Effect of Fat and Protein Content of Milk from Individual Cows on the Specificity Rates of Antibiotic Residue Screening Tests

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ABSTRACT

The effect of high concentrations of milk protein and milk fat on the specificity rates of several antibiotic residue screening tests was evaluated in this study. Milk was sampled from 60 Jersey and 30 Holstein cows at one milking and analyzed for \( \beta \)-lactam residues using four antibiotic residue screening tests. Cows selected were not treated with an antibiotic for at least 30 d prior to sample collection, and milk was visibly normal. Before milk collection, quarter foremilk was aseptically sampled for mastitis pathogen analysis. Milk subsamples were analyzed for fat and protein contents, and somatic cell counts (SCC). Ten Jersey and four Holstein cows were infected with one or more mastitis pathogens. Concentrations of milk fat (4.78 vs. 3.39%) and protein (3.81 vs. 3.00%) were greater for Jersey cows compared with Holstein cows. Milk SCC averaged 148,000/ml and did not differ by breed. The specificity rates were greater than 0.9 for three of the four screening tests. Across breeds, there was an increased probability of a false-positive outcome for the Penzyme test (Coulter Food Science, Milwaukee, WI), with increased milk protein content and decreased SCC. Increased milk fat content was associated with an increased probability of false-positive outcomes for the CITE Snap test (IDEXX Laboratories, Inc., Westbrook, ME). High concentrations of milk protein and milk fat can adversely affect antibiotic residue test performance, but the degree of the effect is dependent upon the analytical method of the screening test.

(Key words: antibiotic residue testing, milk, Jersey cows, Holstein cows)

Abbreviation key: MF = milk fat, MP = milk protein.

INTRODUCTION

Establishing management practices that reduce the risk of antibiotic residues in the milk supply is an essential component of human food safety and the production of high quality milk. Screening milk for violative levels of antibiotic residues can prevent adulterated milk from entering the human food supply. The FDA has established a list of acceptable \( \beta \)-lactam antibiotic residue screening tests for use with bulk tank and tanker truck milk to assist in the regulation of commingled milk (Smucker, 1996; USHHS, USFDA, 1989). While the use of these tests to screen commingled milk provides assurances for maintaining human food safety, testing bulk tank milk for antibiotic residues does not prevent bulk tank contamination on the farm.

The use of antibiotic residue screening tests and implementation of good management practices on dairy farms have been positively correlated with reductions in the occurrence of antibiotic residues in milk (McEwen et al., 1991). Recently, Sischo et al. (1997) reported that the use of antibiotic residue screening tests for evaluating individual cow’s milk was associated with a reduction in the risk of residue violations. In addition, the Milk and Dairy Beef Residue Prevention Protocol of the Dairy Quality Assurance Program recommends that milk from individual cows be tested for antibiotic residues following extra-label use of an antibiotic (Boeckman and Carlson, 1999). Testing milk from antibiotic-treated cows following an appropriate milk-withholding period allows the dairy producer to make informed decisions about milk withholding and reduces the risk of antibiotic contamination of commingled milk. Several screening tests used to evaluate commingled milk are also used on the farm or at the milk plant to screen milk from individual cows. Therefore, an evaluation of factors present in individual cow’s milk that may affect test performance is warranted.

Test performance varied when \( \beta \)-lactam antibiotic residue screening tests were evaluated with milk from individual cows (Andrew et al., 1997; Bishop et al., 1987; Cullor, 1992; Seymour et al., 1988; Sischo and Burns, 1993; Van Eenennaam et al., 1993). For example, false-positive rates varied from 0 to 83% across studies, depending on the quality of milk analyzed and the screening test evaluated (Andrew et al., 1997; Bishop et al., 1987; Norell et al., 1994; Seymour et al., 1988; Tyler et al., 1992). Increased concentrations of fat, protein, somatic cells, free fatty acids, and lactoferrin in milk have been associated with increased false-positive...
rates for several screening tests (Carlsson et al., 1989; Egan and Meaney, 1984; Norell et al., 1994; Van Eenennaam et al., 1993). Holstein cows have been the primary source for milk used to evaluate antibiotic residue screening tests. Because Jersey cows produce milk with higher concentrations of milk fat (MF) and milk protein (MP) compared with milk from Holstein cows, there may be an increased risk of false-positive outcomes when Jersey milk is screened for antibiotic residues. Therefore, the objective of this study was to evaluate the specificity rate of several antibiotic residue screening tests using milk containing wide variations in concentrations of MF and MP.

