Milk Antitrypsin as a Marker of Bovine Mastitis—Correlation with Bacteriology

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ABSTRACT

Milk antitrypsin reflects leakage of protein of blood plasma into milk. Mastitis detection can be based on measurement of the trypsin-inhibitor capacity in milk; the "Eflab Mastitis Test" procedure is based on this principle. The procedure was developed to screen for subclinical mastitis; high capacity and sensitivity permits whole herds to be analyzed on a quarter basis to identify inflamed quarters during a single sampling.

Milk antitrypsin was measured on 2174 quarter milk samples by a colorimetric procedure. The Multiskan MC reader was hooked to desk computer to interpolate antitrypsin from the standard curve and to analyze results. The analysis of inflammation was based on antitrypsin as well as computerized interquarter evaluation. These measures were compared with bacteriologic study of milk to set criteria for automatic identification of mastitis in individual quarters. The comparison against the lowest teat antitrypsin gave better indication of infection than antitrypsin as such. By strict criteria that identified inflammations caused by major pathogens, 18.8% of uninfected quarters were identified as false positive. The explanation is that a single bacteriological culture is not reliable for detecting mastitis, because there are temporary causes of tissue irritation other than bacteria that may be identified during standard bacteriologic examination.

INTRODUCTION

Culturing milk samples has been a standard method of examination for mastitis. By definition, mastitis is inflammation of the mammary gland. Accordingly, any screening method for mastitis should be based on demonstration of inflammatory changes of mammary secretion. An inflammatory reaction in the mammary gland may originate from mechanisms other than infection. Bacteriologic study of milk has not been fully reliable for detecting mastitis. A single bacteriologic examination of quarter milk samples does not identify all infected quarters, and it is difficult to avoid contamination during sampling. Chronically infected quarters give false-negative results in a single bacteriologic test, even though the majority of mastitis cases are due to colonization of the mammary gland by pathogenic bacteria (18). Successive samplings improve the bacteriologic result (11).

A positive diagnosis of mastitis should fulfill two criteria, a positive bacteriological test and an inflammatory change (8, 20). Cell counts generally have been used for the latter purpose. The two criteria often are used separately. By screening cows with an indirect test first and then culturing the positive reactors, the number of samples for bacteriologic study can be kept at a minimum and costs of mastitis control programs reduced. Measures of somatic cell concentrations have been used as indicators of probable infection, and cell counting has become the classic method of screening for mastitis.

One of the principles of detecting inflammation within the mammary gland is to assess the integrity of the mammary epithelium. Mastitis is associated with increased permeability between blood and milk compartments which can
be seen as leakage of blood plasma proteins into the milk. Theoretically, measurement of any plasma protein in milk can be an indicator of mastitis, provided this protein is not transferred actively across mammary epithelium. Proteins in blood plasma with low molecular weight move preferentially across the blood-milk barrier (6). Milk BSA (bovine serum albumin) concentration has been suggested as a sensitive indicator for mastitis (4). The analysis is usually by radial immunodiffusion technique (10).

Milk α₁-antitrypsin (α₁-protease-inhibitor) recently was suggested as an indicator of mastitis (6). Milk antitrypsin shows close agreement with BSA and somatic cell counts (16). Antitrypsin has the advantage over BSA that it can be quantified by its trypsin-inhibitor capacity by colorimetric procedures (15). Automation of this method is under way including computer analysis of degree of inflammation. The present investigation was to determine thresholds of antitrypsin activity to be used in computerized diagnosis of mastitis. Maximum agreement with bacteriological study was the criterion for setting limits for normal and abnormal secretion.

MATERIALS AND METHODS

Premilking samples (n=2174) were collected from quarters of udders during herd surveys on selected herds with problems of chronic mastitis. The 544 cows were of the Finnish Ayrshire breed. Another 100 “normal” milk samples were selected from another series to establish limits for normal milk. Criteria for selecting these samples were negative bacteriological results from two consecutive samplings, somatic cell count < 300,000/ml, and BSA content <.25 mg/ml. In addition to these criteria, milk samples taken within 1 mo of parturition were not included in the material (to avoid effects of colostral antitrypsin).

