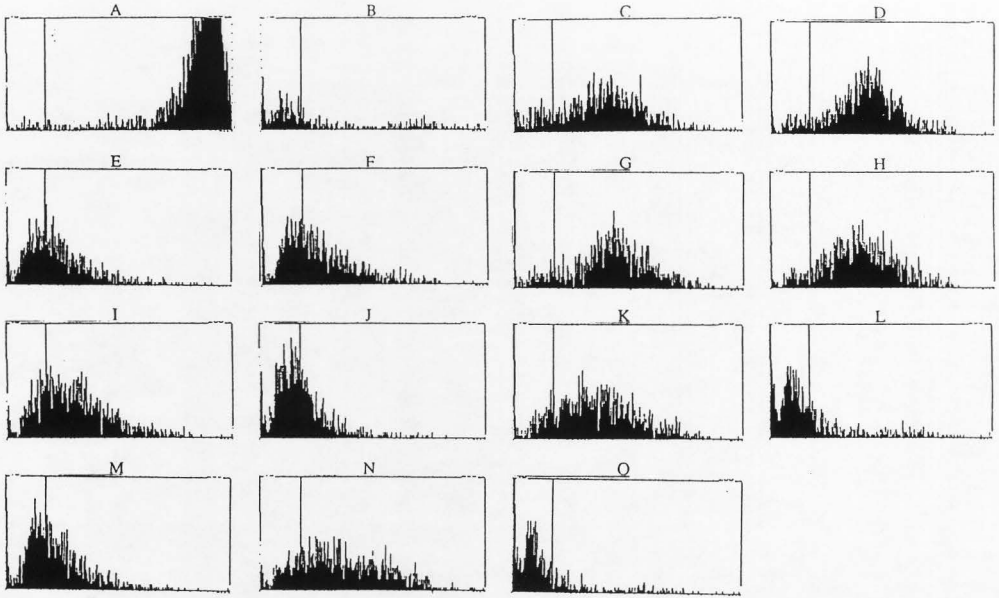

Fig 9 -- Flow cytometry graphic results of hyperimmune mouse serum (A), FOX/NY culture fluid (B), and monoclonal antibodies LG50, LG51, LG56, LG57, LG60, LG61, LG63, LG65, LG66, LG68, LG69, LG70, and LG75 (C-O, respectively) following reaction with ATCC type species *Actinobacillus seminis* (15768).

As8C

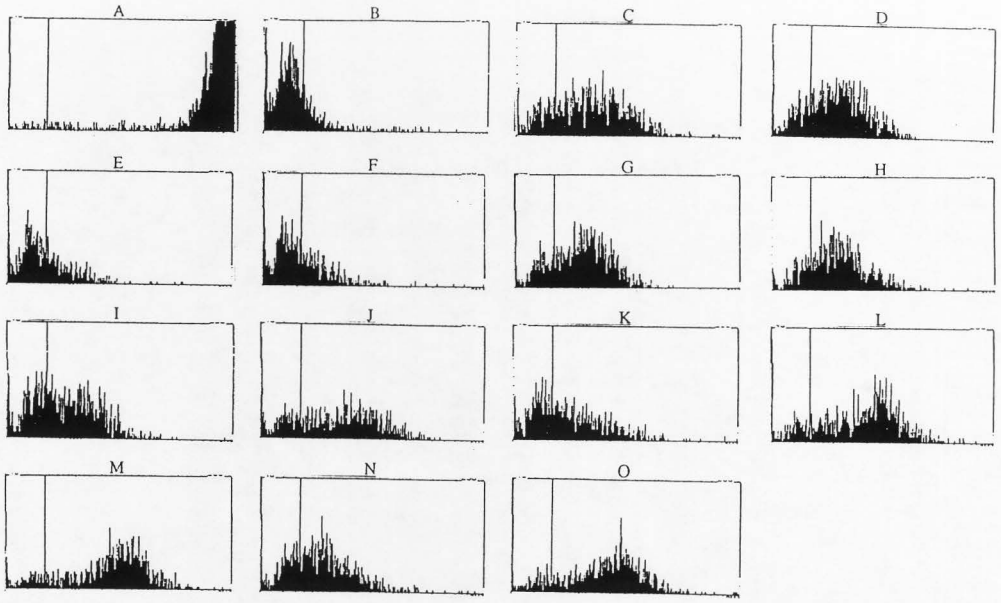

Fig 10 -- Flow cytometry graphic results of hyperimmune mouse serum (A), FOX/NY culture fluid (B), and monoclonal antibodies LG50, LG51, LG56, LG57, LG60, LG61, LG63, LG65, LG66, LG68, LG69, LG70, and LG75 (C-O, respectively) following reaction with bacterial isolate As8C.

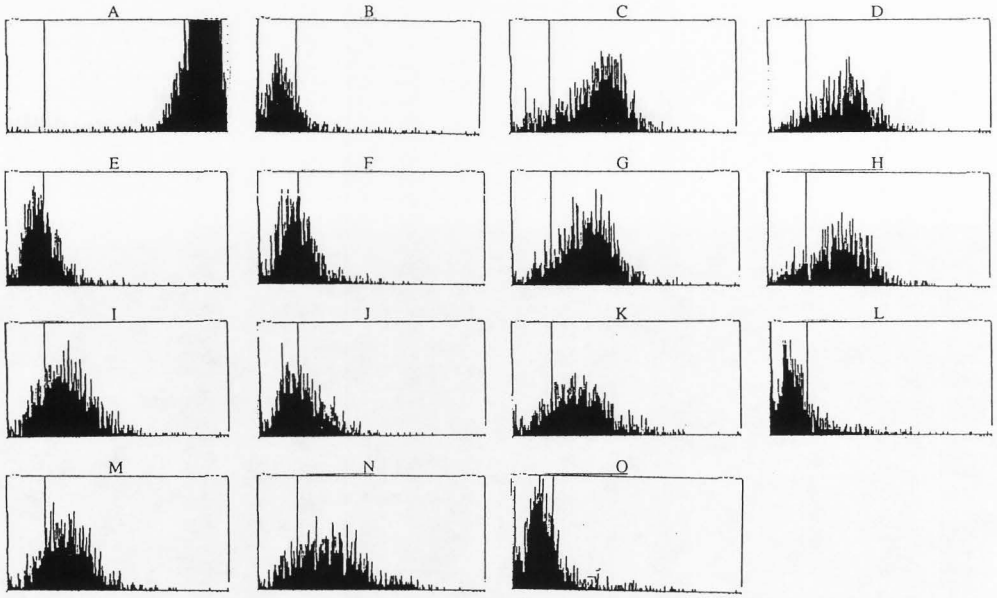

Fig 11 -- Flow cytometry graphic results of hyperimmune mouse serum (A), FOX/NY culture fluid (B), and monoclonal antibodies LG50, LG51, LG56, LG57, LG60, LG61, LG63, LG65, LG66, LG68, LG69, LG70, and LG75 (C-O, respectively) following reaction with bacterial isolate 4101.

84-832(5)

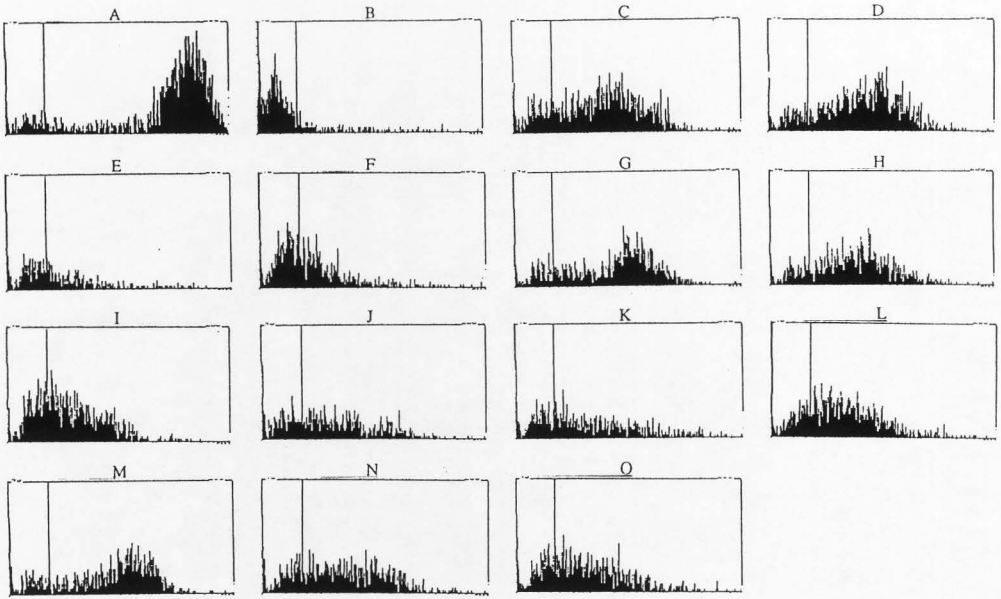

Fig 12 -- Flow cytometry graphic results of hyperimmune mouse serum (A), FOX/NY culture fluid (B), and monoclonal antibodies LG50, LG51, LG56, LG57, LG60, LG61, LG63, LG65, LG66, LG68, LG69, LG70, and LG75 (C-O, respectively) following reaction with bacterial isolate 84-832(5).

