Enzyme-Linked Immunosorbet Assay and Microbiologic Culture for Diagnosis of *Staphylococcus aureus* Intramammary Infection in Cows

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ABSTRACT

Recent reports have indicated that the relative sensitivity and specificity of the ELISA test for detection of intramammary infection of cows with *Staphylococcus aureus* is not as high as originally reported. It has been suggested that antibodies measured by enzyme-linked immunosorbet assay (ELISA) more closely reflect previous infection status rather than current infection status, and that the delay in antibody formation following infection and the persistence of antibodies after elimination of infection may be responsible for some of the discrepancy observed between ELISA and bacterial culture results conducted on the same milk sample. This study \( \text{n} = 209 \) cows was undertaken to determine if an ELISA for *S. aureus* intramammary infection more closely reflects previous infection status than it does current infection status, and to ascertain whether correction of this time-delay factor substantially improves calculated values of ELISA relative sensitivity and specificity. Receiver-operator curves were constructed to compare different time-related definitions of microbiologic culture results used for comparison with ELISA results. A greater degree of curvature in receiver-operator curves indicated that ELISA results did more closely reflect culture results performed on milk samples taken 1 and 3 weeks previously. Insignificant improvement in sensitivity and specificity occurred when the database was limited to cows \( \text{n} = 140 \) with milk production greater than 13.6 kg/day. However, values of sensitivity were all less than or equal to 90%, and values of specificity were all less than 54%.

Key words: Mastitis, ELISA, receiver-operator curves, sensitivity, specificity, dairy cows, *Staphylococcus aureus*

Mastitis caused by the contagious bacterium *Staphylococcus aureus* is particularly refractory to therapy due to antibiotic resistance and to the bacterium’s ability to form microabscesses in the udder. Cows with intramammary infection due to *S. aureus* (IMI-SA) often become chronic sources of IMI-SA for other cows in the herd (17). Control is best achieved through sanitary milking technique, teat disinfection, dry cow therapy, the culling of carrier cows, and segregation of infected cows (9). Segregation and culling both require accurate identification of infected carrier cows. Such identification is often accomplished through serial bacteriologic milk cultures, but the availability of an easier and less expensive diagnostic method is desirable.

The enzyme-linked immunosorbet assay for *Staphylococcus aureus* (ELISA-SA) antibodies (ProStaph I, ProScience Corp., Sterling, VA) in milk was developed as an inexpensive screening test to identify cows with IMI-SA (1). Some Dairy Herd Improvement Association groups perform the ELISA assay on monthly milk samples collected for production testing. Original reports claimed the ELISA-SA had a sensitivity of 93% and a specificity of 99% when compared with microbiologic culture performed on the same milk sample (2, 10). Subsequent studies reported lower sensitivity and specificity (5). At issue has often been the selection and interpretation of the definitive test for IMI-SA which is used to evaluate ELISA-SA. Currently, dairy producers and consultants continue their efforts to decide how ELISA technology might best be used to identify cows with IMI-SA.

The sensitivity and specificity of a diagnostic test measure the degree to which a diagnostic procedure being evaluated, the ELISA-SA in this case, accurately depicts the disease state being investigated (IMI-SA). Any discrepancy between the absence or presence of the disease and the results of the diagnostic test is interpreted as a false positive or false negative, and diminishes the calculated sensitivity or specificity of the diagnostic test. Often a truly definitive determination of the disease state is not available. In such instances, epidemiologists calculate relative sensitivity and relative specificity by comparing the diagnostic test being evaluated with an accepted standard test (18). To the extent that the standard test is incorrect, the calculated values of relative sensitivity and relative specificity will be biased downwards from their correct values. In function, improving the accuracy of the standard test will remove this bias and increase the observed sensitivity and specificity of the diagnostic test being studied.

It has been suggested that one reason for frequent
disagreement between the ELISA-SA and microbial culture is that microbial cultures reflect the current infection status of the udder, whereas the ELISA-SA measures antibodies induced by IMI-SA and produced 1 or more weeks in the past; e.g., an ELISA-SA determination from milk obtained on 21 January may really be reflecting IMI-SA status on 1 January. If the underlying biological hypothesis is that antibody titers follow infection by 1 to 3 weeks, ELISA-SA results should best be compared with culture results of milk samples from 1 to 3 weeks in the past.