**MATERIALS AND METHODS**

**Animal Selection and Sampling Protocol**

Milk from 60 Jersey and 30 Holstein cows was sampled from five herds, the University of Connecticut herd and four commercial herds in Connecticut during each farm’s p.m. milking schedule. Cows were selected for individual milk collection if they had not been treated with an antibiotic for at least 30 d before sampling and were greater than 14 DIM. Only milk from cows that were free from visual signs of clinical mastitis were selected. Depending on the number of Jersey cows that met the criteria, 10 to 20 cows were sampled at each farm. Milk from at least three Holstein cows was concurrently collected and analyzed with the milk from Jersey cows for each sample collection day.

Two streams of foremilk from each quarter were evaluated for visual signs of clinical mastitis using a strip cup before milking preparation. The teats were prepared for milking following the farm’s general operating procedures. On three farms, teats were predipped with an iodine-based product and dried with paper towels. At two farms, teats were washed with water and dried with individual towels. Following teat preparation, a 5-ml foremilk sample was collected from each quarter using aseptic procedures and cooled to 5°C within 2 h of collection. After milking, a total composite milk sample (200 ml) was collected from DHIA meters for each cow. The milk sample was divided into two aliquots and cooled to 5°C within 2 h of collection.

**Mastitis Pathogen Analyses**

Quarter foremilk samples were analyzed for SCC and mastitis pathogens at the University of Connecticut Mastitis Laboratory to determine the IMI status of each cow sampled. Somatic cell counts were enumerated by direct microscopic leukocyte counting. Breed smears were prepared with milk films of 0.01 ml spread over 1 cm² of a slide. Fixed smears were stained with Newman-Lampert (methylene blue) stain. Cell counting was in accordance with counting methods described in Standard Methods for the Examination of Dairy Products (Marshall, 1992). A cow was considered infected if foremilk contained a leukocyte count of equal to or greater than 1 × 10⁶/ml. However, cows with quarter foremilk samples that were positive for Staphylococcus aureus were considered infected at 2 × 10⁵ leukocytes/ml and higher. For identification of pathogens, approximately 0.05 ml of sample was spread onto blood agar. Blood agar plates were incubated at 37°C for 18 h. Milk samples were then incubated at 37°C for 3.5 h. Approximately 0.01 ml of milk was inoculated onto blood agar and 0.01 ml onto mannitol sugar agar following the 3.5-h milk incubation period. These plates were also incubated at 37°C for 18 h. The bacteriological status of milk samples was described by diagnostic procedures recommended by the National Mastitis Council (1987).

**Milk Composition and Antibiotic Residue Analyses**

One of the two aliquots from the total composite milk was analyzed for MF and MP by infrared spectroscopy (DAIRY ONE, DHI Milk Testing Laboratory, Ithaca, NY). Within 24 h of milk sample collection, the subsequent cooled subsample was analyzed for antibiotics using four commercially available, β-lactam antibiotic residue screening tests (Table 1). These screening tests represent the range in analytical principles for the currently available tests. For each day of analysis, standard positive (Charm Sciences, Inc., Malden, MA; Penicillin G Standard Control, 0.008 IU/ml) and negative control samples (Charm Sciences, Inc., Malden, MA; Negative Control Milk) were analyzed along with the milk samples for all screening tests to verify test accuracy.

**Statistical Analyses**

A specificity rate (defined as the rate of truly negative samples that were found to be negative by the screening test) and a 95% confidence interval were calculated for each screening test within each breed (Andrew et al., 1997). The false positive rate was calculated as one minus the specificity rate for each screening test and for each breed. A specificity rate of 1.0 indicated that no false-positive outcomes were observed for the test. Using logistic regression (Cox and Snell, 1989; SAS, 1990), factors associated with the rate of false-positive outcomes were evaluated for each test that had more than one false-positive outcome. The model was specified as logit (p) = log(p/(1 – p)) = α + β = x, where α is the intercept parameter, β is the vector of the slope parameter, and x is the explanatory variable. Somatic
cell counts were converted to SCS. The explanatory variables were MF, MP, SCS, and pathogen status. The score statistic was used to evaluate the model. A statistically significant model was determined at \( P < 0.05 \). The effects of farm location and breed were not significant \( (P > 0.05) \).