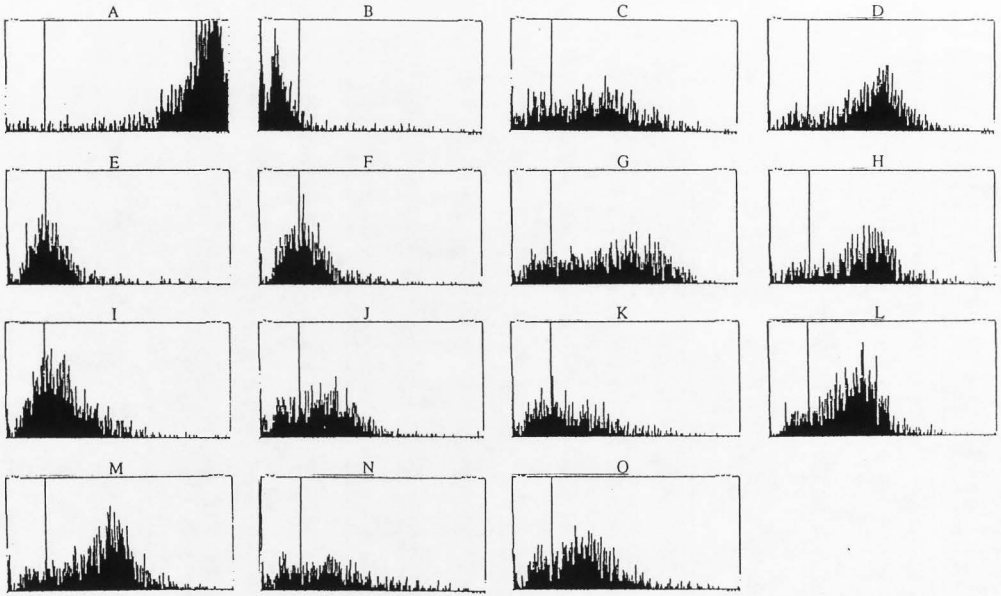

Fig 13 -- Flow cytometry graphic results of hyperimmune mouse serum (A), FOX/NY culture fluid (B), and monoclonal antibodies LG50, LG51, LG56, LG57, LG60, LG61, LG63, LG65, LG66, LG68, LG69, LG70, and LG75 (C-O, respectively) following reaction with bacterial isolate 84-832(9).

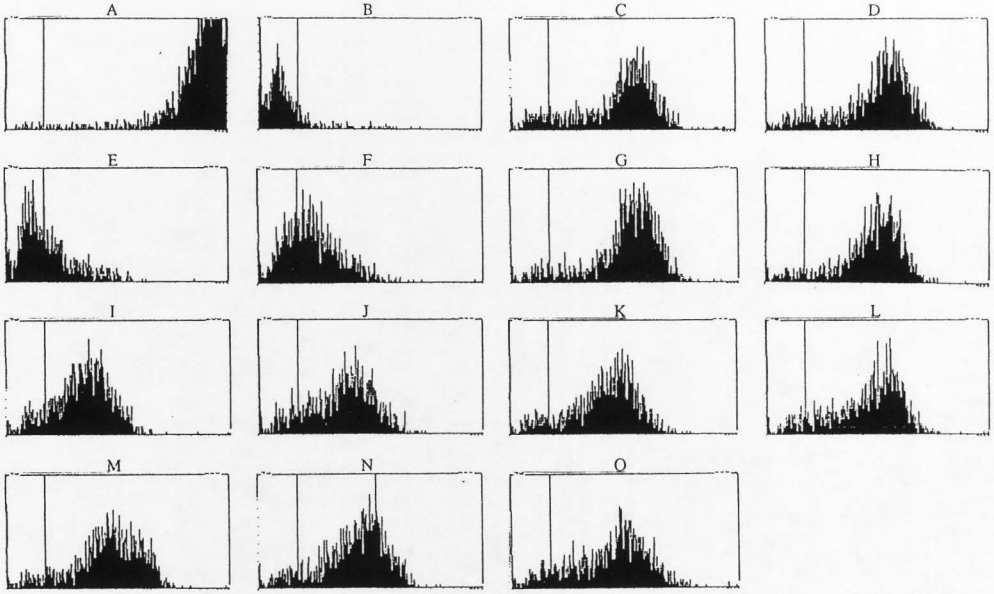

Fig 14 -- Flow cytometry graphic results of hyperimmune mouse serum (A), FOX/NY culture fluid (B), and monoclonal antibodies LG50, LG51, LG56, LG57, LG60, LG61, LG63, LG65, LG66, LG68, LG69, LG70, and LG75 (C-O, respectively) following reaction with bacterial isolate 86-618.

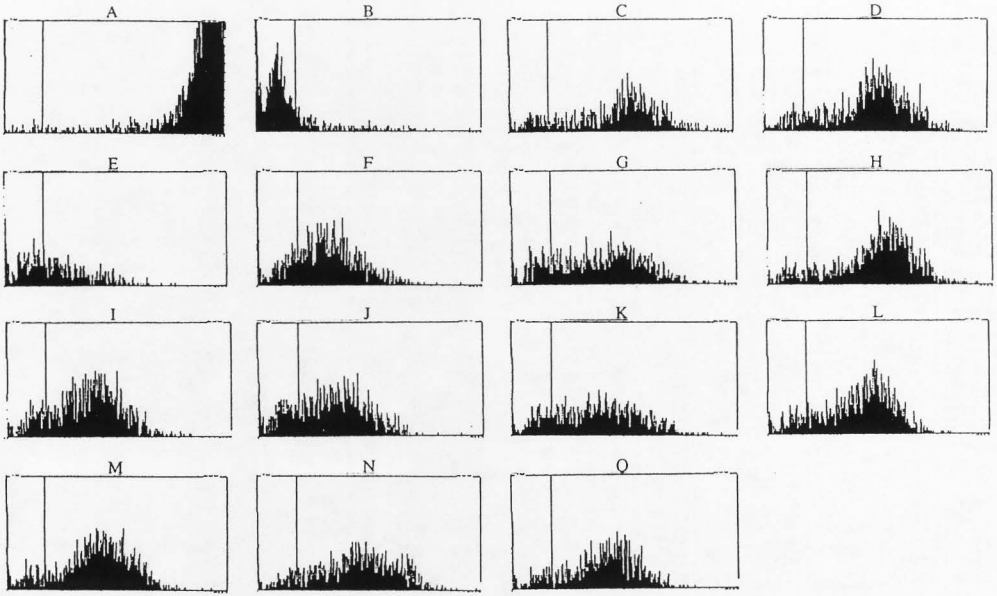

Fig 15 -- Flow cytometry graphic results of hyperimmune mouse serum (A), FOX/NY culture fluid (B), and monoclonal antibodies LG50, LG51, LG56, LG57, LG60, LG61, LG63, LG65, LG66, LG68, LG69, LG70, and LG75 (C-O, respectively) following reaction with bacterial isolate 86-722.

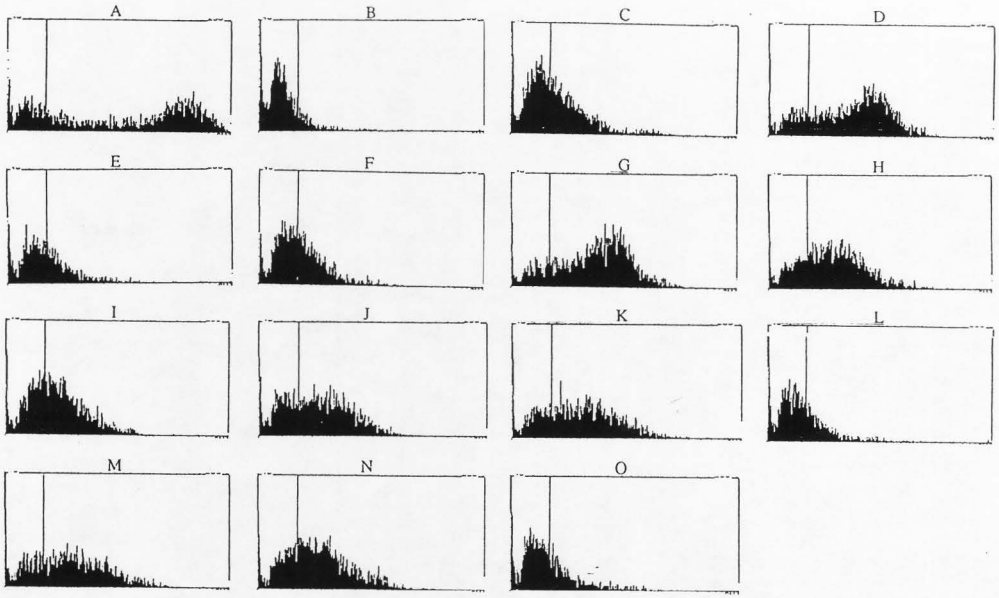

Fig 16 -- Flow cytometry graphic results of hyperimmune mouse serum (A), FOX/NY culture fluid (B), and monoclonal antibodies LG50, LG51, LG56, LG57, LG60, LG61, LG63, LG65, LG66, LG68, LG69, LG70, and LG75 (C-O, respectively) following reaction with bacterial isolate 86-723.