The purpose of this study was to evaluate the relative sensitivity and specificity of the ELISA-SA when compared to three different standard tests of IMI-SA: (i) microbiologic culture of the same milk sample tested by ELISA-SA, (ii) microbiologic culture of a milk sample obtained from the same cow 1 week previously, and (iii) microbiologic culture of a milk sample obtained from the same cow 3 weeks previously.

This objective was addressed by receiver-operator curve (ROC) analysis. Shortly after the invention of radar, it was learned that very sensitive radar reception would detect birds and other airborne entities which were not airplanes. If radar reception was too insensitive, aircraft would be missed. This trade-off between false-positive results (calling a bird an airplane) and false-negative results (failing to detect an incoming aircraft) were analyzed as ROC curves to optimize radar instrumentation and interpretation. Since this beginning, ROC have been widely used by epidemiologists studying the trade-off in sensitivity and specificity of different definitions of a positive diagnostic test. An ROC shows relative sensitivity (true-positive rate) on the vertical axis and 100 minus relative specificity (false-positive rate) on the horizontal axis (4, 16, 18, 19). Receiver-operator curves with greater curvature indicate a superior trade-off between sensitivity and specificity, and a greater concordance between the diagnostic test being evaluated and the definitive or standard test used for comparison.

MATERIALS AND METHODS

Four commercial herds with a history of S. aureus infection were selected for this study on the basis of their proximity to our clinic and their owners' willingness to participate in the study. Milk samples from each quarter of the udders of all lactating cows were aseptically collected at 3, 1, and 0 weeks before the scheduled cessation of lactation (dry-off). Milk samples were refrigerated and plated within 24 h. A 0.05-ml sample of milk from each quarter was streaked for isolation on an entire blood agar plate. Bacterial colony types were Gram stained and tested for catalase production (11). Identified staphylococci were further tested for coagulase production; coagulase-positive staphylococci were presumptively identified as S. aureus. A cow was considered culture positive for IMI-SA if at least one quarter was infected.

Only milk samples obtained at dry-off were submitted for ELISA-SA testing. Equal amounts of milk from quarter milk samples were mixed together to create a composite milk sample for each cow. Composite milk samples were frozen and mailed with dry ice to the Wisconsin Dairy Herd Improvement Cooperative (WDHIC Appleton Laboratory, Appleton, WI) for ELISA-SA testing. The ELISA results for each sample were recorded as the percentage of optical density (OD%) of a standard positive control which was provided by the manufacturer. By definition, the standard positive control had an OD% of 100. Optical density was read at 410 nm.

All data was entered in a spreadsheet (Excel, Microsoft Corp.) and transferred to a statistical program for analysis (SAS-PC, SAS Institute, Cary, NC). Four different definitions of a standard test for IMI-SA were evaluated:

1. S. aureus culture positive at dry-off (same sample as tested by ELISA-SA).
2. S. aureus culture positive 1 week prior to dry-off.
3. S. aureus culture positive 3 weeks prior to dry-off.
4. S. aureus culture positive at any of the above three times.

A range of 18 different cutoff values for defining a positive ELISA test were considered between an OD% of 20 (20% of the optical density of the standard positive control) and 1,000 (10 times the optical density of the positive control). At each OD% cutoff value, relative sensitivity and specificity were calculated for each of the four definitions of a S. aureus culture-positive result. These calculated values of sensitivity and specificity were then used to create receiver-operator curves for each of the four culture definitions of IMI-SA. Receiver-operator curves with greater curvature indicated greater concordance between the ELISA-SA result and the culture result and also indicated that the diagnostic test being evaluated (ELISA-SA) and the standard test (microbiologic culture) were more likely to be measuring the same biologic phenomena.

There is some evidence that the ELISA-SA is less accurate in cows with milk production of <13.6 kg/day (13). The biological explanation for this limitation is that antibody is concentrated when milk production is low. Recalculated ROC were generated after excluding cows with production of <13.6 kg/day at their last monthly production test. Using an OD% of 100 as a definition of an ELISA-SA positive result, cows producing <13.6 kg/day were compared by chi-square analysis with cows making ≥13.6 kg/day.

