Comparison of tear proteins of llamas and cattle

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Objective—To analyze and compare contents of the preocular tear films of llamas and cattle.

Animals—40 llamas and 35 cattle.

Procedure—Tear pH was determined by use of a pH meter. Total protein concentration was determined by use of 2 microtiter methods. Tear proteins were separated by use of electrophoresis and molecular weights of bands were calculated. Western blot immunoassay was used to detect IgA, lactoferrin, transferrin, ceruloplasmin, α_1-antitrypsin, α_1-amylase, and α_2-macroglobulin. Enzyme electrophoresis was used to detect proteases.

Results—The pH of llama and cattle tears were 8.05 ± 0.01 and 8.10 ± 0.01, respectively. For results of both methods, total protein concentration of llama tears was significantly greater than that of cattle tears. Molecular weights of tear protein bands were similar within and between the 2 species, although llama tears had a distinct 13.6-kd band that was not detected in cattle. Lactoferrin, IgA, transferrin, ceruloplasmin, α_1-antitrypsin, α_1-amylase, α_2–macroglobulin, and proteases were detected in both species.

Conclusions and Clinical Relevance—Llama tears have significantly greater total protein concentration than cattle tears, whereas pH is similar between species. Because little variation was detected within species for the number and molecular weight of protein bands, pooling of tears for analysis is justified. Results suggest that lactoferrin, ceruloplasmin, transferrin, α_1-antitrypsin, α_2-macroglobulin, α_1-amylase, and IgA are present in the tears of llamas and cattle. (*Am J Vet Res* 2000;61:1289–1293)

Results of a retrospective study and anecdotal information suggest that llamas and cattle have different susceptibilities to ocular diseases.[1,2] Llamas appear to be resistant to infectious keratoconjunctivitis and, to our knowledge, outbreaks of diseases resembling "pink-eye" have not been reported in llamas. Results of the 13-year retrospective study also indicate that cattle seem to be much more susceptible to ocular or periocular **squamous cell carcinoma (SCC)** than llamas[1]; SCC was not reported in llamas but was the most commonly reported ocular disease in cattle.[1,2] Reasons for the apparently different susceptibilities to ocular disease between llamas and cattle are unknown but could be attributable, in part, to differences in ocular defense mechanisms.

The preocular tear film plays an important role in defense of the ocular surface. Mechanical flushing action by tears helps prevent invasion of the cornea and conjunctiva by microorganisms. In addition, tears contain numerous proteins, immunoglobulins, and enzymes that provide specific and nonspecific antimicrobial actions.[3] Because of the importance of the preocular tear film in prevention of ocular disease, the objective of the study reported here was to analyze and compare contents of preocular tear film of llamas and cattle.

Materials and Methods

Collection method—Tears were collected from both eyes of 40 llamas and from 1 eye of 36 Holstein cows by placing a 50-µl borosilicate pipette in the lower conjunctival fornix. All animals had no signs of disease of the external ocular structures. In most cows, tears were obtained by capillary action, whereas some aspiration was required to collect llama tears. Care was taken to collect tears as atraumatically as possible. Each animal's tears were placed in a separate tube, centrifuged for 2 minutes to remove debris and mucus, and frozen at –20 C for future use. For some assays, tears were pooled by species.

Determination of pH and total protein concentration—The pH of pooled llama tears and pooled cattle tears were determined by use of a pH meter.[a] Total protein concentration of each sample from each animal was determined by 2 methods: the **bicinchoninic acid (BCA)** microtiter plate protocol[b] and another microtiter plate method.[c] In both protein determination methods, **bovine serum albumin (BSA)** standards with protein concentrations ranging from 6.25 to 200 µg/ml were used for controls. Whole tears were diluted to a 1:40 concentration with **phosphate buffered saline solution (PBSS)**. All plates were read with an ELISA reader[d] at a wavelength of 600 nm after 5 minutes of incubation at 20 C. Controls were fitted to a standard curve by use of statistical computer software[e] and protein concentrations in tears were extrapolated from the curve.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)—For interspecific and intraspecific comparisons of tear proteins, SDS-PAGE was performed on the tears of each llama and each cow; this method separated the proteins on the basis of their molecular weights. Precast 8 and 12% slab gels and 4 to 20% gradient vertical slab gels were used in an electrophoresis mini-cell[f] with a 4% (wt/vol) acrylamide stacking gel and 12% (wt/vol) acrylamide resolving gel. Whole tear samples from each individual were run in separate gel columns, under reduced (with 2-mercaptoethanol) and nonreduced conditions for each animal. Gels were electrophoresed for 90 minutes at 125 V, stained with Coomassie blue stain, soaked in 5% glycerol solution, and dried using an automated gel dryer.[g]

