Research Summaries I

"Beef and General"

Moderator—Don Hansen

HPLC Comparison of Tear Film Protein Pattern in Normal and Infectious Bovine Keratoconjunctivitis Affected Cattle

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Abstract

Purpose: This project was undertaken to establish the tear protein pattern in normal cattle and changes in the pattern at different stages of IBK, and disease of great economic importance.

Methods: Non-stimulated tears were atraumatically collected with a 50µl capillary tube. The study included normal and infected animals in three stages of naturally occurring IBK. Analysis was performed with a TSK 3000 SWXL size exclusion HPLC column at a flow rate of 0.3ml/min with 0.5M NaCl, 0.1M NaH_3PO_4, pH 5. Quantitation was completed with computerized integration of peaks.

Results: In all animals 7 major peaks were found at retention times of 17, 19, 21, 27, 29, 33, and 39 minutes. During the active course of the disease, peaks 1, 2, 3, 5, 6, and 7 showed various changes. Separation for different bacterial culture result within the group had statistically significant differences.

Conclusions: The tear protein pattern in IBK affected cattle versus normal shows distinct changes. In addition to the stage of the disease changes are markedly influenced by the type of recovered organism. The first three peaks that changed in size during the course of the disease are likely to contain immunoglobulins.

Introduction

Infectious bovine keratoconjunctivitis (IBK), commonly known as pinkeye, is the most important infectious bovine ocular disease affecting cattle worldwide.[1-3] It is diagnosed primarily by clinical signs of excessive lacrimation, photophobia, and blepharospasm, corneal ulcerations and conjunctivitis. Although spontaneous recovery can occur from any stage of the disease, the corneal lesions tend to progress and can lead to corneal rupture, permanent blindness, and phthisis bulbi. The associated pain and vision loss reduce weight gain and milk production in beef and dairy cattle. Weaning weights of affected animals are between 17 and 18 kg lower than those of their healthy herd mates.[4]

Moraxella bovis is the most commonly isolated infectious agent.[5] It seems to be the primary etiologic agent, but additional factors like UV radiation, mechanical irritants, concurrent infection with mycoplasma or infectious bovine rhinotracheitis virus are necessary to produce clinical disease.[6-13]

Research on the overall pathophysiology of IBK has been limited, while some virulence factors like pili have been intensely investigated.[14-16] Pili are important for establishing infection, because attachment of the bacteria to epithelial cells as a first step in the disease process allows reproduction.[17]

Studies in humans stress the importance of the precorneal tear film and its role in the ocular defense mechanism.[18,19] Immunoglobulins and other proteins in the tears such as lactoferrin, transferrin, and lysozyme have a major impact on ocular immunity.[20,21]

The objective of this study was to investigate the tear film pattern and confirm the hypothesis, that the normal bovine tear protein pattern changes during the course of IBK. We hypothesized that a protein could be identified that is responsible for recovery from the dis-

ease. In order to evaluate disease induced changes, the tear protein pattern of normal, healthy cattle had to be established first.

High performance liquid chromatography was chosen as the initial technique for tear film evaluation. After establishment of the method, repeatable data could be generated in a short period of time, giving an overview of the different molecular weight protein peaks with the potential for quantification.

Materials and Methods

Animals used in this study were Angus or Amerifax purebred cattle under 15 months of age. Animals were selected according to the following criteria.

Group 1

Normal animals with no clinical signs of ocular disease. (n=36)

Group 2

Peracute stage with epiphora, blepharospasm, and/or ulceration smaller or equal to 1mm diameter. (n=22)

Group 3

During the **acute** stage of the disease signs included epiphora, blepharospasm, and ulceration greater than 1mm diameter. (n=51)

Group 4

Recovered animals were healed with or without corneal scaring 4-6 weeks after the initial sampling. (n=36)

The herd included in this study was selected because of its history of endemic IBK (Ohlde's Cattle Company, Palmer, KS). The cow-calf pairs were kept on range pasture with mineral supplementation. During the peak fly population the animals were sprayed or dusted with an insect repellent at approximately 7 day intervals.

All samples were collected between 1-26-93 and 11-12-93. The author performed all procedures in the following order: external eye exams, grouping of the animals, tear sample collection, microbiology swab, and corneal fluorescein staining.

Tear samples were collected atraumaticaly with 50µl glass pipets from the inferior lacrimal lake avoiding contact to the cornea. All samples were stored at -20°C until analysis. Animals showing clinical signs of disease with corneal ulceration were treated with a 1ml subconjunctival injection of Azimycin^{R} at the time of diagnosis.

