**Effects of Milk Antimicrobial Proteins on Incidence of Mastitis in Dairy Cattle**

*M.D. Person, C.N. Person, T.D. Lester, and R.W. Rorie*

### Story in Brief

Dairy cattle vary considerably in their susceptibility to mastitis, perhaps due to innate levels of milk antimicrobial proteins. This study evaluated the relationship between level of antimicrobial proteins in milk and incidence of mastitis. Milk samples were collected from 81 Holstein cows with at least 3 consecutive months of Dairy Herd Improvement (DHI) records for somatic cell count (SCC). Composite milk samples were analyzed for the antimicrobial proteins, lysozyme, glucosaminidase, lactoferrin and lactoperoxidase. Based on SCC history and criteria established by DHI, mastitis status of each cow was categorized as either new (SCC > 200,000 for the first time during current test date), chronic (SCC ≥200,000 on 2 or more consecutive test dates), previous (classified as chronic previously, but current test SCC < 200,000), or no infection (SCC < 200,000). Levels of each of the milk antimicrobial proteins were then compared for cows in the 4 mastitis categories. Cows with new or chronic mastitis infections had higher (P ≤ 0.001) glucosaminidase levels than cows with previous or no infection. Likewise, lactoferrin levels were higher (P ≤ 0.25) for cows with new or chronic mastitis than for those with previous or no mastitis. No relationship was found between either lactoperoxidase or lysozyme milk levels and the mastitis categories (P = 0.61 and 0.64, respectively). Results suggest that glucosaminidase and lactoferrin increase in response to mastitis infection, rather than innate high levels of these proteins prevent mastitis since cows without mastitis had lower levels of these proteins than cows with infection.

### Introduction

Bovine mastitis is the most costly single disease currently affecting dairy cattle. The loss in milk production, along with the expense of treatment and prevention of mastitis, results in a cost of more than 2 billion dollars per year for the dairy industry. While advances have been made in controlling mastitis, most of the treatments are still ineffective. Selection of cows for low somatic cell count has been used to reduce the incidence of mastitis, but this can also result in selection of non desirable traits. With worldwide concern about the use of antibiotics in animal agriculture, the development of new selection techniques are needed to reduce reliance on antibiotic treatment of mastitis. One possible solution could be selection of cows with higher innate levels of antimicrobial proteins in their milk. This approach has the potential to reduce the use of antibiotics and their possible exposure to consumers, while improving the health of dairy cattle and profitability of dairy operations. Therefore, the objective of this research was to evaluate the use of antimicrobial proteins in milk as a selection method for the determination of cows less susceptible to mastitis.

### Experimental Procedures

Composite milk samples were collected from 81 Holstein cows with at least 3 consecutive months of Dairy Herd Improvement (DHI) records. The milk samples were frozen at -20°C until assayed for lysozyme, glucosaminidase, lactoferrin and lactoperoxidase. The cows were divided into 4 categories based upon their SCC history for the previous 3 recording dates. (1) no infection: animals with SCC < 200,000 for at least 2 consecutive test dates during the current lactation: (2) new infection: animals with a SCC ≥ 200,000 for the first time during the current lactation: (3) chronic infection: a cow with a SCC > 200,000 on 2 or more consecutive test dates during the current lactation: (4) previous infection: a cow with a SCC > 200,000 for 2 consecutive test dates during the current lactation but not above 200,000 on the current test date.

N-acetyl-β-D-glucosaminidase activity was determined as previously described (Kitchen et al., 1980), but adapted to a microplate reader (Mattila and Sandholm, 1985). Briefly, duplicate 10 µL milk samples were mixed with 40 µL of substrate solution consisting of 2 mM 4-methylumbel-lifer-N-acetyl-β-D-glucosamide (Sigma Inc., St. Louis, Mo.) in a 0.25 M citrate buffer (pH 4.6) in 96-well microtiter plates. Plates were incubated at 25°C for 15 min, and then the reaction was stopped by adding 150 µL of 0.2 M glycine-NaOH buffer. The fluorescence of the released 4-methyl umbellifereone was measured using a fluorescent microplate reader (FLx800™, BioTek Instruments, Inc., Winooski, Vt.) at an excitation wavelength of 355 nm and an emission wavelength of 480nm. Glucosaminidase activity (U) was then determined for each sample by comparing the reading to a standard curve (0 to 100 U) consisting of 0.15 mM 4-methyl umbellifereone (Sigma) in citrate buffer.

Lactoferrin was measured, using a bovine lactoferrin ELISA kit (Bethyl Laboratories, Inc, Montgomery, Texas). The ELISA was performed using the manufacturer’s directions. The optical density of the bound substrate, TMB, to the enzyme was measured at 450 nm using a microplate spectrophotometer (Spectromax 250, Molecular Devices Corp., Sunnyvale, Calif.). The samples were then compared against a standard curve (0 to 500 ng/mL) to calculate the ng/mL of lactoferrin.