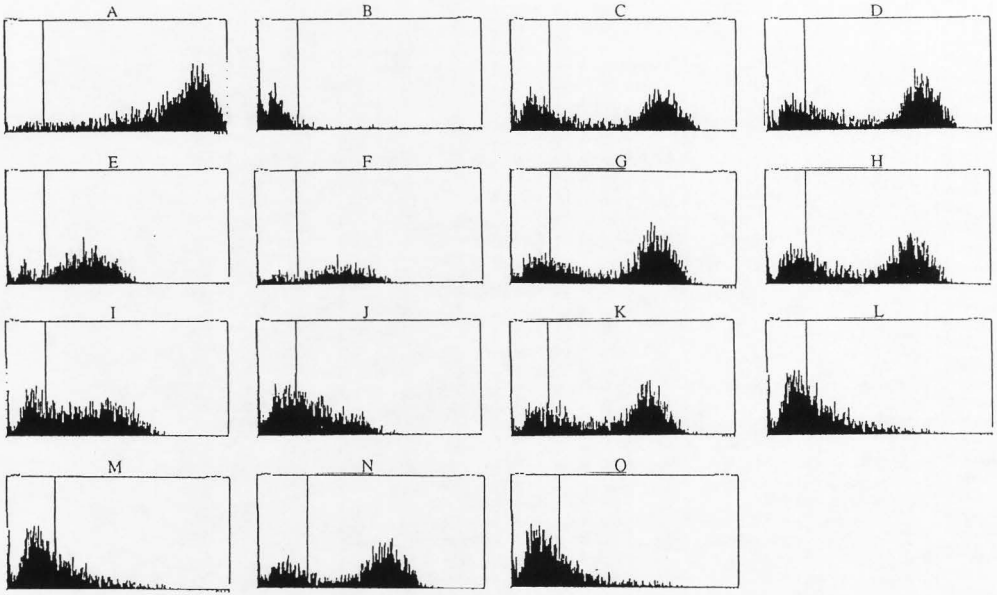

Fig 17 -- Flow cytometry graphic results of hyperimmune mouse serum (A), FOX/NY culture fluid (B), and monoclonal antibodies LG50, LG51, LG56, LG57, LG60, LG61, LG63, LG65, LG66, LG68, LG69, LG70, and LG75 (C-O, respectively) following reaction with bacterial isolate 86-724.

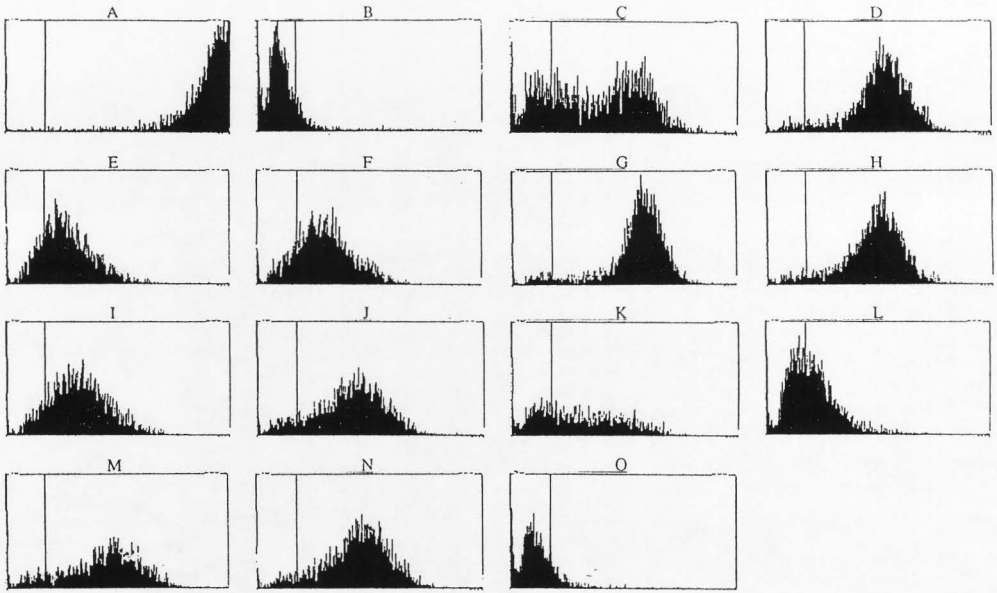

Fig 18 -- Flow cytometry graphic results of hyperimmune mouse serum (A), FOX/NY culture fluid (B), and monoclonal antibodies LG50, LG51, LG56, LG57, LG60, LG61, LG63, LG65, LG66, LG68, LG69, LG70, and LG75 (C-O, respectively) following reaction with bacterial isolate 86-731A.

86-814A

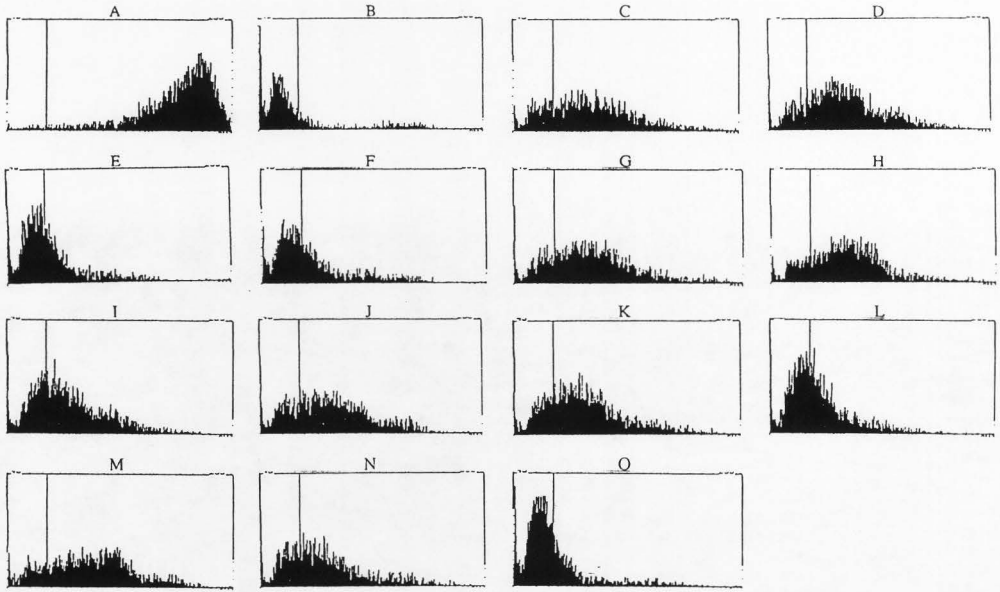

Fig 19 -- Flow cytometry graphic results of hyperimmune mouse serum (A), FOX/NY culture fluid (B), and monoclonal antibodies LG50, LG51, LG56, LG57, LG60, LG61, LG63, LG65, LG66, LG68, LG69, LG70, and LG75 (C-O, respectively) following reaction with bacterial isolate 86-814A.

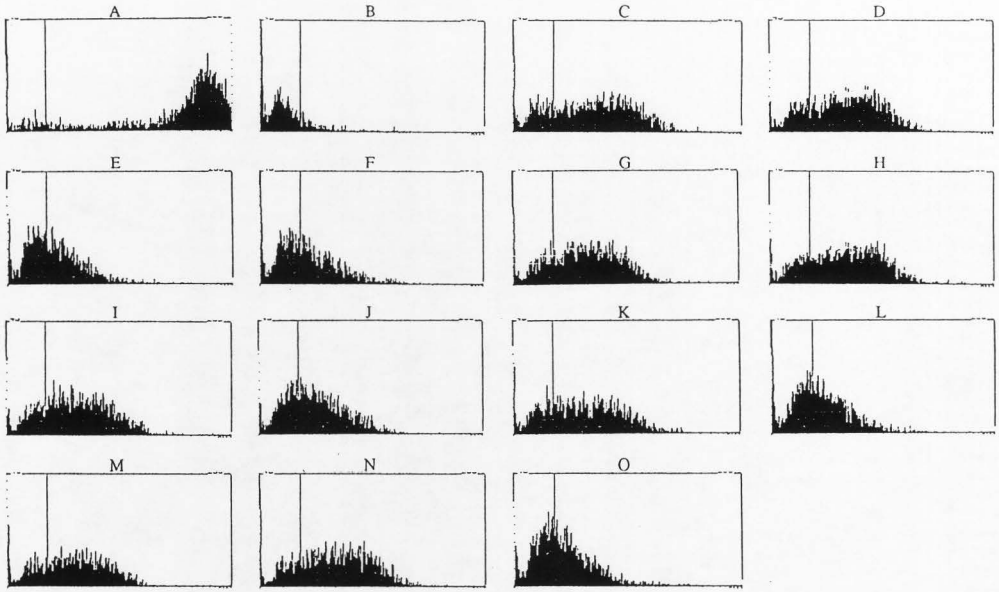

Fig 20 -- Flow cytometry graphic results of hyperimmune mouse serum (A), FOX/NY culture fluid (B), and monoclonal antibodies LG50, LG51, LG56, LG57, LG60, LG61, LG63, LG65, LG66, LG68, LG69, LG70, and LG75 (C-O, respectively) following reaction with bacterial isolate R75.

