Application of a Monoclonal Antibody-Based Enzyme-Linked 
Immunosorbent Assay for Detection of an Inflammatory 
Response Antigen in Subclinical Mastitic Milk Samples

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A monoclonal antibody to a 23.5-kDa bovine inflammatory antigen present in high levels in mastitic milk has been used in an antigen-capture enzyme-linked immunosorbent assay (ELISA) to screen milk samples from herds of cattle for subclinical mastitis. The results from 20 herds with a total of 2,612 quarter samples are presented. Good correlation was observed between the ELISA level and the milk cell count (MCC). The results demonstrated an average of 5% false negatives (1.8% associated with isolates of Staphylococcus aureus and/or Streptococcus spp.) and 7.7% false positives for each herd in relation to mastitic (>400,000 cells per ml) or nonmastitic (<400,000 cells per ml) MCCs.

The standard methods for detecting subclinical mastitis in dairy herds are bacterial culture of milk and measurement of the milk cell count (MCC) (3, 5). The main problems associated with these parameters are the fluctuation of both in some infections and the submission of poorly collected contaminated samples. In recent years, various enzyme tests have been used in the detection of mastitis (6); of these, the estimation of N-acetyl-β-d-glucosaminidase (NAGase) has been the most widely examined and has been adapted to a microtiter technique by using a fluorescent substrate (1, 7).

The monoclonal antibody (MAB) used in the present study was obtained indirectly during work involving the development of MABs to Staphylococcus aureus. The MABs obtained were directed not toward S. aureus antigens but toward a bovine inflammatory antigen present in high levels in infectious mastitic milk irrespective of the pathogen. An antigen-capture enzyme-linked immunosorbent assay (ELISA) was developed by using the MAB and tested in a microtitre plate system (8).

MATERIALS AND METHODS

MAB production. Cells from an S. aureus strain isolated from milk of a cow with clinical mastitis were scraped off blood agar, washed twice with saline, and finally suspended in 0.03% formal saline. This antigen was used to immunize a BALB/c mouse until a precipitin line was produced between the mouse serum and clinical S. aureus-infected milk in a double immunodiffusion test. Precipitin lines were cut out of the agar and washed several times in distilled water. A homogenized preparation was used to immunize another BALB/c mouse until a precipitin line was again evident in a double immunodiffusion test with the serum against the same antigen. The spleen cells from this mouse were fused with NSO myeloma cells (2) 3 days after a final intrasplenic inoculation. The hybridoma cells were maintained in RPMI 1640 medium supplemented with 20% gamma globulin-free horse serum (GIBCO).

The culture fluids of approximately 200 actively growing hybridomas were screened by an ELISA in microtitre plates (Dynatech). The supernatant of ammonium sulfate (50% saturation)-fractionated skim milk from a clinical S. aureus mastitic milk sample was found to be a satisfactory microtiter well-coating antigen to detect antibody in the serum of the sacrificed mouse. This antigen, together with a similar antigen prepared from normal milk, was used to screen the hybridomas. Those producing a high reading with the mastitic milk-derived antigen were cloned twice by limiting dilution. Ascites fluid was prepared from the cloned MABs by intraperitoneal injection of each line into BALB/c mice 4 days after priming with Freund's incomplete adjuvant (9). The developed ascites fluid was removed approximately 10 days later and stored at −70°C.