Lactoperoxidase activity was determined as described by ISO (2002), but adapted to microtiter plates. Briefly, duplicate 5 µL milk samples were mixed with 200 µL reagent solution consisting of 2,2-anizniodi (ABTS) (Sigma), hydrogen peroxide solution and a disodium hydrogen phosphate buffer. The lactoperoxidase within the milk sample oxidizes the ABTS resulting in a change in optical density. Optical density of the samples was read at 420 nm, at 15 sec and again at 2 min using a microplate spectrophotometer (Molecular Devices). Lactoperoxidase activity (mU/mL) was calcu-

---

1 All authors are associated with the Department of Animal Science, Fayetteville.
lated using the formula provided by ISO, where the change in optical density over time was representative of lactoperoxidase content. Lysozyme activity was determined, using an EnzChek® lysozyme assay kit (E-22013, Molecular Probes Inc., Eugene, Ore.). Samples were diluted by combining 25 µL of 1× reaction buffer with 25 µL of milk sample. Fifty microliters of substrate stock suspension containing 1.0 mg Micrococcus lysodeikticus, labeled with fluorescein in 1 mL deionized water was added to each sample and incubated for 30 min at 37°C. Fluorescence intensity was measured using a fluorescent microplate reader (BioTek Instruments) at excitation wavelength of 494 nm and an emission wavelength of 518 nm. Lysozyme activity (U/ml of milk) was determined for each sample by comparing the reading to a standard curve (0 to 250 U). Milk antimicrobial protein levels were compared for cows in the 4 mastitis categories using analysis of variance. When significant, means were separated by Student’s t test. The statistical analysis was performed using JMP 6 statistical software (SAS Institute Inc., Cary, N.C.).

**Results and Discussion**

The relationship of milk antimicrobial proteins to mastitis incidence in dairy cows is presented in Table 1. Cows with new or chronic mastitis levels had higher (P ≤ 0.001) glucosaminidase levels than cows with previous or no infection. Glucosaminidase is a lysosomal enzyme secreted in large quantities in the mammary gland in response to mastitis. Our results concur with the results of Kitchen et al. (1976), who reported that glucosaminidase was a good marker for subclinical mastitis, if not better in some aspects than the Wisconsin Mastitis Test. Urech et al. (1999) also found that glucosaminidase levels could be used to identify cows with mastitis infection. Lactoferrin levels were also higher in cows classified as new or chronic (P ≤ 0.025) than in those with previous or no infection. These results are in agreement with Harmon et al. (1975) and Hagiwara et al. (2003) who both reported that lactoferrin concentrations were much higher in the milk of cows with mastitis than normal cows. Lactoferrin is an iron-binding protein found in cow milk at a concentration of approximately 20 mg/L. Guo-Hua et al. (2004) reported lactoferrin has an affinity for biological membranes, affecting the integration of biomembranes in bacteria, and in turn, increasing sensitivity of bacteria to antibodies and lysozyme.

No relationship was found between either lactoperoxidase or lysozyme milk levels and the mastitis categories (P = 0.625 and 0.683, respectively). Lactoperoxidase has no antimicrobial activity itself, as it must be in a system with hydrogen peroxide and thiocyanate. When this system is active, it forms hypoiodite ions, which is a strong antimicrobial agent. Lactoperoxidase levels vary widely among cows and are affected by such factors as feed and stage. Lysozyme is active against many bacteria and kills by disrupting the formation of a glycosidic bond between the 2 components of peptidoglycan found in the bacterial cell wall. Contrary to our findings, Antanas et al. (2005) reported that cows with higher lysozyme levels in their milk had lower somatic cell counts.

**Implications**

Our results suggest that glucosaminidase and lactoferrin increase in response to mastitis infection, rather than innate high levels of these proteins prevent mastitis, since cows without mastitis had lower levels of these proteins than cows with infection. It is possible that a cow’s ability to produce these proteins quickly in response to mastitis might reduce the severity and duration of infection.

**Literature Cited**


**Table 1. Relationship of milk antimicrobial proteins to mastitis incidence in dairy cows.**

<table>
<thead>
<tr>
<th>Antimicrobial protein¹</th>
<th>Types of mastitis infection</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>New</td>
<td>Chronic</td>
<td>Previous</td>
<td>None</td>
<td>P value</td>
</tr>
<tr>
<td>Glucosaminidase</td>
<td>17.41⁺</td>
<td>18.09⁺</td>
<td>9.23⁺</td>
<td>10.23⁺</td>
<td>≤ 0.001</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>133.96⁺</td>
<td>114.96⁺</td>
<td>81.04⁺</td>
<td>83.79⁺</td>
<td>≤ 0.025</td>
</tr>
<tr>
<td>Lactoperoxidase</td>
<td>0.96⁺</td>
<td>1.07⁺</td>
<td>1.04⁺</td>
<td>1.04⁺</td>
<td>0.612⁺</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>94.17⁺</td>
<td>86.48⁺</td>
<td>87.08⁺</td>
<td>90.32⁺</td>
<td>0.641⁺</td>
</tr>
</tbody>
</table>

¹Glucosaminidase values are U of activity. Lactoferrin values are mg/L of milk. Lactoperoxidase and lysozyme values are U/L of milk.

⁺⁺Means in a row with no letter in common differ (P< 0.05)