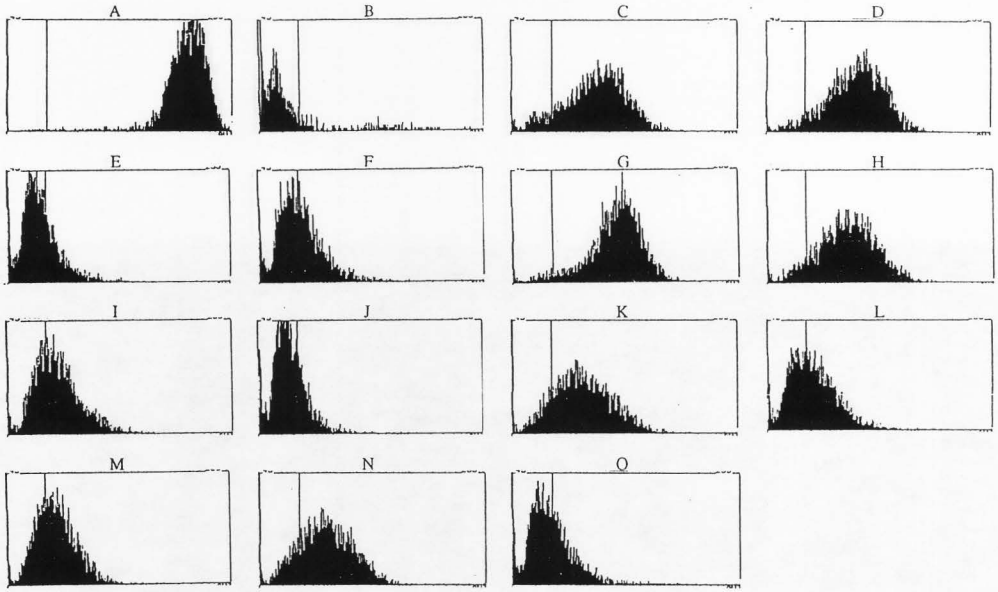

Fig 21 -- Flow cytometry graphic results of hyperimmune mouse serum (A), FOX/NY culture fluid (B), and monoclonal antibodies LG50, LG51, LG56, LG57, LG60, LG61, LG63, LG65, LG66, LG68, LG69, LG70, and LG75 (C-O, respectively) following reaction with bacterial isolate A11.

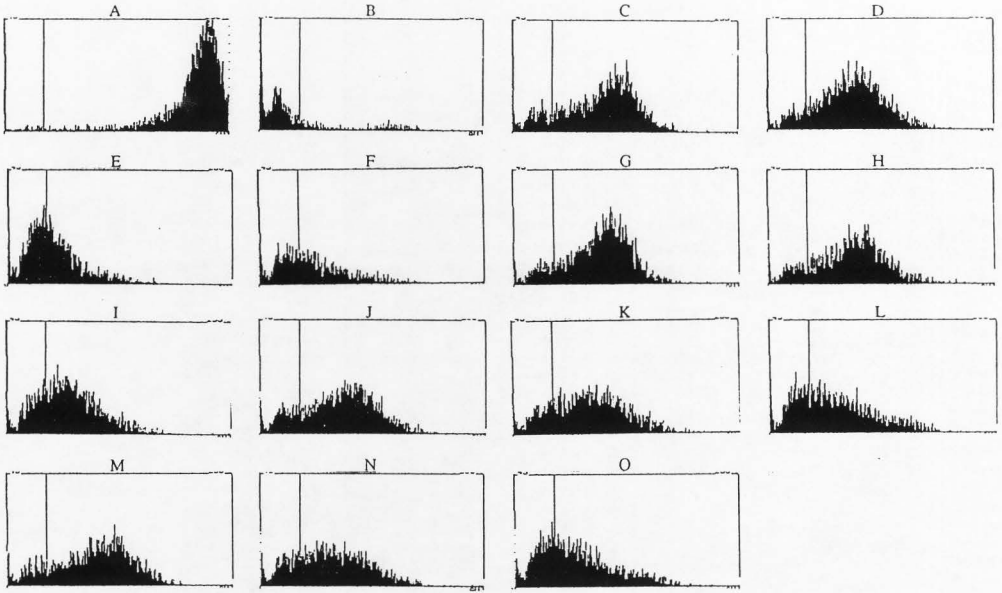

Fig 22 -- Flow cytometry graphic results of hyperimmune mouse serum (A), FOX/NY culture fluid (B), and monoclonal antibodies LG50, LG51, LG56, LG57, LG60, LG61, LG63, LG65, LG66, LG68, LG69, LG70, and LG75 (C-O, respectively) following reaction with bacterial isolate A46.

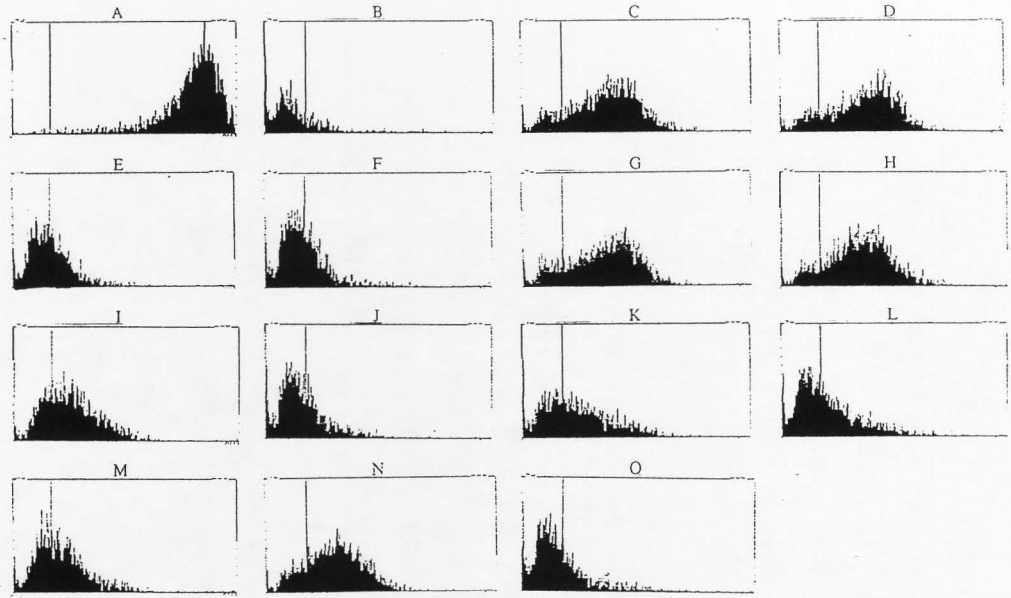

Fig 23 -- Flow cytometry graphic results of hyperimmune mouse serum (A), FOX/NY culture fluid (B), and monoclonal antibodies LG50, LG51, LG56, LG57, LG60, LG61, LG63, LG65, LG66, LG68, LG69, LG70, and LG75 (C-O, respectively) following reaction with bacterial isolate A22.

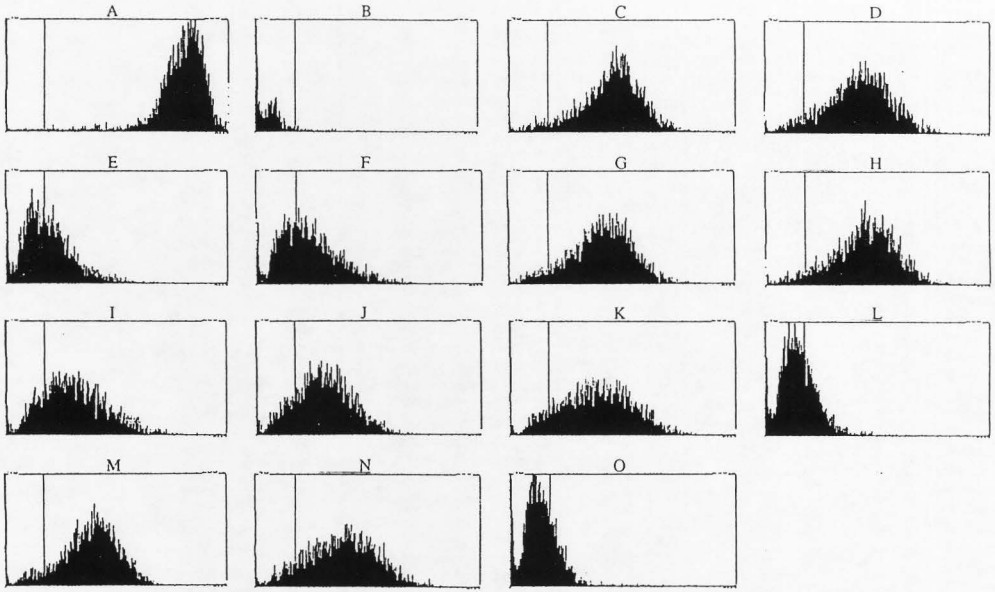

Fig 24 -- Flow cytometry graphic results of hyperimmune mouse serum (A), FOX/NY culture fluid (B), and monoclonal antibodies LG50, LG51, LG56, LG57, LG60, LG61, LG63, LG65, LG66, LG68, LG69, LG70, and LG75 (C-O, respectively) following reaction with bacterial isolate 84-833.