Swabs for bacterial and mycoplasmal cultures were taken (Becton Dickinson Microbiology Systems, Cockeysville, Maryland). The culturettes were transported on ice packs during the summer months. *M. bovis* was identified by standard procedures. *Mycoplasma* was grown on modified Hayflick's medium and identified by colony morphology.

At least 20 samples per disease group were chosen for protein analysis. Group 1-4 contained 23, 20, 22, and 20 samples respectively. Decisions for testing these samples were determined by microbiology results. In the normal group 1, animals with no growth cultures were favored. *Moraxella* and *Mycoplasma* positive animals were preferred for groups 2 and 3. For group 4 follow-up samples of animals included in groups 2 and 3 were chosen to reduce individual variations in tear proteins.

The chromatography followed a modified procedure as previously described.[22] A high performance liquid chromatograph with integrated computerized data acquisition system was used with an improved Progel™ TSK Column G3000 SWXL (Supelco, Bellefonte, PA) and a Perkin Elmer LC 295 UV/Vis Detector (Perkin Elmer, Norwalk, CT).[23] The flow rate was 0.3ml/min for increased peak separation.

Whole tears were centrifuged to remove particle contaminants and mucous. The sample volume for all analysis was 10µl of whole tears. Purified proteins and a size exclusion chromatography protein mixture were run under identical conditions to preliminarily identify peak proteins. Whole human tears were run under identical conditions to facilitate comparison of bovine tears to published data on human tears. The area under the peak was quantitated by computer integration.

All statistical evaluation was done with the Statistical Analysis System (SAS). One way analysis of variance was performed for comparisons of overall groups, groups separated by bacterial culture, and retention times of the different detectors. All analysis for sex differences used chi square test procedures. Results with a $P \leq 0.05$ were considered to be significant.

Results

Bacteriology results are shown in Table 1. For the 1993 calf crop a statistically significant larger percentage ($P=0.013$) of the females showed positive bacterial cultures. Only 1 out of 11 females had no bacterial growth after conjunctival culture. The majority of the negative cultures were recovered from males (87.5%).

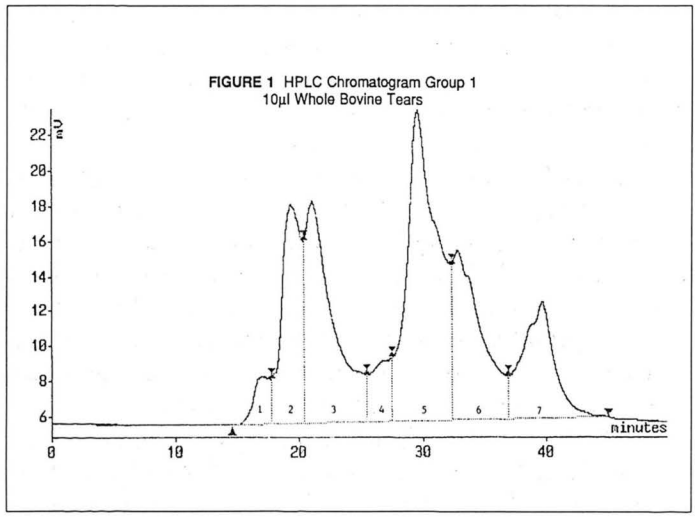
Seven major peaks with consistent retention times could be found in all four groups. Retention times for peak 1-7 were 17, 19, 21, 27, 29, 33, and 40 minutes respectively (Figure 1). The retention times of standard proteins are shown in Table 2.

The human tear protein chromatogram showed five major peaks at retention times of 20.7, 29.3, 33.6, 37.2, and 40.1 minutes. The human tear chromatogram at 0.3ml/min flow rate had the same pattern as the 0.m/min chromatogram of an earlier publication.

Table 1. Bacterial Distribution, Groups 1-4

Aerobic Culture Results	Group 1 % (number)	Group 2 % (number)	Group 3 % (number)	Group 4 % (number)
no growth	25.00 (9)	22.73 (5)	29.41 (15)	19.44 (7)
no specific growth			3.92 (2)	16.67 (6)
α Stretptococci	19.44 (7)	9.09 (2)	17.65 (9)	8.33 (3)
non-hemolytic Staphylococci	33.33 (12)	22.73 (5)	19.41 (15)	36.11 (13)
Staphylococcus epidermidis			1.96 (1)	
non-hemolytic E. coli	5.56 (2)	4.55 (1)	1.96 (1)	13.88 (5)
Bacillus	13.89 (5)		3.92 (2)	5.56 (2)
Gram -		18.18 (4)	1.96 (1)	
Pseudomonas	16.67 (6)	4.55 (1)		5.56 (2)
Aeromonas				2.78 (1)
Acinetobacter			1.96 (1)	
Enterobacter agglomerans				2.78 (1)
Hemophilus somnus			3.92 (2)	
Pasteurella multocida			1.96 (1)	
Klebsiella pneumoniae				2.78 (1)
Branhamella	2.78 (1)		1.96 (1)	2.78 (1)
Moraxella bovis		4.55 (1)	7.84 (4)	2.78 (1)
Moraxella sp.		9.09 (2)	11.76 (6)	
Mycoplasma		18.19 (4)	11.76 (6)	11.11 (4)

