False-Positive Outcome and Drug Residue in Milk Samples Over Withdrawal Times

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ABSTRACT

This study was conducted to identify false-positive outcomes and drug residues in milk samples over withdrawal times and to determine whether the positive results were caused by drug residues or natural inhibitors. A total of 73 milk samples over withdrawal times after the last intramammary infusion were collected from each treated quarter of cows and tested using the Delvotest SP assay. Reading time was 150, 165, and 180 min, and results of samples were recorded according to the color of the well containing the control milk sample. There were 24, 20, and 12 positive samples at the reading times of 150, 165, and 180 min, respectively. All 24 positive milk samples were heated at 82°C for 5 min and retested to verify that the positive results were caused by drug residues or natural inhibitors. Twenty-one samples that exhibited positive results were negative after heat treatment, and drug residues were not identified by LacTek and Charm tests. However, 3 samples that exhibited positive results from heat treatment of 82°C were positive for drugs. In our study, most positive results (89%) in the milk samples over withdrawal times were false-positive results by natural inhibitors. Moreover, the heat treatment is a fast, simple, and inexpensive method to remove false-positive results and has no effect on positive samples containing drugs. We suggest that heat treatment before screening tests is an effective way to reduce false-positive results in the milk samples.

(Key words: Delvotest SP assay, heat treatment, withdrawal time, natural inhibitor)

INTRODUCTION

Because the presence of drug residues in milk is undesirable, a drug residue-free milk supply is an important

issue in the dairy industry. The test for drug residues in milk is performed by several methods, such as microbial growth inhibition assays, microbial receptor assays, receptor binding assays, immunologic assays, enzymatic assays, and chromatographic analysis (Mitchell et al., 1998). The Delvotest assay, one of the microbial growth inhibition assays, is a simple, sensitive, and broadly drug-detecting test system. This assay is based on the rapid growth and acid production of the test organism, *Bacillus stearothermophilus* var. *calidolactis* (Katz, 1982; Kelley, 1982). Several studies have reported that false-positive results occurred on samples containing no drug in the test using the Delvotest assay. These false-positive results are high in the milk sample taken from individual cows having mastitis or colostrums (Oliver et al., 1984; Cullor et al., 1992; 1994; Tyler et al., 1992; Sisco and Bruns, 1993; Van Eenennaam et al., 1993; Andrew, 2001; Gibbons-Burgener et al., 2001). Tyler et al. (1992) found that the percentage of false-positive results from the Delvotest P assay was 45% in the milk samples taken from cattle with experimental endotoxin-induced mastitis. Van Eenennaam et al. (1993) reported, in the case of cattle with naturally occurring clinical mastitis, that 37.7% false-positive results from the Delvotest P assay were identified. However, a false-positive result is low in bulk tank and tank-lorry samples because false-positive samples can be diluted in the larger volume of a bulk tank or tank-lorry (Kang and Kondo, 2001). Moreover, Halbert et al. (1996) reported that false-positive results of the Delvotest assay in the milk samples taken from cows having no clinical mastitis was very low.

High levels of natural inhibitors are present in mastitic milk and in colostrums, and they can cause false-positive results in the microbial growth inhibition assays (Kosikowski and O'Leary, 1963; Harmon et al., 1975; 1976; Nickerson, 1985; Carlsson and Björch, 1987; Carlsson et al., 1989; Hillerton et al., 1999). Carlsson et al. (1989) found that a false-positive result in the Delvotest assay correlated with an increase in lactoferrin and lysozyme concentrations. Not only natural inhibitors, but incubator type (Suhren and Beukers, 1999), component of milk (Andrew, 2000; 2001), and

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method of sample collection (Andrew et al., 1997) have an influence on the result of the Delvotest assay. In a previous study, we identified the importance of reading time in the Delvotest assay. The occurrence of a false-positive result was high when the plate was cultured for 150 min rather than 165 and 180 min (Kang and Kondo, 2001).

Drugs are widely used in treatment of various bacterial infections in dairy cattle, including mastitis. The recommended withholding period following treatment should be followed to avoid drug residue in milk. However, sometimes drugs can be contained in milk over withdrawal times, resulting in positive test results for drug residues (Mercer et al., 1970; Allison, 1985; Booth and Harding, 1986; Seymour et al., 1988a; 1988b; Oliver et al., 1990; McEwen et al., 1992). Moreover, natural inhibitors in the milk of cows with mastitis are increased and kept at high concentrations for several days (Harmon et al., 1975; 1976; Nickerson, 1985). The increased natural inhibitors can lead to false-positive results in the use of bioassays based on bacterial growth inhibition, inclusive of the Delvotest assay, on the milk samples over withdrawal times. Therefore, it is important to evaluate that the positive results in milk over withdrawal times are caused by drug residues or natural inhibitors.

This study was performed to identify false-positive outcomes and drug residues in the milk samples over withdrawal times. Our study also evaluated that the positive results were due to drug residues or natural inhibitors.

MATERIALS AND METHODS

Experimental Cows and Sample Collection

Among lactating Holstein cows with clinical mastitis, 64 cows treated by intramammary infusion were used and cows treated by multiple routes of administration (e.g., intramammary plus intramuscular or intravenous administration) with more than one drug were excluded. The treatment of cows was carried out by the farmer or veterinarian after sampling the affected quarter and after milking. Before administering drug to quarters, teat ends were thoroughly disinfected with a pledget moistened with 70% alcohol for a few seconds. The affected quarters of cows were infused with the contents of syringe per quarter according to the manufacturer's recommendation. The types and withdrawal times of drugs used in this study were based on the drug label instructions and are presented in Table 1. A total of 73 foremilk samples over withdrawal times were collected from treated quarters of cows after the last drug treatment. The collected milk samples were

Table 1. Types and withdrawal times of drugs used in this study.