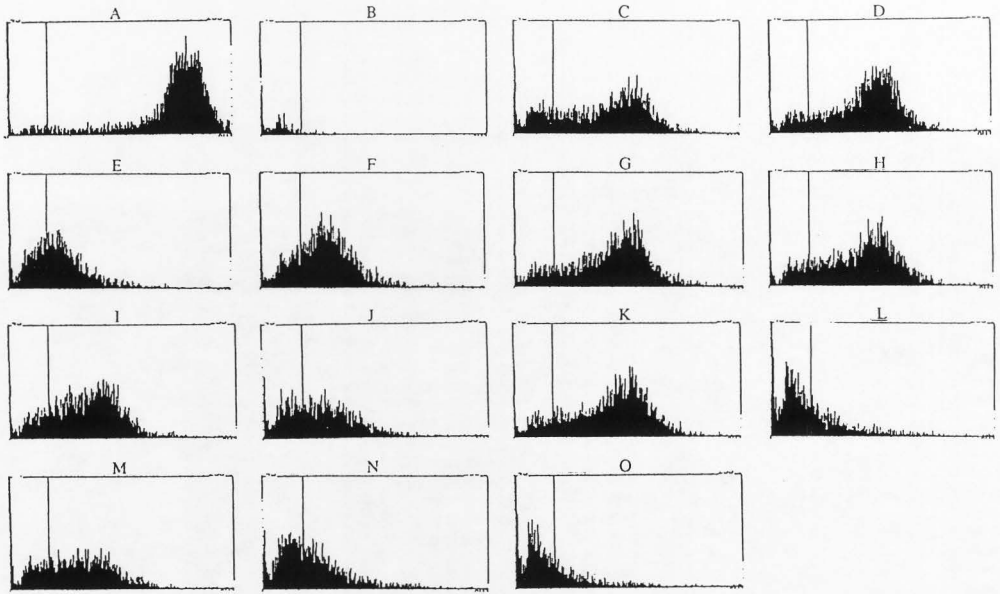

Fig 25 -- Flow cytometry graphic results of hyperimmune mouse serum (A), FOX/NY culture fluid (B), and monoclonal antibodies LG50, LG51, LG56, LG57, LG60, LG61, LG63, LG65, LG66, LG68, LG69, LG70, and LG75 (C-O, respectively) following reaction with bacterial isolate B30.

B30-2

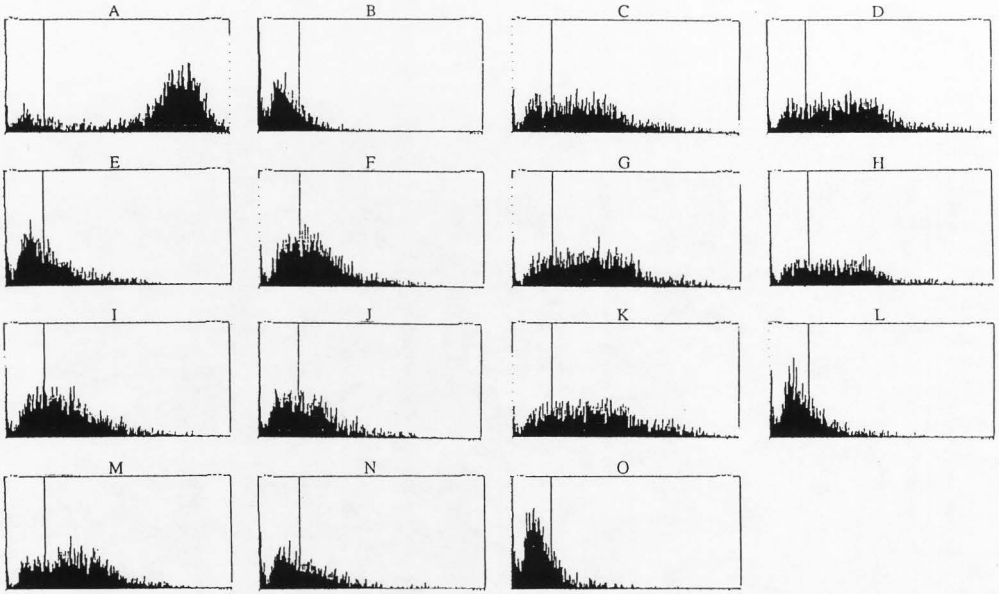

Fig 26 -- Flow cytometry graphic results of hyperimmune mouse serum (A), FOX/NY culture fluid (B), and monoclonal antibodies LG50, LG51, LG56, LG57, LG60, LG61, LG63, LG65, LG66, LG68, LG69, LG70, and LG75 (C-O, respectively) following reaction with bacterial isolate B30-2.

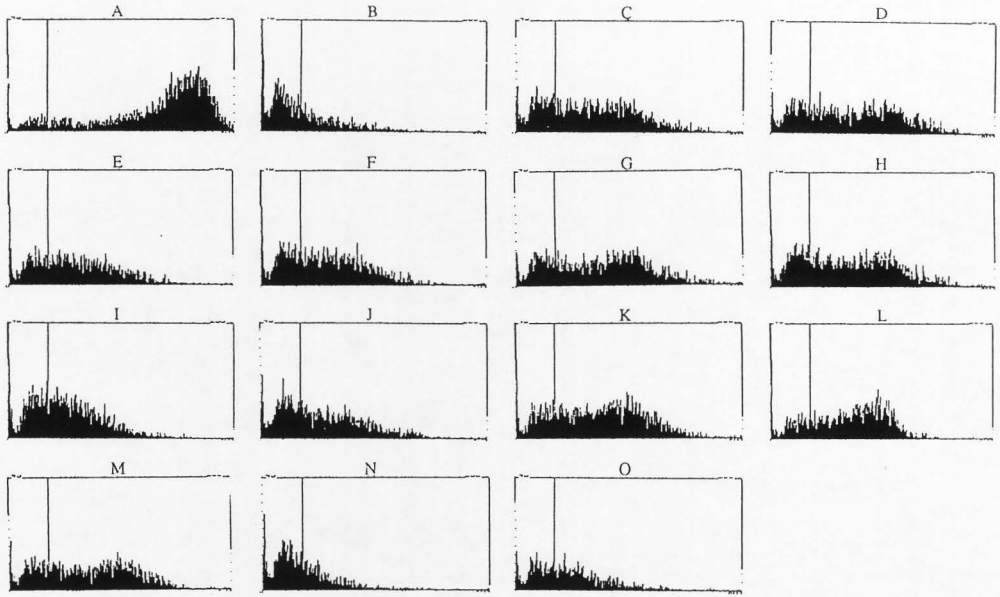

Fig 27 -- Flow cytometry graphic results of hyperimmune mouse serum (A), FOX/NY culture fluid (B), and monoclonal antibodies LG50, LG51, LG56, LG57, LG60, LG61, LG63, LG65, LG66, LG68, LG69, LG70, and LG75 (C-O, respectively) following reaction with bacterial isolate B111.