Bacteriological Assay

Milk samples were collected under maximum asepsis. Washed and dried ends of teats were cleansed with a swab dipped in 70% alcohol. The first three streams were rejected before samples were collected. Samples were kept cool and cultured on blood agar plates within 24 h according to standard methods for milk bacteriological culturing (9).

Antitrypsin Assay

Measurement of trypsin-inhibitor capacity was essentially as in (15, 16) but by the kit produced by Eflab (Pulttitie 9-13, 00810 Helsinki 81, Finland. US marketing: Flow Laboratories, 7655 Old Springhouse Road, McLean, VA 22102). The principle of this assay is depicted in Figure 1.

Milk samples (300 μl) were mixed with three volumes of the clearing solution (16.7% polyethylene glycol and 1% dimethylformamide in .1 M Tris-.02 M CaCl₂ buffer, pH 8.2). This coprecipitates casein, cream, and interfering α₂-macroglobulin, resulting in a clear supernatant upon centrifugation (10,000 × g/1 min). Supernatants (100 μl) were transferred to wells in rows 3 to 12 of 8 x 12 well, flat-bottomed microtitration plates. The trypsin working solution (100 μl, trypsin content 2.5 μg/ml) was mixed with the supernatants, and substrate was added (100 μl N-benzoylarginine-p-nitroanilide, 1 mg/ml distilled water). The first row of each plate was reserved for zero blanks and the first four wells in the second row for 100% controls (contained trypsin and substrate but no antitrypsin). The other four wells in the second row contained standard milk samples (with a relative antitrypsin content of 1.0), trypsin, and substrate. The α₁-protease-inhibitor content of this “standard milk” was .009 mg/ml as related to the trypsin-inhibitor capacity of pure α₁-protease-inhibitor obtained from Boehringer Mannheim GmbH, West Germany.

Plates were left to incubate at room temperature for 3.5 h after which they were measured at 405 nm (absorbance of the chromogen released by trypsin) as well as at 510 nm (to correct for possible turbidity). The Multiskan reader was hooked to a desk computer (Olivetti L1 M20). The computer had been programmed to command the Multiskan reader to correct sample absorbances for turbidity, to interpolate antitrypsin from internal standard curves, and to compare antitrypsin with the reading of the lowest individual quarter milk (interquarter evaluation).

The antitrypsin assay is an extremely sensitive method for detecting inflammation in the udder. Therefore, the system is particularly useful for detecting chronic subclinical mastitis. The sensitivity and repeatability of the assay has been optimized. To keep the antitrypsin
Figure 1. Principle of the milk antitrypsin assay. The milk is precipitated with the clearing solution. The supernatant is mixed with trypsin, and part of the trypsin becomes inactivated by the antitrypsin in milk. The excess trypsin releases a chromogen from BAPNA (N-benzoylarginine-p-nitroanilide), which is measured at 405 nm. By including the 100% control (without any antitrypsin) and 0% blank (without trypsin), a computer-fit program can be formed to interpolate the antitrypsin from the internal standard curve.
scale fixed from plate to plate and day to day, the same milk pool was used as a standard on each plate (stored freeze-dried). Antitrypsin of the standard was set consciously as close to the decision level as possible. The interfering effect of possible turbidity in the milk supernatants was eliminated by absorbance at two wavelengths automatically measured, one of which was for the chromogen and the other for correction for possible turbidity. With these adjustments repeatability of the assay at antitrypsin 1 to 2 was 6% (coefficient of variation). The output of the antitrypsin assay procedure depends largely on the centrifugation step.

Calculations

Antitrypsin and the interquarter ratios (antitrypsin/lowest quarter) were variables for inflammation in search for cuts-off that would give most separation between infected and uninfected quarters. By logarithmic transformation, the distribution of the 100 "healthy" samples became "normal," and so natural logarithms were used throughout the study. The average logarithmic (ln) antitrypsin of the selected 100 "healthy" milk samples was given the numeric 1.0 (ln=0), and all the other quantities were related to this. The standard milk sample throughout the experiments also had been standardized to 1.0. Measured antitrypsin and interquarter ratios were tested alone and in combination under a matrix analysis using stepwise procedures to analyze separation between bacteriologically positive and negative samples. The criterion for most separation was that the sum of the false percentages within bacteriologically positive and negative samples be minimum. Positive bacteriologic results were used to mark the mastitis population although not all the inflammations were caused by bacteria.