Figure 1.



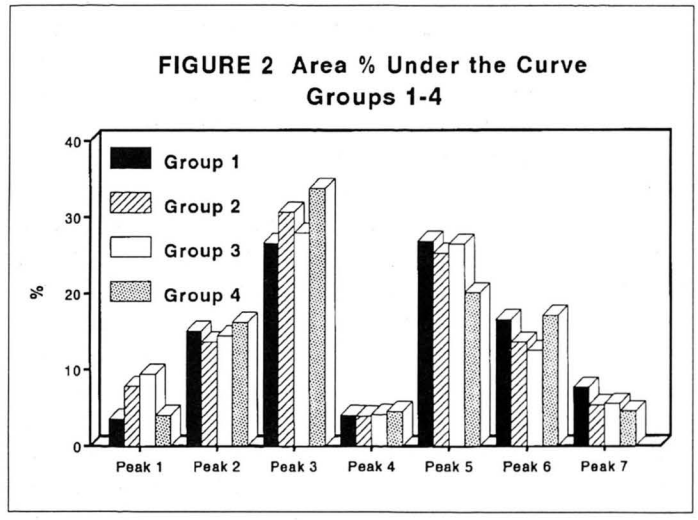
The mean area% under the chromatogram, disregarding different culture results, showed a multitude of differences for the four groups (Figure 2).

The normal group 1 calves, combined active disease groups 2/3, and the recovered group 4 were evaluated separately for the influence of bacterial growth on mean area% under the curve for peak 1-7. Effects of Gram+, Gram-, and no growth cultures were compared. In group 1 peak 2 had a lower Gram+ value (14.5) than the no growth (16.2). In peak 5 Gram+ had a lower value (23.7) than both Gram- (30.3) and no growth (29.7).

Table 2. Standard Protein Retention Times (minutes)

PROTEINS	RT
bovine IgM	17.567
human secretory IgA	20.157
bovine IgG	21.280
human IgA	22.326
thyroglobulin	22.753
bovine gamma globulin	26.473
bovine transferrin	28.940
bovine lactoferrin	29.186
bovine albumin	29.487
chicken ovalbumin	30.700
equine myoglobin	33.653
chicken lysozyme	36.727
vitamin B-12	37.947

Figure 2.



For the combined active disease groups the only difference between Gram+, Gram-, and no growth cultures was in peak 4. Gram+ were lower (3.2) than no growth (4.6).

In the recovered group peak 3 showed the only significant variation. Gram+ (37.7) tear samples were higher than Gram- (24.2). The value for no growth cultures were also higher but were not significantly in-

creased.

Samples positive for *Moraxella* did not show any HPLC detectable significant differences when compared to samples positive for both *Moraxella* and *Mycoplasma*.

Discussion

The normal ocular flora resulting from our study confirmed published findings from bovine as well as other species for a predominance of Gram+ organism. *Staphylococcus* and *Streptococcus* are most commonly isolated.

In the past the label IBK has been widely used for any case of keratitis and conjunctivitis, especially when rapid transmission occurred within the herd. Confirmation of a causative organism in a field situation is rarely done. Our study confirmed, that in a high percentage of the clinically typical cases, *M bovis* could not be isolated from the corneal lesions. Other organisms or none at all were recovered. Insufficient culture technique cannot completely account for the low recovery rate of *Moraxella*. At the time of sampling the organism might have been cleared from the eye. On the other hand primary low *Moraxella* involvement might explain the low protective value of vaccines and insufficient response to antibiotic treatment in field situations.

Most *Mycoplasma* isolations were closely related to the time and location of *Moraxella* recovery.

The effect of the animals sex on ocular flora in the normal calves showed a surprising predisposition. Starting with even sex distribution, 90.9% of all female calves had positive bacterial cultures versus 38.5% in the male.