Characterization of antigen. MAB 1B3 was used on a protein A-based affinity chromatography column (Immuno Pure IgG Orientation Kit; Pierce, Beijerland, The Netherlands) to purify the specific antigen. Clarified whey from S. aureus mastitic milk dialyzed in 10 mM Tris-HCl buffer (pH 7.5) was passed through the prepared column. The column was washed with the buffer, and the antigen was eluted with 0.1 M glycine buffer (pH 2.8). Eluted fractions were collected in equal volumes of 1 M Tris-HCl (pH 9).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with the eluted material on a 12.5% acrylamide resolving gel with a 5% acrylamide stacking gel, and the sample was electrophoresed under both reducing and nonreducing conditions. Strips were removed for protein staining, and the remainder of the gel was blotted onto nitrocellulose. Strips of nitrocellulose were blocked with 2% bovine serum albumin in 0.01 M phosphate-buffered saline with 0.001 M EDTA and 0.5% Tween 80 and incubated at 37°C, first with dilutions of biotinylated 1B3 and then with streptavidin-peroxidase. Each incubation was followed by washing with five changes of 0.01 M phosphate-buffered saline (pH 7.2) with 0.05% Tween 20. The final peroxidase substrate used was 0.5 mg of 3,3′-diaminobenzidine tetrahydrochloride (Sigma) per ml in 0.02 M Tris-HCl buffer (pH 7.2) with 0.3 μl of H2O2 (30% solution) per ml. ELISA. The MABs were used in an antigen-capture ELISA on microtitre trays with 100-μl volumes for each reagent per well. The optimum dilution for each reagent was established by titration. The immunoglobulin G fraction from ascites fluid of MAB 1B3 was purified by the caprylic acid method (8) and used to coat the wells in 0.05 M...
carbonate buffer overnight at 4°C. All further incubations were at 37°C, and after each incubation stage, the wells were washed with five changes of 0.01 M phosphate-buffered saline (pH 7.2) containing 0.05% Tween 20. After the wells were coated, duplicate test samples of undiluted milk were incubated for 2 h and then incubated with biotinylated (4) 1B3 for 1 h, streptavidin-peroxidase (Sigma) for 1 h, and the substrate for 10 min. The substrate used consisted of o-phenylenediamine dihydrochloride (Sigma; 0.4 mg/ml) and 0.4 μl of 30% H₂O₂ per ml in 0.1 M citrate phosphate buffer (pH 5.0). The substrate reaction was stopped by the addition of 50 μl of 2.5 M H₂SO₄ per well, and the A₄₉₂ was measured on an ELISA plate reader (Titertek).

**Test samples.** The antigen-capture ELISA was initially evaluated with mastitic milk samples submitted to our laboratory for diagnosis. These included infections caused by *S. aureus*, *Streptococcus* spp., *Escherichia coli*, *Haemophilus somnus*, *Mycoplasma californicum*, and *Mycoplasma bovis*. Normal milk and colostrum samples from animals that had recently calved were also tested.

Subsequent evaluation of the test in herd surveys to detect subclinical mastitis was carried out with quarter milk samples collected by farmers or their veterinary practitioners in sterile bottles supplied by our laboratory. Samples from 20 herds were examined.

**Bacteriology.** Milk specimens streaked on blood agar were incubated at 37°C for 24 h, and any observed growth was identified by standard bacteriological methods. In the absence of growth after 24 h, samples with high MCCs were incubated for a further 24 h.

**MCCs.** Samples were fixed by incubation with formalin (0.2 ml of 10% formalin in 10 ml of milk) overnight at 30°C. The cell level was measured electronically with a Coulter Counter. An MCC of 400,000 cells per ml was taken as the cutoff point, with milk samples with levels above this point regarded as mastitis positive. ELISA-negative samples with MCCs of >400,000 cells per ml were considered false negatives, and conversely ELISA-positive samples with MCCs of <400,000 cells per ml were considered false positives.

**NAGase test.** The NAGase test was carried out on duplicate 20-μl samples from all milk specimens by the method described by Ball and Greer (1).

**Statistical analysis.** The statistical correlations between the nontransformed MCCs and the ELISA levels and between the MCCs and the NAGase levels of all milk specimens from each herd were analyzed with a computer program (Stat 80; Hewlett Packard) and expressed as correlation coefficients. The microplate reader gave variable readings, on repetition, of the high (>2.00 absorption units) ELISA levels; these 36 results were considered to artificially lower some of the correlation coefficients and were omitted from this analysis.

**RESULTS**

Only 2 of the approximately 200 hybridomas examined produced an ELISA reaction specifically with the antigen derived from *S. aureus* mastitic milk. Both were stable and were cloned.