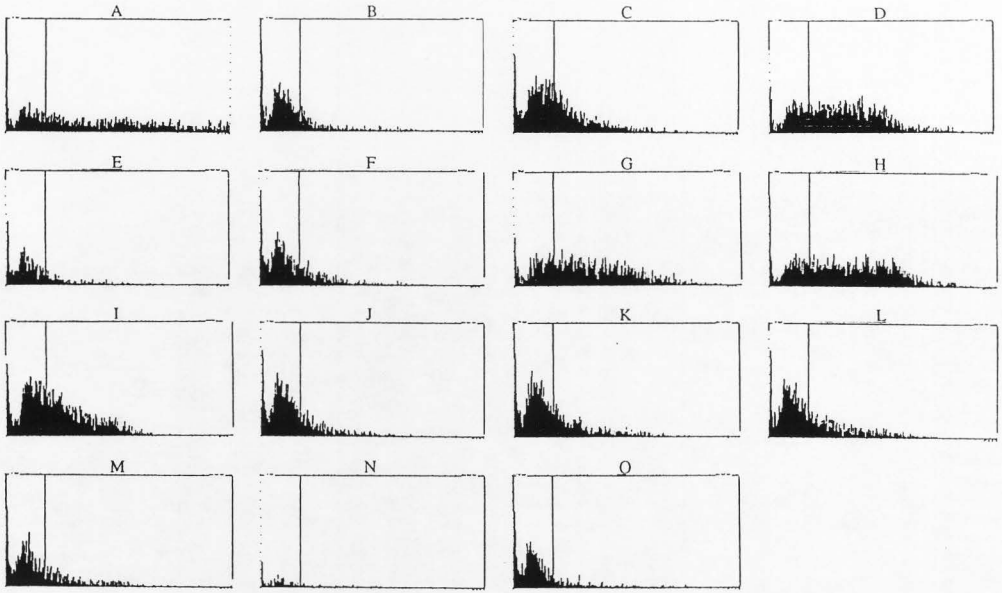

Fig 28 -- Flow cytometry graphic results of hyperimmune mouse serum (A), FOX/NY culture fluid (B), and monoclonal antibodies LG50, LG51, LG56, LG57, LG60, LG61, LG63, LG65, LG66, LG68, LG69, LG70, and LG75 (C-O, respectively) following reaction with bacterial isolate Y136.

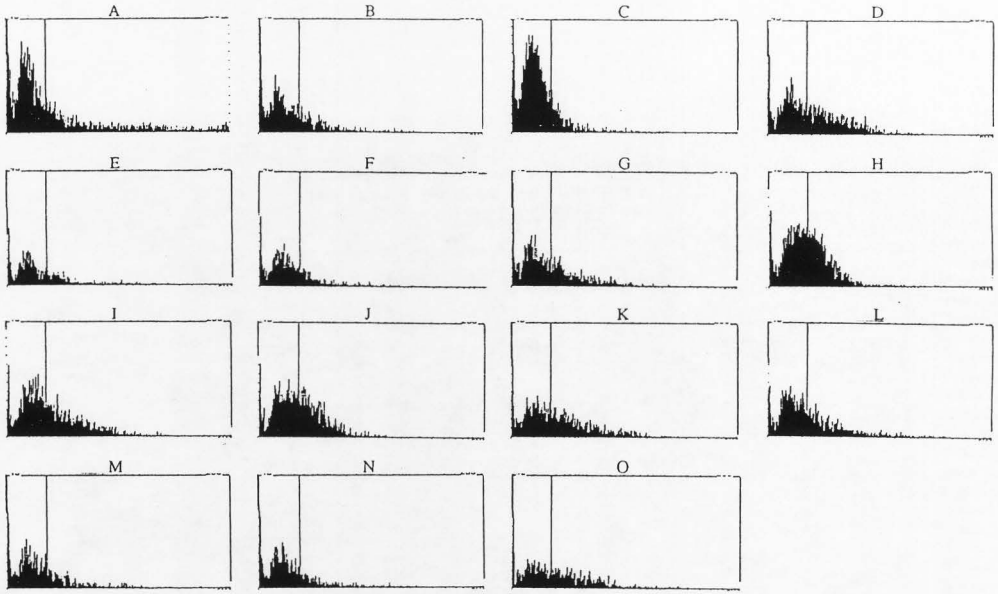

Fig 29 -- Flow cytometry graphic results of hyperimmune mouse serum (A), FOX/NY culture fluid (B), and monoclonal antibodies LG50, LG51, LG56, LG57, LG60, LG61, LG63, LG65, LG66, LG68, LG69, LG70, and LG75 (C-O, respectively) following reaction with bacterial isolate S6.

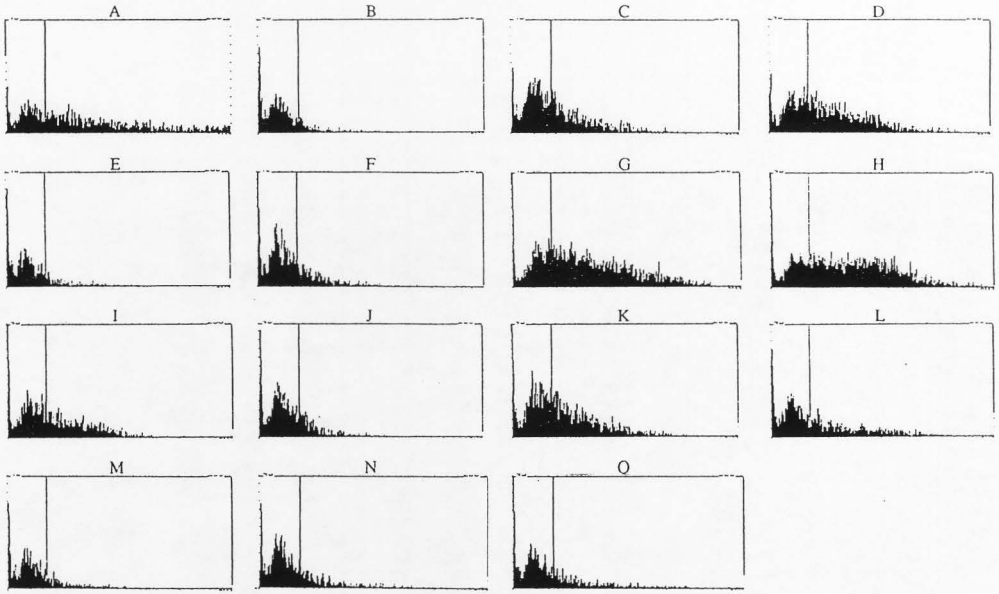

Fig 30 -- Flow cytometry graphic results of hyperimmune mouse serum (A), FOX/NY culture fluid (B), and monoclonal antibodies LG50, LG51, LG56, LG57, LG60, LG61, LG63, LG65, LG66, LG68, LG69, LG70, and LG75 (C-O, respectively) following reaction with bacterial isolate S15.

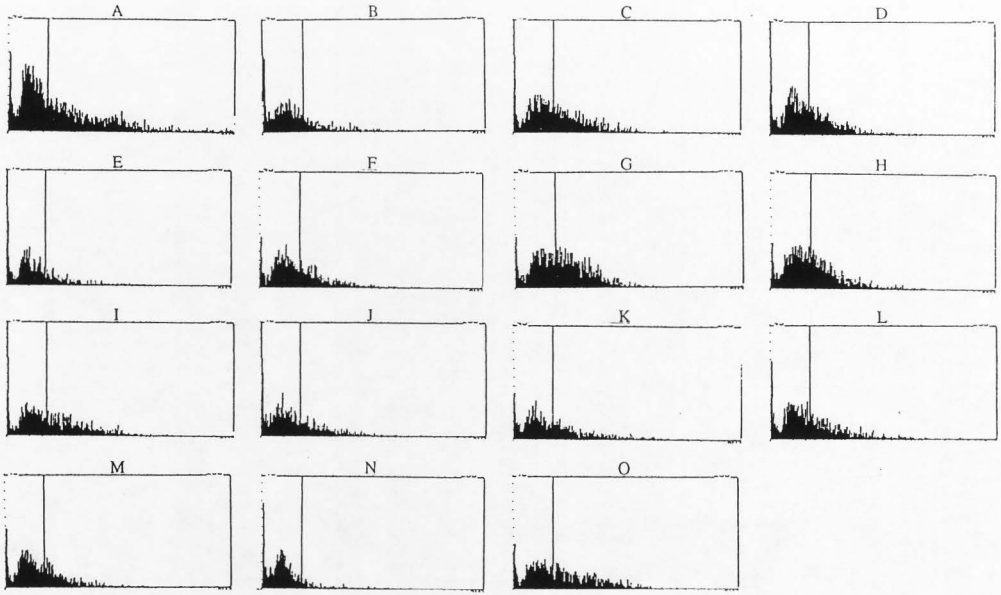

Fig 31 -- Flow cytometry graphic results of hyperimmune mouse serum (A), FOX/NY culture fluid (B), and monoclonal antibodies LG50, LG51, LG56, LG57, LG60, LG61, LG63, LG65, LG66, LG68, LG69, LG70, and LG75 (C-O, respectively) following reaction with bacterial isolate D107.