The proteins contained within the different peaks are indicated by published human findings and retention times of purified proteins. The first three major peaks in the bovine tear protein chromatogram seem to represent the immunoglobulins IgM, IgA, and IgG respectively. Both of the iron containing proteins lactoferrin and transferrin and bovine albumin elude within the bovine peak 5. Peak 6 containing tear specific prealbumin has a 33.6 minute retention time in humans and a 33.0 minute retention time in the bovine. Lysozyme can be detected in the two last human peaks at 37.2 and 40.1 minutes. Chicken lysozyme eluded at 36.7 minutes. The bovine chromatogram contains a peak at 39.6 minutes. The three mentioned species seem to have differences in their lysozyme retention times and therefore molecular weight and structure. Although earlier studies could not detect lysozyme in bovine tears there might still be small amounts within this last peak.

Both decreases and increases in area% are found during both active disease and recovery. The Ig concentrations change during the progression of the disease, as expected for an infectious process. Peak 1, likely representing IgM, increased during the active stages of the disease more than twofold, as it would be expected from IgM's dominant role in the primary immune response. For the recovered animals this value drops almost to the original value.

The IgA containing fractions of peak 2 decrease during the disease but are increased after recovery suggesting a specific increased immunity. Reduction in area% under the curve can be caused by a decreased production or by dilution with constant lacrimal gland production during stimulated tear flow. It has been shown in humans that reflex tears, as they can be expected in an irritated eye, have decreased concentrations of the proteins that are constitutively secreted like IgA. Antigen-antibody reaction and the subsequent elimination of the complex will also account for lower concentrations during the active disease process.

Peak 3 containing IgG is effected differently since it derives mostly from serum decreasing with increased stimulus. It decreases somewhat in the severe stage of the disease, but shows a general rise in recovery.

Peak 5 is markedly decreased after recovery from the disease. This can be caused by a decrease in one or more of its proteins. The proteins within this peak come from different origins. Albumin and transferrin are serum-derived whereas lactoferrin is locally produced. The reason for this drop is not known, but may lie in decreased production. Protein synthesis can be decreased either with exhaustion of one of the substrates or the involved enzymes or coenzymes.

Peak 6, mostly likely containing tear specific prealbumin, decreases during the active course of the disease to reach a higher than normal value in the recovered animal. It has been shown that the concentration of tear specific prealbumin as one of the main lacrimal gland proteins is relatively unaffected by stimulated tear flow. This indicates an unknown separate mechanism to decrease tear concentration.

Peak 7, which might contain lysozyme, decreases during the active course of disease and reaches the lowest value in the recovered animal, reduced by almost 50% compared to the value in the normal animal. Lysozyme is also locally produced in the lacrimal gland and shows only minor reduction in stimulated tears. The mechanism of reduction is not known and again it can be speculated that the reason is depletion of substrates. Another possibility is, that a decreased concentration in the normal animal predisposes for IBK.

Another question that could be raised is whether the increased proteins are of host or bacterial origin. The number of proteins within one peak has not been identified and might contain additional proteins of bacterial origin. Specific changes for Gram+ and Gram- versus no growth samples emphasize this thought.

Peak 2 and 5 show variation for normal animals. Peak 2 is smaller for Gram+ bacteria than for no-growth cultures. This may be due to immunoglobulin complexes or lower immune stimulation compared to Gram- organism. Peak 5 is much smaller for Gram+ than for both Gram- and no growth samples. A drop of the iron binding proteins decreases the amount of protein bound iron. More free iron becomes available for bacterial growths and therefore promotes infection. Then the difference should also be seen during the active course of the disease, which is not the case. With more than one protein contained within this peak, speculations are difficult because increase and decrease of the proteins can neutralize each other.

During the active disease stages of IBK peak 4 shows the only statistically significant difference. All samples with bacterial growth have decreased area% under the curve, but the difference is only significant for Gram+ cultures. None of our standard proteins eluted at that retention time.

In IBK-recovered animals the only difference is in peak 3, where Gram- organisms caused a lower concentration of IgG suggesting a lower stimulation of the IgG immune response.

In contrast to bacterial infections *Mycoplasma* did not change the tear protein pattern. *Moraxella* positive samples viruses *Moraxella* and *Mycoplasma* samples had no statistically significant differences that could be detected with HPLC.

The HPLC tear protein profile for the normal calf has been established, though changes due to subclinical infections have been detected. A multitude of changes during and after recovery from IBK were identified. The logical next step from here is to investigate the number and identity of the proteins within each peak. Further research to define mechanisms from host as well as Gram+ and Gram- bacteria and their role in the disease process is indicated.

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