Affinity chromatography with MAB 1B3 purified an antigen which immunoblotted on nitrocellulose following PAGE under nonreducing but not reducing conditions (Fig. 1). Protein staining of the slab gel revealed three bands in the sample electrophoresed under nonreducing conditions. The MAB-specific antigen had a molecular mass of approximately 23.5 kDa; the molecular masses of the nonspecific bands were approximately 75 and 66 kDa.

Both MABs could be used in the antigen-capture format, either singly or on either side of the sandwich. MAB 1B3 produced higher readings in the test than the other MAB and was selected for further work. Initial examination of mastitic milk samples with the antigen-capture test established that the MABs were not specific for *S. aureus* antigen. An antigen was detected in milk from cows with mastitis caused by a range of organisms but was absent from normal milk and colostrum.

The application of the test to a herd survey of 20 farms is summarized in Table 1. Apart from the high ELISA readings (>2.00 absorption units), only small variations in the duplicates of each sample tested were found. Background levels were at first estimated from normal milk samples used as negative controls. Since these were consistently below 0.060, it was later assumed that levels below 0.06, which were always present on test plates, were from normal milk, and such samples were regarded as controls. Any reading of >0.100 was taken as positive.

Background bacterial contamination was a problem in the milk samples from some farms, and the rightmost column of Table 1 was included to give a measure of this for each herd. The presence or absence of *S. aureus* and *Streptococcus* spp. is also indicated in Table 1 for the false negatives for the same reason. The percentage of samples that were ELISA positive ranged from 5.4 to 67.1% (average, 22.7%) and was similar in most herds to the percentage that were MCC positive, which ranged from 7.4 to 50.8%. The exceptions were herds 2, 9, and 10, in which the number of ELISA positives was approximately twice that of the MCC positives, and herd 3, in which the number of ELISA positives was approximately one-half that of the MCC positives.

The range of ELISA false negatives was 0 to 7.2% (average, 1.8%) for samples with bacteria and 0 to 17.2% (average, 3.2%) for those without bacteria. The range of ELISA false positives was 0 to 36.1% (average, 7.7%).

Table 2 lists the regression analysis results of the MCC
TABLE 1. Analysis of ELISA results in relation to MCCs for samples from 20 herds

<table>
<thead>
<tr>
<th>Herd no.</th>
<th>No. of samples</th>
<th>No. (%) with MCCs &gt;400,000 cells/ml</th>
<th>No. (%) of ELISA positives</th>
<th>No. (%) of false negatives</th>
<th>No. (%) of false positives</th>
<th>No. (%) with low MCCs (&lt;400,000 cells/ml) with bacteria</th>
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<tr>
<td>1</td>
<td>271</td>
<td>20 (7.4)</td>
<td>22 (8.1)</td>
<td>3 (1.1)</td>
<td>2 (0.7)</td>
<td>5 (1.8)</td>
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<td>21 (10.0)</td>
<td>41 (19.5)</td>
<td>0</td>
<td>1 (0.5)</td>
<td>21 (10.0)</td>
</tr>
<tr>
<td>3</td>
<td>192</td>
<td>63 (32.8)</td>
<td>30 (15.6)</td>
<td>6 (3.1)</td>
<td>33 (17.2)</td>
<td>6 (3.1)</td>
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<tr>
<td>4</td>
<td>251</td>
<td>29 (11.5)</td>
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<td>2 (0.8)</td>
<td>12 (4.8)</td>
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<tr>
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<td>143</td>
<td>32 (22.4)</td>
<td>34 (23.8)</td>
<td>2 (1.4)</td>
<td>3 (2.1)</td>
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<tr>
<td>6</td>
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<td>14 (16.7)</td>
<td>13 (15.5)</td>
<td>1 (1.2)</td>
<td>0</td>
<td>6 (7.1)</td>
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<tr>
<td>7</td>
<td>63</td>
<td>32 (50.8)</td>
<td>35 (55.5)</td>
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<tr>
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<td>20 (18.0)</td>
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<td>2 (1.8)</td>
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<td>36 (18.7)</td>
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<tr>
<td>10</td>
<td>130</td>
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<td>88 (67.7)</td>
<td>2 (1.5)</td>
<td>2 (1.5)</td>
<td>47 (36.1)</td>
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<td>109</td>
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<tr>
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<td>1 (0.7)</td>
<td>10 (7.5)</td>
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<td>4 (7.1)</td>
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<tr>
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<td>7 (7.5)</td>
<td>5 (5.4)</td>
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<td>4 (3.6)</td>
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<td>1 (1.3)</td>
<td>1 (1.3)</td>
<td>15 (20.0)</td>
</tr>
<tr>
<td>20</td>
<td>82</td>
<td>34 (41.5)</td>
<td>31 (37.8)</td>
<td>1 (1.2)</td>
<td>5 (6.1)</td>
<td>3 (3.6)</td>
</tr>
</tbody>
</table>