Follow-Up Study of False Positives

After criteria for mastitis were decided from antitrypsin and interquarter evaluations, a representative number of quarters having false positive results but negative bacteriology (n=60 quarters) were followed for 12 days by re-analysis of milk samples every 3 days through measuring antitrypsin and bacteriologic tests.

RESULTS

Of the 2174 quarter milk samples, 215 showed positive bacterial cultures during the first screening (25% of cows). Results of milk cultures are in Table 1. Positive bacteriologic cultures were associated with increased antitrypsin.

Percentage distributions of antitrypsin among bacteriologically positive and negative samples are in Figure 2. The selected "normal"
Figure 2. Percentage distribution of antitrypsin (logarithm) (top panel) and interquartile ratios (lower panel) in 2174 quarter milk samples.
Milk samples (n=100) all fell within the antitrypsin range below 2.0 (ln<.69). In all the 2174 quarter milk samples, 11.9% of the bacteriologically negative samples exceeded the maximum of this “normal range” (ln>.69, Figure 2). The respective percentage for bacteriologically positive samples was 61.0%.

In the whole unselected material, rear quarters showed higher antitrypsin than front quarters (geometric means 1.46 and 1.42). Furthermore, 61% of the bacteriologically positive samples were from rear quarters. When bacteriologically positive samples were excluded from the material, the difference in antitrypsin between front and rear quarters disappeared.

The interquarter ratio gave more separation between infected and uninfected quarters than antitrypsin (Figure 2, lower panel).

When results were calculated in a cumulative distribution pattern separately for bacteriologically positive and negative samples, one could identify the cut-off for antitrypsin and the interteat ratio that would give maximum separation between infected and uninfected quarters. Maximum separation between bacteriologically determined groups was at antitrypsin 1.6 (ln=.5). At this cut-off, 32.6% of bacteriologically positive samples were classified “false negative,” and 15.9% of bacteriologically negative samples as “false positive.” The most separation was by the interquarter ratio 1.13 (ln=.12). When the interquarter ratio remains below the decision ln, the sample is classified healthy. By these criteria, 88.5% of the bacteriologically positive samples were classified mastitic, but 18.8% of the bacteriologically negative samples were classified false positive (Table 2). In all samples taken within 2 wk of parturition the ln antitrypsin exceeded the respective interquarter ratio by more than 1.5 ln units. According to the analysis, the desk computer was programmed to use the following criteria to distinguish whether secretion was “normal” or “abnormal” and to obtain a rough estimate of severity of inflammation. 1) Samples with an antitrypsin <1 (ln<0) are labeled “healthy.” 2) Samples with interquarter ratio below 1.13 (ln<.12) are labeled “healthy.” 3) When the difference between ln antitrypsin and the respective inter-quarter ratio is >1.5, the sample is labeled “colostral residue?" 4) All other samples are identified “mastitic.”

For an arbitrary grading of inflammation, the determined antitrypsin measures (antitrypsin and interquarter ratio) were combined by their logarithmic means (“balanced antitrypsin”). After material in criteria 1 to 3 were excluded, bacteriologically positive material was divided into three equal groups (33% of the samples in each group). The highest third was labeled “severe mastitis***,” the middle third “mastitis**,” and the lowest third “mild mastitis.” Cut-offs for these criteria for “balanced antitrypsin” appeared to be at ln .46 and 1.5.