**RESULTS AND DISCUSSION**

As expected, mean MF and MP concentrations were higher for milk from Jersey cows than from milk from Holstein cows (Table 2), and were typical of breed averages for these components (King and Wiggans, 1983). In contrast, the ranges in percentage MF and MP were similar across breeds. Somatic cell counts did not differ between the two breeds (Table 2). The average SCC for the milk tested was low at 148,000/ml and may not have been indicative of the average SCC present in milk across a wide range of farms. However, the SCC ranged from \( 9 \times 10^3 \) to \( 1142 \times 10^3 \)/ml across the breeds. This range in SCC was representative of the variation expected within and across farms (Miller et al., 1999).

Although milk sampled was free of visual signs of mastitis, 10 Jersey cows and four Holstein cows were infected with one or more mastitis pathogens (Table 3). The infection rate was 16.7 and 13.3% for Jersey and Holstein cows, respectively, and did not differ by breed. Three Jersey cows were infected with two organisms, and one Holstein cow was infected with four mastitis pathogens (\( S. aureus \), \( Streptococcus dysgalactiae \), \( Corynebacterium bovis \), and coliforms). There were four Jersey cows and one Holstein cow infected with \( S. aureus \), and each cow was infected in one quarter. Also, \( S. aureus \) was cultured in cows across three herds. Coliform infections were more prevalent in Jersey cows. Three quarters from Holstein cows were infected with \( C. bovis \), and there were no \( C. bovis \) infections in Jersey cows. The infection rates and pathogens isolated were consistent with the results of mastitis pathogen surveys (Wilson et al., 1999). There was no association \( (P > 0.10) \) between infection status and screening test outcome for all antibiotic screening tests evaluated. In a previous study, increased coliform count was associated with an increase in the false-positive rate for the Penzyme test (Andrew et al., 1997). Van Eenennaam et al. (1993) and Norell et al. (1994) reported an association between the incidence of clinical mastitis and screening test false-positive rates when visibly abnormal milk was sampled prior to antibiotic treatment; however, we did not determine whether the false-positive outcomes were related to bacterial infection or the host immune response. Although several cows were infected with mastitis pathogens in the present study, the cows did not exhibit clinical mastitis, and SCC were not markedly elevated (Table 2). This finding may explain the lack of an association between infection status and screening test false-positive rates. This relationship needs to be further elucidated because an infection can persist following a clinical cure, and

<table>
<thead>
<tr>
<th>Test kit</th>
<th>Manufacturer</th>
<th>Analytical principle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Delvotest SP</td>
<td>Gist Brocades Food Ingredients, Inc. (Menomonee Falls, WI)</td>
<td>Inhibition of bacterial growth</td>
</tr>
<tr>
<td>Penzyme Milk Test</td>
<td>Cultor Food Science (Milwaukee, WI)</td>
<td>Enzymatic</td>
</tr>
<tr>
<td>CITE Snap</td>
<td>IDEXX Laboratories, Inc. (Westbrook, ME)</td>
<td>Antibiotic-antigen capture system</td>
</tr>
<tr>
<td>Charm Cowside</td>
<td>Charm Sciences, Inc. (Malden, MA)</td>
<td>Displacement of competitive-binding penicillin</td>
</tr>
</tbody>
</table>

**Table 2.** Milk fat and protein contents and SCC for Jersey and Holstein cows that were sampled for the evaluation of antibiotic residue screening tests for milk from individual cows.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mean</th>
<th>SD</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jersey (n = 60)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Milk fat (%)</td>
<td>4.78</td>
<td>0.97</td>
<td>2.0</td>
<td>7.1</td>
</tr>
<tr>
<td>Milk protein (%)</td>
<td>3.81</td>
<td>0.36</td>
<td>3.1</td>
<td>4.63</td>
</tr>
<tr>
<td>SCC (( \times 10^3 )/ml)</td>
<td>150</td>
<td>206</td>
<td>10</td>
<td>1023</td>
</tr>
<tr>
<td>Holstein (n = 25)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Milk fat (%)</td>
<td>3.39</td>
<td>0.82</td>
<td>2.4</td>
<td>6.3</td>
</tr>
<tr>
<td>Milk protein (%)</td>
<td>3.00</td>
<td>0.49</td>
<td>2.5</td>
<td>4.8</td>
</tr>
<tr>
<td>SCC (( \times 10^3 )/ml)</td>
<td>147</td>
<td>256</td>
<td>9.0</td>
<td>1142</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Jersey</th>
<th>Holstein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of cows infected ( n )</td>
<td>60</td>
<td>30</td>
</tr>
<tr>
<td>All pathogens</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td>Total number of quarters infected ( n )</td>
<td>240</td>
<td>120</td>
</tr>
<tr>
<td>( Staphylococcus aureus )</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>( Streptococcus dysgalactiae )</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>Coliforms</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>( Corynebacterium bovis )</td>
<td>0</td>
<td>3</td>
</tr>
</tbody>
</table>

### Table 4. Specificity rates for antibiotic screening tests using milk from Jersey (n = 60) and Holstein cows (n = 30).