Molecular weights were determined for each protein band separated from each sample of nonreduced llama tears

Received Jun 1, 1999.
Accepted Sep 21, 1999.

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The authors thank S. Stingley-Salazar, J. Schoenbeck, and Dr. A. Pfaff for technical assistance and Dr. Eric Monnet for statistical assistance.

and nonreduced cow tears by comparing migration distance of the unknown bands to the prestained, known molecular weight standards[h] that were run with each gel. Migration distance of each protein band was measured on the 12% gels, because there appeared to be more differences between llama and cow tears in the vicinity of the low molecular weight proteins than in the vicinity of the high molecular weight proteins, and 12% gels separated low molecular weight bands well. The molecular weights of each standard were plotted on a linear regression curve against distance migrated, and molecular weight of each unknown band was obtained by extrapolation from the curve.

N-terminal sequence and amino acid analysis— Proteins from the SDS-PAGE gels were transferred onto nitrocellulose membranes. Membranes were stained with copper stain[h] to identify the desired band to cut from the membrane and destained for analysis. A 13.6-kd band that was present in llama tears but not cattle tears was manually cut from each gel and subjected to N-terminal sequencing and amino acid analysis. The protein band was hydrolyzed in 6*M* HCl at 165 C for 2 hours in the presence of dithiodiproprionic acid. The N-terminal amino acid sequence was obtained by the Edman degradation method and a gas phase amino acid sequencer.[i]

To identify tear proteins represented by the amino acid sequence, we used the **Basic Local Alignment Search Tool (BLAST)**, a research database produced by the National Center for Biotechnology Information that is available over the internet. The database contains thousands of amino acid sequences and will match an unknown amino acid sequence to the closest known protein.

Western blot immunoassay—To obtain gels for use in the western blot immunoassay, we used a slightly different method of gel electrophoresis than the one used initially to separate protein bands from tears. The second method more effectively separated proteins with higher molecular weights. To control the test, pooled llama and pooled cattle tears were run on the same gels. Tears were placed on precast 11% agar running gels with 5% stacking gels. Gels were loaded with 20 ml of diluted tears (25 ml of tears diluted with 175 ml of 2X SDS). Gels were run in 1X SDS running buffer at 20 V for approximately 35 minutes. Protein bands on each gel were measured and the distance of migration was calculated and compared with known molecular weight standards[j] that were run with each gel. Three runs of each gel were performed for each species and mean values of the 3 migration distance measurements were determined.

Protein bands were then transferred onto nitrocellulose membranes[h] by use of a semidry electrophoretic transfer cell.[j] After transfer, gels were soaked away from the membrane in transfer buffer. Membranes were dried and saved for immunoblotting. Gels were stained with silver stain[k] or blue stain[l] reagent to ensure that proteins were transferred properly.

For western blot immunoasssay, membranes obtained by use of gel electrophoresis were rinsed in PBSS with 0.05% Tween 20 and nonspecific binding sites were blocked with PBSS with 5% BSA. The blot was incubated with the primary antibody (goat anti-human α_1-antitrypsin,[m] rabbit anti-human α_1-amylase,[m] goat anti-human ceruloplasmin,[m] goat anti-human transferrin,[m] goat anti-human α_2-macroglobulin,[n] rabbit anti-human lactoferrin,[m] or goat anti-mouse IgA[n] antibody). Blots were washed in PBSS with 5% BSA and sodium azide for 1.5 hours and incubated with secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit IgG or horseradish peroxidase-conjugated rabbit anti-goat IgG). Membranes were washed 3 times in PBSS with 0.05% Tween 20 for 10 minutes each, incubated with PBS with 0.05% Tween 20, and incubated in a working solution.[o] Blots were exposed to radiographic film until a clear band developed.