The bacteriologic distribution of the 11.5% of samples that were left outside the “mastitic” group is in Table 3. The bacteriological test showed a high proportion of “minor udder pathogens” in this group. In all the bacteriologically positive samples there were no statistically significant differences between bacterial species, as far as they affected antitrypsin or ratio. This was because the number of strains

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**Table 2. Bacteriological analysis of milk samples labeled “mastitic” or “normal” according to the interquarter evaluation.**

<table>
<thead>
<tr>
<th>Bacteriological test</th>
<th>Correct diagnosis (%)</th>
<th>Incorrect diagnosis (%)</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive (isolated)</td>
<td>88.5</td>
<td>11.5</td>
<td>Many of these belong to “minor pathogens” (Table 3)</td>
</tr>
<tr>
<td>Negative (not isolated during a single examination)</td>
<td>81.2</td>
<td>18.8</td>
<td>Single bacteriological test probably failed to isolate the infective organism</td>
</tr>
</tbody>
</table>
TABLE 3. Bacteriological distribution in the quarters becoming labeled falsely healthy according to criteria 1 to 4.1

<table>
<thead>
<tr>
<th>Strain</th>
<th>(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>41</td>
</tr>
<tr>
<td><em>Micrococcus</em></td>
<td>17</td>
</tr>
<tr>
<td><em>Streptococcus uberis</em></td>
<td>7</td>
</tr>
<tr>
<td>Coryneforms</td>
<td>21</td>
</tr>
<tr>
<td><em>Staph. epidermidis</em></td>
<td>7</td>
</tr>
<tr>
<td>Other streptococci</td>
<td>7</td>
</tr>
</tbody>
</table>

1 n=29.

in each species was too small. With bacteriologically negative samples, 18.8% of these fell into the category of "false positives" when the strict criteria 1 to 4 were used to isolate the bacteriologically positive samples. The follow-up study of these "false positives" (n=60) showed 40% with high antitrypsin and negative bacterial cultures during repeated samplings, which means that the cause of tissue irritation remained unresolved. In 15%, repeated bacterial testing was positive during one or more samplings. In these instances the single bacterial test apparently had failed to isolate ineffective organisms. In the remaining 45%, antitrypsin declined to normal during the 12-day follow-up study, indicating that there had been a brief bacteriologically negative tissue irritation at first sampling.

DISCUSSION

A high-capacity and inexpensive monitoring system will enable the veterinarian and the dairy producer to maintain a constant record of mastitis in the herd. The present assay system was developed to identify inflamed quarters in a herd during a single sampling. The system enables cows to be analyzed by quarters of udders permitting a built-in security factor to be incorporated in the system. Automatic interquarter evaluation gives this additional security.

When comparing figures obtained with the present methodology of using antitrypsin as a marker for mastitis in combination with bacteriological results, one should be aware of the different nature of the two variables. Agreement of 100% between the measures cannot be expected. However, positive bacteriologic results can be used to "mark" the population of mastitic samples in establishing criteria for another test such as an antitrypsin assay.

Mastitis means inflammation of the mammary gland, and isolation of bacteria from milk samples not contaminated externally means infection. The inflammatory reaction results in increased permeability between blood and milk compartment which is seen as increased plasma protein in the milk.

The primary objective of a method intended for the diagnosis of mastitis must rely on accurate determination of inflammation as such. The present method based on milk antitrypsin has shown correlation with somatic cell counts; excellent agreement with BSA was observed as well (16). The antitrypsin and BSA determinations seem to have the same diagnostic value for mastitis. Principal advantages of the present assay are automatic interquarter evaluation and accuracy.

The diagnostic accuracy of any test to identify mastitis depends on separation of "mastitic" and "nonmastitic" samples. The dividing line between health and disease may be set so that only obvious or severe disease is identified (the result being a test with high specificity and low sensitivity). Or it may be set to detect even those with mild or subclinical disease (low specificity and high sensitivity). The latter approach commonly is used in screening tests for mastitis. The present criteria for antitrypsin determination were set for maximum separation between bacteriologically positive and negative samples. This results in the best possible compromise between specificity and sensitivity.