<table>
<thead>
<tr>
<th>Screening test</th>
<th>Number positive</th>
<th>Specificity rate</th>
<th>Lower CI&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Upper CI&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Number positive</th>
<th>Specificity rate</th>
<th>Lower CI&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Upper CI&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Delvotest SP&lt;sup&gt;3&lt;/sup&gt;</td>
<td>1</td>
<td>0.98</td>
<td>0.91</td>
<td>1.0</td>
<td>0</td>
<td>1.0</td>
<td>0.88</td>
<td>1.0</td>
</tr>
<tr>
<td>Penzyme&lt;sup&gt;4&lt;/sup&gt;</td>
<td>28</td>
<td>0.53</td>
<td>0.41</td>
<td>0.66</td>
<td>7</td>
<td>0.77</td>
<td>0.59</td>
<td>0.88</td>
</tr>
<tr>
<td>Charm Cowside&lt;sup&gt;5&lt;/sup&gt;</td>
<td>1</td>
<td>0.98</td>
<td>0.91</td>
<td>1.0</td>
<td>0</td>
<td>1.0</td>
<td>0.88</td>
<td>1.0</td>
</tr>
<tr>
<td>CITE Snap&lt;sup&gt;6&lt;/sup&gt;</td>
<td>4</td>
<td>0.93</td>
<td>0.84</td>
<td>0.97</td>
<td>0</td>
<td>1.0</td>
<td>0.88</td>
<td>1.0</td>
</tr>
</tbody>
</table>

<sup>1</sup>Lower 95% confidence interval.
<sup>2</sup>Upper 95% confidence interval.
<sup>3</sup>Gist Brocade Food Ingredients, Inc., Menomonee Falls, WI.
<sup>4</sup>Cultor Food Science, Milwaukee, WI.
<sup>5</sup>Charm Science, Malden, MA.
<sup>6</sup>IDEXX Laboratories, Westbrook, ME.

This may occur at a time when antibiotic residue tests would likely be used.

All positive control and negative control milk samples were identified correctly by the screening tests. For the CITE Snap screening test (IDEXX Laboratories, Inc., Westbrook, ME), there were four samples of milk from Jersey cows that failed to develop the diagnostic color spot. These test failures were recorded as positive test results for this study. Except for one screening test, the specificity rates were greater than 0.9 for each test across the two breeds (Table 4). These results are in agreement with previous studies evaluating screening tests for detecting violative levels of antibiotics in milk when visibly normal milk was tested (Andrew et al., 1997; Sechen et al., 1998; Sischo and Burns, 1993). However, for all screening tests evaluated, the specificity rates were numerically lower when milk from Jersey cows was tested compared with testing milk from Holstein cows. This suggests that screening milk that contains high concentrations of protein and fat may increase the rate of false-positive outcomes for specific antibiotic residue detection screening tests. The FDA has established an acceptable specificity rate of 0.9 or greater with a 95% confidence interval for screening tests used for commingled milk (Smucker, 1996). The specificity rates for the P enzyme test were 0.53 and 0.77 for the Jersey and Holstein milk, respectively. Except for the P enzyme test, the antibiotic residue screening tests used with individual cow’s milk met the FDA requirements for testing commingled milk.

The effect of concentration of milk components on the probability of a false-positive outcome for each test was calculated with all of the data across breeds to provide a wide range in SCS, MF, and MP. Milk subsamples collected from five Holstein cows were not properly preserved and were not analyzed for MF, MP, and SCC. Therefore, the logistic regression analysis included 25 Holstein and 60 Jersey cows. An increase in MP was associated with an increased probability of a false-positive outcome for the P enzyme test (Figure 1). The logistic regression model was $\logit(p) = -2.29 + 0.576$ MP and was significant ($P = 0.03$). This is in agreement with a previous study evaluating the effect of MP and SCS on test performance for the P enzyme test using milk from Holstein cows (Andrew et al., 1997). The P enzyme test is an enzymatic assay, and it is possible that MP may interfere with the assay, particularly if there is a cross reactivity of MP for active sites on the enzyme. In addition, milk proteins derived from serum, including lactoferrin and lysozyme, are elevated in milk from cows with an IMI (Kitchen, 1981). Lactoferrin and lysozyme have antimicrobial properties that may affect the performance of antibiotic residue screening tests that are based on bacterial inhibition (Carlsson et al., 1989). In the present study, there was only one false-positive outcome for the Delvotest-SP (Gist Brocades...
Figure 2. Predicted probabilities (–) of false-positive outcomes and upper and lower 95% CI (– – –) for the Penzyme test as somatic cell score increased in milk from 60 Jersey and 25 Holstein cows. Bars = number of observed positive responses.