RESULTS

The ROC for the different microbiologic definitions of IMI-SA are shown in Figures 1 to 3 for the entire database of 209 cows. The ROC for culture at dry-off is plotted on each figure for purposes of comparison. The curves for cultures 1 and 3 weeks before dry-off show a greater curvature than that for culture at dry-off, indicating a superior trade-off in sensitivity versus specificity and a superior concordance of ELISA-SA results with IMI-SA determined at 1 and 3 weeks before ELISA-SA testing. This improved sensitivity/specificity trade-off is primarily realized in the high OD% section of the curve, above the OD% of 85 to 100 commonly used to define suspect or positive results. Less difference is seen in the lower OD% range of the curves.

Table 1 shows the sensitivity and specificity for each of the three culture definitions of IMI-SA. Results are shown for OD% of 85 and 100 as a definition of a positive test. Also shown are sensitivity and specificity measures calculated by including only those 140 cows with milk production >13.6 kg/day at their last monthly production test.

Additional ROC curves analogous to those shown in Figures 1 to 3 were developed after restriction to those 140 cows with production >13.6 kg/day. One of these curves (Fig. 4) shows that milk culture at 1 week before dry-off was more reflective of ELISA-SA at dry-off than was the milk culture at dry-off. Other ROC for the restricted database,
FIGURE 1. Receiver-operator curve for coagulase-positive Staphylococcus ELISA at dry-off compared with culture results at 0 and 1 week before dry-off (n = 209 cows).

FIGURE 2. Receiver-operator curve for coagulase-positive Staphylococcus ELISA at dry-off compared with culture results at 0, 1, or 3 weeks before dry-off (n = 209 cows).

FIGURE 3. Receiver-operator curve for coagulase-positive Staphylococcus ELISA at dry-off compared with culture results at dry-off and at 0, 1, or 3 weeks before dry-off (n = 209 cows).

FIGURE 4. Receiver-operator curve for coagulase-positive Staphylococcus ELISA at dry-off compared with culture results at 0 and 1 week before dry-off. Only cows with milk production greater than 13.6 kg/day are included (n = 140 cows).

TABLE 1. ELISA percent sensitivity (Se) and percent specificity (Sp) using four different standard culture definitions of S. aureus infection.

<table>
<thead>
<tr>
<th>Standard Culture Definition</th>
<th>All cows (n = 209)</th>
<th>Only cows (n = 140) making &gt;13.6 kg milk/day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>At OD% 85</td>
<td>At OD% 100</td>
</tr>
<tr>
<td></td>
<td>Se  Sp</td>
<td>Se  Sp</td>
</tr>
<tr>
<td>1. Culture positive at dry-off</td>
<td>94 43</td>
<td>97 46</td>
</tr>
<tr>
<td>2. Culture positive 1 wk before dry-off</td>
<td>95 42</td>
<td>97 45</td>
</tr>
<tr>
<td>3. Culture positive 3 wks before dry-off</td>
<td>96 43</td>
<td>93 44</td>
</tr>
<tr>
<td>4. Culture positive at any of the above three culture times</td>
<td>92 44</td>
<td>95 48</td>
</tr>
</tbody>
</table>
ELISA FOR STAPHYLOCOCCUS AUREUS

analogous to Figures 2 and 3, showed virtually no difference from the ROC curve for culture at dry-off.

Considering only culture-positive milk samples at dry-off, 85% (28/33) of cows producing >13.6 kg/day were positive (at OD% > 100) on ELISA-SA compared with 72% (13/18) for cows making under 13.6 kg/day (P = .28, by chi-square). When the microbiologic culture was performed at 1 and 3 weeks previously, comparable probabilities were P = .28 and P = .68, respectively. When considering only culture-negative milk samples at dry-off, 48% (51/107) of cows making over 13.6 kg/day were positive (at OD% > 100) on ELISA-SA compared with 61% (31/51) for cows making under 13.6 kg/day (P = .12, by chi-square). When the microbiologic culture was measured at 1 and 3 weeks previously, comparable probabilities were P = .11 and P = .40, respectively. These results indicate that restriction of testing to cows making 13.6 kg or more of milk did not significantly improve test accuracy, although there was some evidence that culture-negative cattle were more likely to test positive on ELISA-SA when milk production was low.