Gels for protease analysis—To detect proteases, precast gels for protease analysis[p] were used. Ten percent and 12% gels were electrophoresed, according to a published protocol.[p] Twenty microliters of 1 part tears diluted with 2 parts buffer[h] were loaded into the wells, and the gels were run at 100 V for 80 to 100 minutes. Gels were incubated and stained.[h]

Statistical analyses—For results of both protein assays, paired *t*-tests were used to compare mean total protein concentration of cattle and llama tears. For comparisons of tear proteins separated by SDS-PAGE, mean and SD of the molecular weights of each band were calculated. Differences were considered significant at $P < 0.05$.

Results

Mean ± SD pH of llama and cattle tears was 8.05 ± 0.01 and 8.10 ± 0.01, respectively. Mean total protein concentrations determined by the BCA method were 14.30 ± 9.7 mg/ml (n = 40) in llama tears and 9.70 ± 3.7 mg/ml in cattle tears (36). Mean total protein concentrations determined by the other microtiter method were 7.50 ± 1.9 mg/ml in llama tears and 4.40 ± 1.9 mg/ml in cattle tears. For results of both methods, differences between values for llamas and cattle were significant ($P < 0.001$).

Little variation among individuals within a species was detected in numbers and molecular weights of protein bands obtained by initial gel electrophoresis. The individual proteins separated out much more distinctly under nondenatured conditions (**Fig 1 and 2**); therefore, calculations and further tests were performed with nondenatured tears. By use of the initial gel electrophoresis, 10 individual protein bands were obtained in llama and cattle tears; however, several bands differed between the 2 species (**Fig 3**). In both species, a thick band of high molecular weight (> 100 kd; **Table 1**) suggested that 2 or more proteins that did not separate well may have been present. Thus, when gels were run for immunoblotting, a different technique was

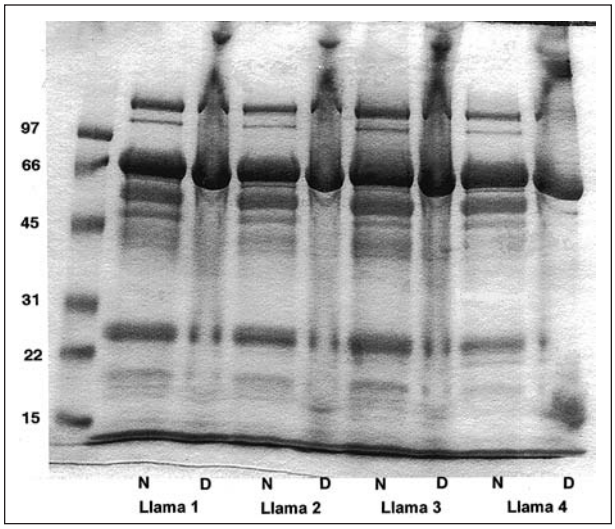

Figure 1—Sodium dodecyl sulfate-polyacrylamide gel electrophoretogram of tears from 4 llamas. Numbers to the left of the figure represent molecular weight (kd) standard markers. Pairs of columns represent denatured (D) and nondenatured (N) tears. Notice consistency of number and location of the protein bands among individual llamas.