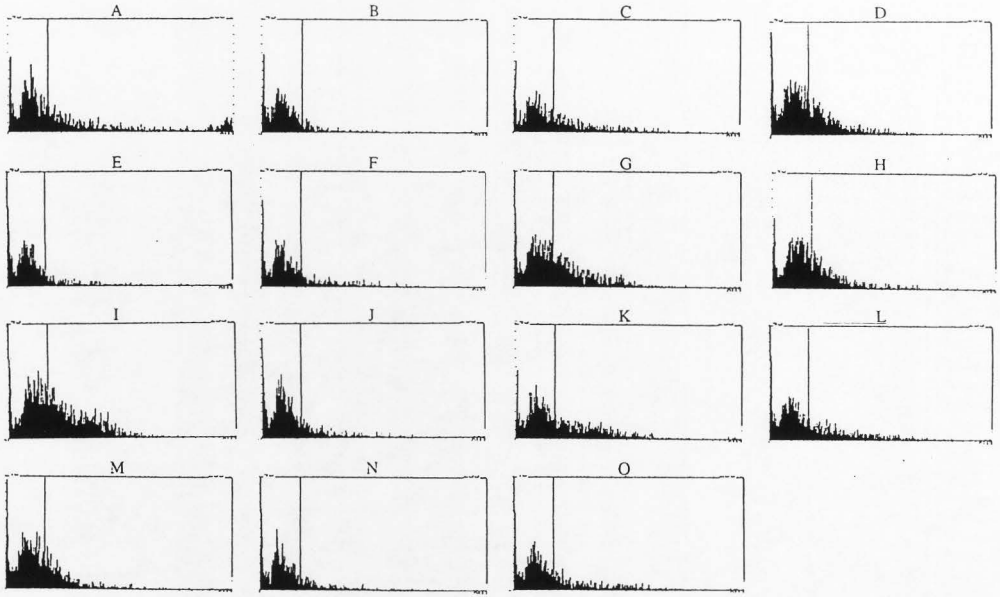

Fig 32 -- Flow cytometry graphic results of hyperimmune mouse serum (A), FOX/NY culture fluid (B), and monoclonal antibodies LG50, LG51, LG56, LG57, LG60, LG61, LG63, LG65, LG66, LG68, LG69, LG70, and LG75 (C-O, respectively) following reaction with bacterial isolate D56.

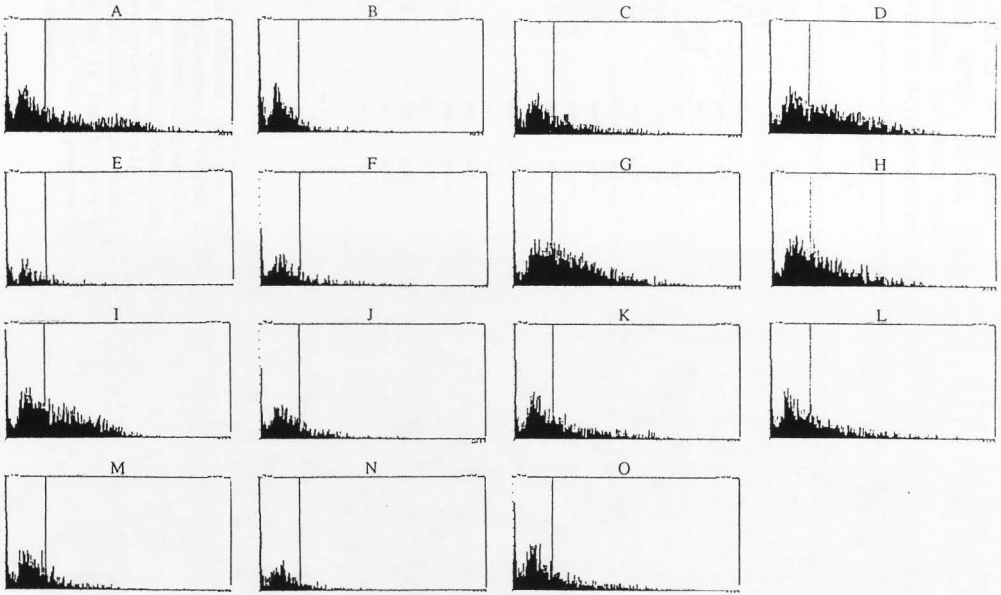

Fig 33 -- Flow cytometry graphic results of hyperimmune mouse serum (A), FOX/NY culture fluid (B), and monoclonal antibodies LG50, LG51, LG56, LG57, LG60, LG61, LG63, LG65, LG66, LG68, LG69, LG70, and LG75 (C-O, respectively) following reaction with bacterial isolate A2.

TABLE 5 -- Bacterial agglutination results, using hyperimmune mouse serum (HMS), FOX/NY culture fluid, and 13 monoclonal antibodies (MoAbs) against 2 ATCC type cultures and 24 field isolates of *Actinobacillus* species

Isolates	HMS*		FOX/NY†	Monoclonal antibodies‡			
	1:1	1:5	10 X	0.5X	1 X	5 X	10 X
29522§	+	+/-	-	-	-	-	-
15768¶	+	+/-	-	-	-	-	-
As8C∏	++	+	-	-	-	-	-
4101	++	+/-	-	-	-	-	-
84-832(5)	++	+	-	-	-	-	-
84-832(9)	++	+/-	-	-	-	-	-
86-618	++	+	-	-	-	-	-
86-722	++	+	-	-	-	-	-
86-723	+/-	+/-	-	-	-	-	-
86-724	++	+/-	-	-	-	-	-
86-731A	++	-	-	-	-	-	-
86-814A	++	+/-	-	-	-	-	-
R75	+/-	-	-	-	-	-	-
A11	++	+/-	-	-	-	-	-
A46	+	+/-	-	-	-	-	-
A22	++	+/-	-	-	-	-	-
84-833	++	+/-	-	-	-	-	-
B30	+/-	+/-	-	-	-	-	-
B30-2	++	+/-	-	-	-	-	-
B111	+	+/-	-	-	-	-	-
Y136	-	-	-	-	-	-	-
S6	-	-	-	-	-	-	-
S15	+/-	-	-	-	-	-	-
D107	-	-	-	-	-	-	-
D56	-	-	-	-	-	-	-
A2	-	-	-	-	-	-	-

*Two different dilutions were used for the positive control (HMS). †Ten times concentrated FOX/NY culture fluid was used as the negative control. ‡ Four different concentrations of hybridoma culture fluids were used as the source of MoAbs. §American Type Culture Collection (ATCC) of *Actinobacillus actinomycetemcomitans*. ¶ATCC of *A seminis*. ∏ The field isolates of *Actinobacillus* from which the 3 protein bands were extracted and against which the 13 MoAbs were produced.

++, +, and +/- = positive reactions ranging from moderately strong to weak, - = negative.

DISCUSSION

Epididymitis is an economically important reproductive disorder in which marked pathological changes occur in the genital tracts of clinically affected rams.[7] *Brucella ovis* was thought to be the main pathogen of ram epididymitis for a long time. However, in purebred virgin ram lambs 6 to 15 months of age, *B ovis* is seldom isolated from the epididymal lesions. Instead, other gram-negative bacterial species are routinely cultured from these lesions.[8] The most common bacterial isolates are *Actinobacillus* species and *Haemophilus somnus*. Healey et al[18,19] speculated and Scanlan et al[15] confirmed that *A seminis* is the most prevalent *Actinobacillus* species involved.

The purpose of this study was to characterize phenotypically *Actinobacillus* and *Actinobacillus*-like species isolated from ram lambs with epididymitis. Information obtained from Healey et al[11,18,19] suggested that isolates of *Actinobacillus* show rather heterologous protein profiles. Conventional methods of bacterial classification depend upon the morphological and biochemical properties of the organisms. With the use of serologic testing, greater accuracy can be achieved in bacterial identification.[24] Monoclonal antibodies are often used as immunologic probes in serology and are very useful in identifying and characterizing surface antigens found on bacterial outer membranes.[17]

The outer membranes of gram-negative bacteria contain large amounts of lipopolysaccharide, phospholipid and protein.[25] The use of outer-membrane proteins in serotyping isolates of related

bacterial strains has been described by Barenkamp et al.[26] Healey et al[18] also reported on the identification of certain field isolates of *Actinobacillus* species cultured from ram lambs with epididymitis. They were able to divide those field isolates into 2 groups, based on results obtained using discontinuous SDS-PAGE and spectrophotometric scans of soluble bacterial proteins. In this study, 2 ATCC type cultures (*A actinomycetemcomitans* and *A seminis*) and 24 field isolates of *Actinobacillus* species were analyzed (Table 1), using SDS-PAGE, ELISA, flow cytometry and bacterial agglutination.

The 2 ATCC type cultures and 24 field isolates of *Actinobacillus* species could be divided into 4 groups based on the similarities and differences in their protein profiles analyzed by the SDS-PAGE. Group 1 isolates included As8C, 4101, 84-832(5), 84-832(9), 86-618, 86-722, 86-723, 86-724, 86-731A, 86-814A, R75, A11, A46, A22, 84-833, B30, B30-2 and B111 (Figs 1-3). These field isolates shared approximately 6 major protein bands among themselves and with those of the 2 ATCC type species, with molecular masses of 94 KD, 64 KD, 61 KD, 53 KD, 40 KD and 27 KD. Three major protein bands at 45 KD, 30 KD and 16 KD were shared by field isolates in group 2 (Y136, S6 and S15; Fig 4). Group 3 isolates (D107 and D56; Fig 4) shared 4 protein bands at 90 KD, 70 KD, 64 KD and 35 KD. Group 4 isolate (A2; Fig 4) had a unique protein profile compared with the bacterial isolates in the other 3 groups. All bacterial isolates cultured from epididymal lesions belonged to the same group (group 1). Cultural and biochemical analysis studied by Scanlan et al[15] indicated that isolates As8C, 4101, 84-832(5), 84-832(9), 86-618, 86-722, 86-723, 86-724, R75, A11, A46, 84-833, B30, B30-2 and B111 were *A*

seminis, while 86-731A, 86-814A and A22 were *A actinomycetemcomitans*.