Total 2,612 516 (19.7) 593 (22.7) 48 (1.8) 84 (3.2) 201 (7.7) 529 (20.2)

* Samples with ELISA levels greater than 0.100 absorption units were considered positive.

b S. aureus, Streptococcus spp., or both.

against the ELISA and the NAGase test. The MCC-ELISA correlation coefficient was >0.7 for most farms (average, 0.80) and was also greater than the MCC-NAGase test correlation coefficient (average, 0.70) for 15 of the 20 herds.

**DISCUSSION**

The results produced with the antigen-capture ELISA using a MAb directed to an antigen associated with an inflammatory response in milk correlated well with the MCC, one of the standard parameters used to detect subclinical mastitis. Although a number of false negatives were detected, these were defined only in terms of an MCC of >400,000 cells/ml. MCCs of >400,000 cells/ml are not necessarily due to an under infection; however, analysis of the results was limited by the poor bacteriological quality of some of the submitted samples and by the limited amount of information available about such factors as lactation stage and milking-machinery efficiency.

The only characterization of the antigen measured by the ELISA was obtained by PAGE and immunoblotting. The evidence from these procedures indicated a protein with a molecular mass of approximately 23.5 kDa which retained the specific monoclonal epitope in SDS solubilizing conditions but not after cleavage into separate polypeptides by mercaptoethanol. It can be speculated that the protein may be one of the intercellular signals involved in an inflammatory response.

A larger number of false negatives was recorded for samples without bacteria than for samples with bacteria. It is possible that those with bacteria are more meaningful, but they must be regarded, for each herd, in the context of the quality of samples, which is estimated in the rightmost column in Table 1. The false negatives for samples without bacteria might have MCCs of >400,000 cells/ml because of intermittent bacterial excretion, antibiotic treatment, or a physiological condition. A large number of the milk samples having MCCs of >400,000 cells/ml in herd 3, which had a high number of false-negative samples without bacteria, were in the MCC range of 400,000 to 600,000 cells/ml. It was postulated that a noninfectious factor, possibly faulty milking machinery, was a contributory cause of these levels.

The number of ELISA-positive samples for the majority of herds was similar to the number of MCC positives. In a similar investigation carried out in our laboratory by the
NAGase test with the same number of herds, the number of NAGase-positive samples was substantially greater than the number of MCC positives (1). In the work with the NAGase test, the cutoff point to distinguish between positive and negative samples was selected in order to reduce the number of false negatives. This resulted in an increase in the number of false positives. In all such comparative tests using a standard, the manipulation of the cutoff point changes the ratio of false negatives to false positives. In the present work, the similar numbers of ELISA and MCC positives for most farms and the relatively small number of false positives indicate a better correlation between the ELISA test and the MCC than between the NAGase test and the MCC. This is also demonstrated by regression analysis (Table 2) in which all the milk samples from each farm (high and normal MCC) are compared.

The generally better correlation between the ELISA and the MCC than between the NAGase and the MCC indicates the potential of the ELISA as a diagnostic test for detecting subclinical mastitis. Work on the development of a more rapid test and the evaluation of the significance of the false negatives and false positives of the present study, using experimental animals, is continuing.

ACKNOWLEDGMENTS

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REFERENCES