Food Ingredients, Inc., Menomonee Falls, WI), which is a microbial inhibition assay. The lack of effect of MP on the bacterial inhibition test may be because the milk samples collected were from cows with visibly normal milk and contained lower concentrations of these proteins than would be expected in milk from cows recovering from mastitis.

A reduction in SCS was associated with increased false-positive outcomes for the Penzyme test (Figure 2). The parameter estimates are \( \logit(p) = 0.371 - 0.241 \) SCS, \( P = 0.008 \). The logistic regression analysis does not establish a direct relationship; therefore, these results may be indicative of another component in milk that was not analyzed but varies in concentration relative to SCC. In contrast, several studies have demonstrated that milk with elevated SCC due to a bacterial infection (Norell et al., 1994; VanEenennaam et al., 1993) or endotoxin challenge (Sechen et al., 1998; Tyler et al., 1992) was associated with increased false-positive rates across a variety of screening assays. Also, milk from cows recovering from mastitis can contain high concentrations of SCC for an extended period following a bacteriological cure (Kitchen, 1981; Paape et al., 1979). Also, SCC can be elevated throughout a lactation if the IMI is not eliminated (Kitchen, 1981). It is unknown if the effect of SCC on test performance is directly due to SCC or is associated with a particular pathogen, or other component that may be elevated in milk from mastitic cows. The variability in the quality of milk sampled contributes to the inconsistent test results when screening tests are used on farm to test milk from cows recovering from mastitis. One way to improve test performance is to screen milk that is visibly normal from cows recovering from mastitis (Andrew et al., 1997; Norell et al., 1994; Sischo and Burns, 1993).

For the CITE Snap test, increased MF content was associated with an increased probability of a false-positive outcome and is described as \( \logit(p) = -5.18 + 0.697 \) MF \( P = 0.02 \). Three of the four false-positive outcomes were a failure of the test and were observed for milk from Jersey cows that contained greater than 5.0% MF. In the CITE Snap assay format, milk moves along a gradient and is mixed with the test analytes. Milk with high fat content may hinder the movement of milk along the gradient and result in a lack of chemical reaction. In addition, FFA have antimicrobial activity and may interfere with screening tests that are based on bacterial inhibition (Egan and Meaney, 1984). This may occur if milk fat is degraded during storage. In the present study, milk samples were cooled within 2 h of collection, which should have limited the extent of lipolysis and improved test performance, particularly for the Delvotest-SP microbial inhibition test. Sample storage and temperature control may be important factors in test performance. Rapid cooling of milk reduces lipolysis and the risk of a false-positive outcome due to potential FFA inhibition.

The screening tests evaluated represent a wide range in analytical principles. The results indicate that, except for one screening test, the antibiotic screening tests evaluated in this study can be used to detect residues without significant losses of saleable milk due to false-positive outcomes for milk from both Holstein and Jersey cows. The sensitivity of the screening tests should be evaluated under similar conditions to determine if there are breed differences in the ability to detect violative concentrations of antibiotics in milk. Sampling visibly normal milk and prompt cooling of milk samples prior to analysis resulted in acceptable specificity rates of greater than 0.9 for three of the four screening tests evaluated. Only two tests were negatively affected by high concentrations of MF and MP that can be expected in milk from Jersey cows.

**CONCLUSION**

Although the screening test performance was greater than 0.9 for all tests except one, there was a trend toward increased false-positive outcomes when the screening tests were used to evaluate milk from Jersey cows compared with testing milk from Holstein cows. Furthermore, increased MP and decreased SCS were associated with an increase in false-positive outcomes for the Penzyme screening test, and increasing MF was associated with an increase in the probability of a false-positive outcome for the CITE Snap test. Based on the results from this study, the Charm Cowside, CITE Snap
test, and Delvotest-SP antibiotic residue screening tests can be used to evaluate the residue status of milk from Jersey cows that may contain high levels of MF and MP. Further work is needed to determine the effects of milk composition on the ability of the tests to detect violative concentrations of antibiotics in milk from Jersey cows.

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