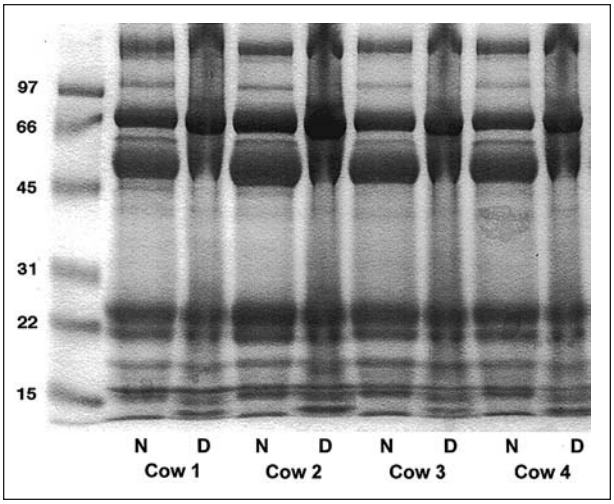

Figure 2—Sodium dodecyl sulfate-polyacrylamide gel electrophoretogram of tears from 4 cows. Numbers to the left of the figure represent molecular weight (kd) standard markers. Pairs of columns represent denatured (D) and nondenatured (N) tears. Notice consistency of number and location of the protein bands among individual cows.

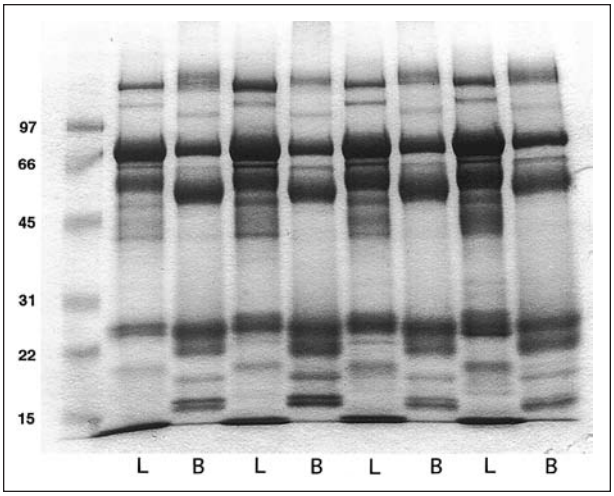

Figure 3—Sodium dodecyl sulfate-polyacrylamide gel electrophoretogram of llama (L) and cattle (B) tears run together on the same gel. Numbers to the left of the figure represent molecular weight (kd) markers. Notice dense protein band at approximately 13 kd in llama tears, which is not evident in cattle tears.

used to better separate proteins of high molecular weight.

On gels used for western blot immunoassay, 11 protein bands were identified in llama and cattle tears. Lower molecular weight protein bands in both gel runs (initial and those used for immunoblotting) were similar in number and molecular weight. There were, however, more bands in the higher molecular weight ranges in both llamas and cattle in the gels run for western blot analysis.

Because differences in protein bands in llama and cattle tears were detected, we attempted to identify the proteins contained in several of the bands. The distinct 13.6-kd band that was prominent in llama tears but absent in cattle tears was subjected to amino acid sequencing. The sequence was obtained and was submitted to BLAST analysis. The best match provided by this analysis was with lysozyme; however, the *P* value of this match was 0.74, indicating poor probability that the protein was lysozyme. Results of further analysis are published elsewhere.[4]

Table 1—Mean ± SD molecular weight (kd) values of protein bands obtained by electrophoresis of llama and cattle tears on 12% precast vertical slab gels

Protein Band	Llama (n = 40)	Cattle (n = 35)
1	13.7 ± 0.8	15.3 ± 0.7
2	18.2 ± 2.4	16.0 ± 0.7
3	23.9 ± 3.0	18.6 ± 0.8
4	43.9 ± 5.1	21.8 ± 0.5
5	50.4 ± 5.5	24.5 ± 1.0
6	57.4 ± 6.4	58.2 ± 2.6
7	62.6 ± 6.9	65.8 ± 3.3
8	70.2 ± 7.7	73.5 ± 3.6
9	91.3 ± 9.8	89.3 ± 4.2
10	100.1 ± 10.3	109.0 ± 6.1

Antibody binding to the protein bands of the western blot gels was obtained in samples from llamas and cattle. The goat anti-mouse IgA bound to the band at approximately 210 to 230 kd in both species. The goat anti-human α_2-macroglobulin, goat anti-human transferrin, and goat anti-human ceruloplasmin all bound in an area corresponding to approximately 94 to 115 kd in both species. In gels of both species, rabbit anti-human lactoferrin, anti-human α_1-antitrypsin, and rabbit anti-human α_1-amylase bound in a band at approximately 57 to 60 kd.