There was relatively large overlapping of antitrypsin among infected and uninfected quarters (Figure 2). However, there is much room for speculation whether the single bacteriologic test was sufficient to detect infected quarters and separate infected from uninfected quarters. In Figure 2, 11.9% of bacteriologically negative samples showed antitrypsin >2 (In> .69), which was the maximum for the "normal range" as determined on preselected milk samples from healthy cows. One would anticipate that these "false positives" must have given a false bacteriological result or had another cause of tissue irritation not identified by bacteriology. Furthermore, the antitrypsin
range from 1 to 2 (ln=0 to .69) must include bacteriologically “false-negative” samples. The difficulty of isolating bacteria seems to stem from milk being a bacteriostatic medium that affects bacterial growth at the initial phase (14). These interpretations have been used to explain why there is generally relatively poor agreement between bacteriological test and markers based on detection of inflammatory changes in milk. Organisms in the teat canal but not in the mammary gland would be expected to contribute to disagreement with bacteriological results. The explanation for tissue irritation during teat canal infections is that the bacteria release toxins that diffuse into the mammary gland causing irritation (19, 21).

Interquarter evaluation can be incorporated in the computer analysis system to verify mastitis provided all quarters are examined in parallel. The basis of the interquarter evaluation is that it is statistically rare that cows have all quarters inflamed at the same time or at least to the same degree. Therefore, the lowest quarter measure can be taken as “healthy.” In healthy cows with bacteriologically negative quarters antitrypsin was equal, and comparison can be against any other normal quarter within the cow.

The criteria (1 to 4) were established to distinguish as accurately as possible infected quarters from the uninfected. According to these criteria, which are based on the analysis of antitrypsin as well as interquarter evaluation, the bacteriologically positive group can be isolated easily (Figure 2). The discriminatory power of the present test alone is much the same as that with somatic cell counting combined with bacteriological examination. The present test with the criteria resulted in 18.8% of “false positives” among bacteriologically negative samples. In the bulk of these uninfected quarters, antitrypsin declined to normal during the follow-up study, indicating relapsing irritation. From experiments on endotoxin-induced mastitis, antitrypsins stabilized to their original amounts within 3 days postinfusion (7), which agrees with the present explanation. There is much room for discussion as to what the cause of tissue irritation was in those samples that retained chronically high antitrypsin but had negative bacteriological results. Additional explanations for negative bacteriology among samples with increased antitrypsin include infection caused by an agent not identified by standard bacteriological procedure. Also, any type of mechanical trauma would lead to leakage of blood proteins into milk without infection.

According to the given criteria, 11.5% of the bacteriologically positive samples remained outside the range of “mastitis.” Half of these milk samples were contaminated by “minor pathogens” and the other half by strains of Staphylococcus aureus (Table 3). “Minor pathogens” cause extremely little tissue irritation. It seems probable that many of the Staphylococci that did not cause tissue irritation have their origin in contamination during the sampling procedure. When milk samples are taken by trained persons, the main contaminants are organisms that most frequently colonize teat ducts, such as staphylococci and “minor pathogens” (11). The main advantage of the present method over other methods is that mastitis, including chronic subclinical mastitis, can be verified during a single sampling with reliability.

Cell counts have not proved satisfactory for detecting mastitis during a single sampling. This is a problem, especially in chronic subclinical mastitis. There is large physiological variation of cell counts. A few influencing factors have been identified, such as timing of milk sampling during the milking procedure, effect of lactation stage and number, sedimentation or flotation of cells, and disruption of cells during storage, “false particles” etc. (1, 2, 5, 6, 12, 13, 17). Subclinical infections by common pathogens often are accompanied by only a marginal increase of cell counts. Thus, the threshold for positive diagnosis has to be set so low that many unaffected cows are diagnosed “false positive.” The current criteria or the diagnosis of subclinical mastitis results in a considerable proportion of false positive diagnosis, 43% on average (3).

The major limitation of the present assay is that colostral and postcolostral samples should be evaluated with care as colostrum has high antitrypsin activity. Traces of colostral antitrypsin can be seen up to 1 mo after parturition (16). However, the automated program identifies colostral samples. It is typical of colostral samples that antitrypsins are high but individual teat measures are equal. If teat measures are not equal, the system identifies mastitis even in the presence of colostral interference.
REFERENCES
1 Blackburn, P. S. 1966. The variation in the cell count of cow's milk throughout lactation and from one lactation to the next. J. Dairy Res. 33:193.