From SDS-PAGE analysis, 3 protein bands (74 KD, 60 KD and 45 KD) were determined to be conserved among most of the bacteria in the first 3 groups (Figs 1-3). These 3 conserved protein bands were isolated from a field isolate of *Actinobacillus* (As8C), and a panel of MoAbs was produced by other members of our research team using the protein bands as antigens. It is unknown whether these 3 bands represent outer membrane proteins, but available evidence suggests that at least MoAbs LG50, LG60 and LG70 do not recognize an outer membrane protein.[27] Thirteen hybridoma cell lines were selected and cloned on the basis of a strong color reaction with the ELISA when their respective MoAbs were incubated with whole cell As8C. All 13 MoAbs belonged to the IgG isotype. Seven produced MoAbs of the IgG1 subisotype (LG51, LG57, LG60, LG61, LG63, LG65 and LG69); 2 produced MoAbs of the IgG2a subisotype (LG68 and LG75); and 4 produced MoAbs of the IgG2b subisotype (LG50, LG56, LG66 and LG70, Table 2). Specificity of each MoAb for 1 or more of the 3 conserved protein bands contained in an *n*-octyl-glucoside extract of As8C was confirmed by the EITB technique. Except for MoAb LG65, which recognized 2 distinct bands having molecular masses of 74 KD and 45 KD (Fig 7), the remaining MoAbs showed specificity for single bands with molecular weights of 74 KD (LG68 and LG75; Fig 7), 60 KD (LG50, LG51, LG56, LG57, LG60, LG61, LG63, LG66 and LG70; Fig 6) and 45 KD (LG69; Fig 7). When the extracted As8C antigens were incubated with HMS, approximately 25 bands were observed. Based on the results obtained from the ELISA, it was concluded that each

MoAb was specific for different epitopes present on the 3 conserved protein bands. For example, MoAbs LG51 and LG56 appeared to recognize a protein band with the same molecular weight (60 KD) using the EITB. When these 2 MoAbs were incubated with all the bacterial isolates and then analyzed by the ELISA, the reaction pattern of these 2 MoAbs suggested that they were not against the same epitope expressed by these isolates (Table 3).

Outer membrane proteins of many gram-negative bacteria are surface expressed and most appear to be antigenic.[28,29] Some exposed proteins stimulate specific immunologic responses and are important in serotyping bacteria.[28,29] The use of MoAbs as serotyping agents allowed us to characterize phenotypically 24 field isolates of *Actinobacillus* species cultured from the semen and lesions of ram lambs with clinical epididymitis. Group 1 isolates could be rapidly screened, using ELISA and flow cytometry. The target epitope for the LG61 MoAb was shared by all isolates tested, whereas epitopes recognized by the remaining MoAbs were present only in isolates from group 1 and a few isolates in the other groups. Using flow cytometry, MoAbs provided both quantitative and graphic results (Tables 4a-4d; Figs 8-33). Each MoAb showed unique reaction patterns against different bacterial isolates. Flow cytometry results were not always consistent with the ELISA results. For instance, when MoAbs LG57 and LG61 were incubated with bacterial isolate As8C and analyzed by the ELISA, these 2 MoAbs were both scored ++ (Table 3). The flow cytometry results for these same 2 MoAbs revealed peak channels of 45 and 57 (Table 4a) and mean values of 75.1 and 86.9 (Table 4b), respectively. Obviously, there

was a considerable difference in the flow cytometry results for MoAbs LG57 and LG61. This suggests that flow cytometry may be a more sensitive assay than the ELISA. It should be possible to prepare a bank of flow cytometry graphs, using a standardized panel of MoAbs, to be used as a reference in the identification of other bacterial isolates belonging to the genus of *Actinobacillus*.

Another rapid assay that can be used to establish bacterial relationships is agglutination. This procedure has been reported by Healey et al[11,17] for *Actinobacillus* species. In the present study, 10 times concentrated hybridoma culture fluids were used as the source of MoAbs. However, none of the MoAbs produced visible bacterial agglutination with either the 2 ATCC type cultures or 24 field isolates of *Actinobacillus* species. Either there were not enough MoAbs to complete the antigen-antibody lattice formation required for bacterial agglutination, or the MoAbs were directed against antigens not located on the bacterial outer membrane.

The outer membrane proteins of most gram-negative bacteria are antigenic, and some of these proteins have vaccinogenic potential.[30,31] It has been demonstrated that some bacterins prepared from outer membrane extracts of pathogenic bacteria had greater efficacy than bacterins prepared from whole cell bacteria.[31,32] The results of this study indicate that there are several different serotypes within the genus *Actinobacillus* that can be isolated from ram lambs with clinical epididymitis. Some species and strains of *Actinobacillus* may be only normal flora of the genital tract, while others may represent pathogens. Further studies are needed to establish the serotypes of *Actinobacillus* that are

responsible for causing ram lamb epididymitis before an efficacious bacterin can be developed.

SUMMARY

The purpose of this study was to characterize phenotypically *Actinobacillus* and *Actinobacillus*-like species isolated from ram lambs with epididymitis. Bacterial antigens of 2 ATCC type cultures (*Actinobacillus seminis* and *A actinomycetemcomitans*) and 24 field isolates of *Actinobacillus* species extracted with the non-ionic detergent *n*-octyl-β-D-glucopyranoside (*n*-octyl-glucoside) were analyzed by discontinuous-gradient SDS-PAGE. The 24 bacterial field isolates were originally cultured from the semen or lesions of ram lambs with clinical epididymitis. The 2 ATCC type species and 24 field isolates could be divided into 4 groups, based on the similarities and differences in their protein profiles, using SDS-PAGE. The first group contained the 2 ATCC type cultures and 18 field isolates of *Actinobacillus* species. The second and third groups consisted of 3 and 2 field isolates, respectively. Few protein bands were shared between isolates across group lines, but protein profiles were similar between isolates within each group. For example, approximately 6 protein bands with molecular masses of 94 KD, 64 KD, 61 KD, 53 KD, 40 KD and 27 KD were shared by isolates in group 1. Isolates in group 2 shared 3 protein bands at 45 KD, 30 KD, and 16 KD, whereas group 3 isolates had about 4 protein bands (90 KD, 70 KD, 64 KD and 35 KD) in common. Only 1 isolate was assigned to group 4. This isolate had a protein profile unique from isolates in the other 3 groups.

A panel of 13 MoAbs, previously prepared against 3 conserved bacterial protein bands (74 KD, 60 KD and 45 KD) derived from field

isolate As8C, was used in the ELISA, flow cytometry and bacterial agglutination test in an attempt to characterize phenotypically *Actinobacillus* species cultured from ram lamb with clinical epididymitis. Specificity of the 13 MoAbs was confirmed by the EITB technique. Twelve MoAbs exhibited single-band staining with the *n*-octyl-glucoside extract of As8C at about 74 KD (LG68 and LG75), 60 KD (LG50, LG51, LG56, LG57, LG60, LG61, LG63, LG 66 and LG70) and 45 KD (LG69), except MoAb LG65, which showed double-band staining at about 74 KD and 45 KD.

Bacterial isolates could be divided into 3 groups based on the ELISA results, and 2 groups based on quantitative results obtained from flow cytometry. Evaluation by the ELISA and flow cytometry indicated that MoAb LG61 was specific for a target antigen shared by all of the isolates tested. The MoAbs LG51 and LG60 also recognized most of the isolates. The remaining MoAbs recognized several bacterial isolates but with decreasing intensity. The flow cytometry graphic data showed a unique reaction pattern for each MoAb-bacterial isolate reaction. These data suggest that there is considerable diversity of bacterial antigens among *Actinobacillus* isolates cultured from ram lambs with clinical epididymitis. It should be possible to prepare a bank of flow cytometry graphs, using a standardized panel of MoAbs, to be used as a reference in the identification of other bacterial isolates belonging to the genus of *Actinobacillus*.

An attempt to agglutinate the bacterial isolates using hybridoma culture fluid containing MoAbs was unsuccessful. However, bacterial agglutination of isolates As8C, 4101, 84-832(5),

84-832(9), 86-618, 86-722, 86-723, 86-724, 86-731A, 86-814A, R75, A11, A46, A22, 84-833, B30, B30-2, B111 and S15 was observed using HMS.

All the bacterial isolates cultured from epididymal lesions showed greater similarities in their protein profiles and in their ELISA and flow cytometry reaction patterns than isolates cultured from semen. If these lesion isolates are truly pathogenic, and the semen isolates represent only normal flora, the development of a bacterin may be simplified considerably. A concerted effort must be made to identify and use protective antigens derived from representative field strains of *Actinobacillus* that are responsible for lamb epididymitis.

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