The 12% protease analysis gels[p] did not reveal any binding or proteolytic activity in any of the protein bands. With use of the 10% gels, 2 bands with proteolytic activity were observed in llama and cattle tears. These bands appeared to be in the same position in the gels of both species.

Discussion

Results of both total protein analysis methods revealed that llamas had a higher concentration of tear protein than cattle; however, the BCA method consistently detected higher total protein concentrations in llama and cattle tears than did the other microtiter assay. This discrepancy is attributable to the different amino acids to which the blue dyes in each assay bind. Tears contain a mixture of many constituent proteins and there are many different amino acids in these proteins; the dye in the Pierce BCA assay may bind to more of the amino acids in tear proteins than does the dye in the other assay. Because of the many different tear proteins, total protein evaluation methods only provide estimates of the actual total protein concentration. For this reason, performance of both assays may have provided a better estimate of total protein concentration than would have been possible with either assay alone. Other researchers commonly perform the nonBCA microtiter plate method on tears, so results of this measurement may be used to compare total protein concentration among species. Davidson et al[5] reported that the total protein concentration of cattle tears, determined by use of the nonBCA microtiter method, was 5.8 ± 2.2 mg/ml, which was similar to our results for cattle.

Tear collection method may greatly influence total

protein concentration in tears.[6] Total protein concentration in humans can decrease as much as 3 mg/ml with increased stimulation to tear flow.[7] Although we took care to collect tears as atraumatically as possible, the tear flow rate in llamas was low and aspiration was required to collect the tears in a timely manner. This may have caused some conjunctival irritation and lower total protein concentration, compared with results obtained without aspiration. Cattle have a large amount of mucus in their tears, which may have bound to some of the tear protein, lowered total protein concentration, and altered the types of proteins detected.

Differences in the numbers and molecular weights of bands detected by use of the 2 gel electrophoresis techniques may be attributed to several factors, most likely of which are the different techniques used to perform the SDS-PAGE gel electrophoresis. For the initial electrophoresis of samples obtained from individual llamas and cows, commercially available precast gels were used, in an attempt to determine the consistency of protein bands among individuals of the same species; this finding determined whether pooled tear samples could be used for later assays. Consistent intraspecific band numbers and sizes were detected, which justified the use of pooled tears. For the western blot analyses, we cast our own gels and used gel concentrations that provided a larger pore size, which aided transfer of proteins to the nitrocellulose membranes and may have allowed better separation of the high and low molecular weight proteins.

Results of western blot analyses suggested that the proteins lactoferrin, ceruloplasmin, transferrin, α_1-antitrypsin, α_2-macroglobulin, α_1-amylase, and IgA were present in tears of llamas and cattle. We chose to evaluate these proteins because they have been detected in the tears of humans and other species[8-11] and because antibodies against them were commercially available. The anticollagenases, α_1-antitrypsin, and α_2-macroglobulin, were of particular interest because of the severe ulcers that often accompany infectious bovine keratoconjunctivitis; perhaps development of these ulcers is influenced by low concentrations or the absence of these tear enzymes.

Lactoferrin, transferrin, and ceruloplasmin are metallic ion chelating proteins found in tears. Lactoferrin is a major tear protein in many species, including cattle,[11] and has a strong binding affinity for iron that is 300 times greater than that of transferrin.[10] Lactoferrin and transferrin both inhibit bacterial growth by competing with bacteria for iron.[10] Lactoferrin is produced in the lacrimal glands.[10,12] Ceruloplasmin is a copper-carrying protein that is commonly found in human tears.[13] In several reports, it is suggested that transferrin can be detected in tears only after mild trauma to the conjunctiva.[10,14] Its presence in the tears of the animals of the study reported here suggested that we may have caused mild conjunctival trauma at tear collection. Each of these 3 proteins was found in the intermediate molecular weight band fractions; these results were similar to molecular weights reported for these proteins by other researchers.[10,11,13,15]