DISCUSSION

Sensitivity values of 86, 84, 59, 84, and 69% and specificity values of 100, 93, 97, 93, and 61% have been reported for the ELISA-SA test (3, 7, 8, 20). In the current study, measures of sensitivity and specificity were generally lower than these previous reports. Sensitivity and specificity may vary due to real differences in the test populations or because of dissimilar procedures and definitions of test results. For example, some analyses defined culture-positive cows on the basis of single milk samples; others used serial samples. Some studies used milk cultures of individual quarters, and others used composite milk samples of all 4 quarters. Different quantities of milk (.01, .025, or .1 ml) were cultured, and different OD were used to define positive ELISA results. Procedures also differed in exclusion of cows with low milk production, cows that had calved less than 30 days previously, or ELISA results yielding borderline optical densities. Prescreening of samples, with exclusion of borderline samples or preferential selection of samples based on prior results or herd prevalence might have been a factor in some studies (6). For example, Poutrel and Sarradin (12) calculated “agreement” with positive and negative samples on the basis of 32 cows previously selected for positive culture results. Selective inclusion of cows with firmly established infections or absence of infection would necessarily improve the calculated values of sensitivity and specificity. This was purposefully done in some studies, and may have occurred unwittingly in others. Differences in chronicity of infection among herds may also have affected results, as possibly did a broad range of management and nutritional differences.

The current study was conducted among cows at the very end of their lactation, which is a good time for dairy producers to make decisions regarding culling of cows chronically infected with S. aureus. Our study showed that sensitivity was relatively unaffected by low milk weights, but there was a suggestive trend that specificity was lower in cows making less than 13.6 kg of milk per day at their last monthly milk test. Decreased specificity in cows with low milk production is presumably due to concentration of antibodies in a low volume of milk. Because all cows in our study were tested at the end of their lactations, low milk production could have contributed to our observed low specificity. Actual milk production at the cessation of lactation would have been somewhat lower than our recorded values, because production was measured at the last available monthly herd production test, which was 1 to 30 days before ELISA-SA testing at the termination of the cow’s lactation.

Ideally, sensitivity should be determined by studying a heterogeneous sample of diseased cows representative of those cows for which the new diagnostic test is designed. Conversely, specificity should be determined by studying a broad range of cows representative of those animals without the disease (14). Too often, measures of sensitivity and specificity which appear on the test package inserts were calculated under very uniform and controlled laboratory conditions that do not challenge the diagnostic test with the diversity found in the general population. One previous researcher conducted a study entirely on Jersey cattle and had to exclude approximately half of the cows in the study because milk production was less than the 13.6 kg/day suggested by the manufacturer as possibly reducing test accuracy. This made the included cows unrepresentative with regard to milk production and other associated characteristics. Some researchers only studied cows with high SCC, poor milk production, or other indications of infection. For example, Hicks et al. (8) showed that the ELISA test had a sensitivity of 80 among high somatic cell count (SCC) milk samples, but only 75 among low SCC samples. Smith (18) describes work-up bias, review bias, and incorporation bias which may affect the determination of sensitivity and specificity when the status of the test and the status of the disease are not independently determined.

The current study is unique in that ELISA-SA results were always obtained at the cessation of lactation, approximately 60 days before anticipated calving. It is certainly possible that the population of cows in late lactation may have a different ELISA-SA sensitivity and specificity when compared with cows in other stages of lactation; this must be considered by anyone comparing our results with those of other studies. However, our study is of particular relevance to those dairy managers who use the ELISA-SA test in late lactation to help make decisions regarding dry-cow therapy and culling of cows chronically infected with S. aureus.

All epidemiological studies have either a stated or an implied induction period between the cause and the hypothesized effect (15). In the evaluation of the ELISA test for S. aureus, the “cause” is the infection and the hypothesized “effect” is the subsequent rise in antibody titer in the milk. Comparing culture results and ELISA-SA results from the same milk sample implies the biologic assumption that antibody response to infection is instantaneous. We believe that it is more appropriate to compare ELISA-SA results to culture results obtained from the same cow at least one to
several weeks previously. The underlying biological assumption with this comparison is that current antibody level reflects the infection status of several weeks earlier.

In the current study, the ROC curves show that a superior trade-off between sensitivity and specificity was observed when ELISA-SA results were compared to culture results obtained 1 or 3 weeks previously. This indicates that ELISA-SA results reflect culture status from 1 to 3 weeks previously to a greater extent than they reflect current culture status. The improvement in sensitivity and specificity, however, was not great, and does not alleviate concerns regarding the applicability of the ELISA-SA diagnostic test.

REFERENCES