The major immunoglobulin in the tears of humans and, probably, most other mammalian species, is IgA.[3,10,15,16,q] In humans, IgA is manufactured primarily in the interstitial plasma cells of the lacrimal glands, and after being coupled with a secretory component, is secreted into the tears as secretory IgA[10,12]; IgA is also produced in the conjunctiva.[3,18] Locally produced secretory IgA forms an important and specific defense mechanism against local microbial invasion. Lal et al[19] detected significantly increased concentrations of IgA in tears of human patients with bacterial, fungal, and viral ulcers, compared with healthy controls.

The antiproteinases α_1-antitrypsin and α_2-macroglobulin were detected in llama and cattle tears. The enzyme α_1-antitrypsin has been identified in normal human tears but in concentrations much lower than in human plasma.[10] Alpha-1-antitrypsin may be secreted by the lacrimal glands, but there is evidence that it may also originate from serum in inflamed eyes.[20] The source of α_2-macroglobulin is unknown.[10] Alpha-1-antitrypsin and α_2-macroglobulin are collagenase inhibitors and their concentrations are increased in tears in eyes with ulcerative keratitis.[20] Alpha-2-macroglobulin, however, was not found in the eyes of 4 healthy humans or 3 human patients with inflammation that did not have ulceration.[20] The importance of finding both of these antiproteinases in the tears of clinically normal llamas and cattle is unknown. Perhaps low concentrations of natural anticollagenases could potentiate the corneal stromal destruction observed in infectious bovine keratoconjunctivitis.

Amylase is detected in human tears in moderate concentrations and is thought to originate in the lacrimal gland.[21] The physiologic role of amylase in tears has not been conclusively established; amylase may play a role in the glycogen metabolism of the corneal epithelium, providing glucose that is necessary for nutrition of the cells of the cornea.[20]

Results of the protease analysis confirmed that some of the protein bands detected by SDS-PAGE gel electrophoresis were proteinases. The substrate of the 10% gels is gelatin, whereas the substrate of the 12% gels is casein. Enzymes in the llama and cattle tears apparently break down substances found in gelatin but not in casein. Anticollagenase and collagenase-like enzymes degrade gelatin, whereas digestive enzymes such as trypsin and chymotrypsin use casein as a substrate. Although the identity of these bands is yet to be determined, it is likely that the proteinases in llama and cattle tears are antiproteinase-like or anticollagenase-like enzymes.

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[a] 220 pH meter, Corning Inc, Corning NY.
[b] BCA microtiter plate method, Pierce Chemical Co, Rockford, Ill.
[c] Bio-Rad microtiter plate method, Bio-Rad Laboratories, Hercules, Calif.
[d] DCP, Los Angeles, Calif.
[e] GraphPad Software Inc, San Diego, Calif.
[f] Mini-Protean II, Bio-Rad Laboratories, Hercules, Calif.
[g] Model 583 gel dryer, Bio-Rad Laboratories, Hercules, Calif.
[h] Bio-Rad Laboratories, Hercules, Calif.
[i] Sequencer model 470 A, Applied Biosystems, Foster City, Calif.
[j] Trans-Blot transfer cell, Bio-Rad Laboratories, Hercules, Calif.
[k] Sliver stain plus, Bio-Rad Laboratories, Hercules, Calif.
[l] Gelcode blue stain, Pierce Chemical Co, Rockford, Ill.
[m] Sigma Chemical Co, St Louis, Mo.

[n]Chemicon International Inc, Temecula, Calif.
[o]Super signal ultra substrate, Pierce Chemical Co, Rockford, Ill.
[p]Zymogram ready gels, Bio-Rad Laboratories, Hercules, Calif.
[q]Gerdes S. *HPLC analysis of the normal bovine tear protein profile and its changes during 3 stages of infectious bovine keratoconjunctivitis.* MS thesis, Kansas State University, Manhattan, Kan, 1994.

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