Types	No. used in treatment	Withdrawal times (d)
Beta-lactams	48	2-5
Tetracyclines	15	3
Sulfonamides	8	2-3
Aminoglycosides	2	3

immediately kept at $<5^{\circ}\text{C}$ (no freezing) and examined within 24 h.

Test of Samples Using the Delvotest SP Assay

A multiplate-type Delvotest SP (Royal Gist-brocades NV, Delft, The Netherlands) was prepared for the examination of samples. A nutrient tablet and 0.1 mL of samples were added to each test well. The plates were incubated in a water bath with a controlled temperature of $64.0 \pm 0.5^{\circ}\text{C}$. Plate results were read at 150, 165, and 180 min. According to comparisons with the color of the well containing the control milk sample, results of each sample were recorded by 4 types: A, purple throughout whole agar; B, slightly more yellow than the control sample; C, moderately more yellow than the control sample; and D, yellow throughout whole agar or the same as the color of the control sample. The UHT market milk was used as the negative control, and it was subjected to the Delvotest SP assay, the Charm test (Charm Science Inc., Malden, MA), and the LacTek test (Idxx Laboratories, Inc., Westbrook, ME) before use as the control sample.

Heating Test for Confirmation of the Presence of Inhibitors

In a previous study (Kang and Kondo, 2001), the method of heat treatment at 82°C for 5 min reported by Kosikowski and O'Leary (1963), and heat treatment at 90 and 100°C for 5 min were used to confirm the presence of natural inhibitors. All methods of heat treatment were effective on the removal of natural inhibitors and had no influence on positive samples. In this study, therefore, heat treatment at 82°C for 5 min was used to confirm the presence of natural inhibitors. After heating at 82°C for 5 min, milk samples were rapidly cooled to 20°C with cold water.

Confirmation of Drugs in Samples and SCC

Charm test and LacTek test were used to confirm the presence of drugs in samples that exhibited positive results (color-type results A to C). Both tests were performed according to the recommendations of the manufacturer.

Table 2. Results of the Delvotest SP assay on the milk samples over withdrawal times.

Reading time (h)	Color results ¹	Before heat treatment	After heat treatment ²
2.50	A	3	3
	B	10	0
	C	11	0
	D	49	70
2.75	A	3	3
	B	4	0
	C	13	0
	D	53	70
3.00	A	2	2
	B	1	1
	C	9	0
	D	61	70

¹A, purple throughout whole agar; B, slightly more yellow than the control sample; C, moderately more yellow than the control sample; and D, yellow throughout whole agar or the same as the color of the control sample.

²The heat treatment was performed at 82°C for 5 min.

The SCC was detected using a Somacount 300 system (Bentley Instruments, Inc., Chaska, MN). Tests were done according to the manufacturer's recommendations.

Statistical Analysis

Somatic cell count results of negative, false-positive, and positive samples are expressed as the mean and standard deviation. All SCC determinations were done in duplicate. Statistical analysis comprised significance testing of the difference between means using a Student's *t*-test at the level of $P < 0.05$.

RESULTS

In the present study, the majority of drugs used for the treatment of mastitis were beta-lactams, penicillins, and cephalosporins. The recommended withdrawal times of drugs ranged from 2 to 5 d (Table 1). Moreover, the color results of the Delvotest SP assay were recorded as 4 types (A, B, C, and D) (Table 2). Hillerton et al. (1999) reported that a partial purple color result (mainly yellow with some purple), which is similar to a color-type C of our study was shown at 8 µg/kg of cephalonium. On this basis, because drugs can be contained in the color-type C, the results of color-type A to C in our study are considered positive results.

Seventy-three milk samples beyond withdrawal times were collected from cows treated by intramammary infusions and tested using the Delvotest SP assay. Before heat treatment at 82°C for 5 min, the number of samples exhibiting positive results (color types A to C) was 24, 20, and 12 at the reading times of 150, 165,

and 180 min, respectively. All 24 positive milk samples were heated at 82°C for 5 min and retested to determine whether the positive results were caused by drug residues or natural inhibitors. Twenty-one milk samples that exhibited color types B and C at a reading time of 150 min were color-type D following heat treatment at 82°C for 5 min. But, 3 samples continued to show positive results after heat treatment at 82°C for 5 min. In LacTek and Charm tests to confirm the presence of drugs, the 3 milk samples that exhibited color-type A and B from heat treatments of 82°C were positive for drug (2 samples in beta-lactams and 1 sample in tetracyclines). But, drugs were not identified in 21 milk samples exhibiting color-type D following heat treatment (Table 2). Therefore, we concluded that the positive results of 21 samples were caused by natural inhibitors contained in milk samples.

In the case of reading time, the occurrence of false-positive results was high when the plate was cultured for 150 min (21 samples) rather than 165 (17 samples) and 180 min (9 samples). These results show that reading time has an important influence on the result of the Delvotest SP assay (Table 2).

After treatment, the SCC of negative, false-positive, and positive samples averaged 2.447, 5.368, and 4.752×10^3 cells/mL, respectively. The SCC in false-positive and positive samples was higher compared with that in negative samples ($P < 0.05$) (Table 3).

DISCUSSION

Intramammary infusion products containing beta-lactams are widely used for the treatment of cows with mastitis (Mitchell et al., 1998). In the present study, beta-lactams showed the highest use frequency for the treatment of mastitis. Moreover, because intramammary infusion can deliver high concentrations of drug directly into the mammary gland, this method was frequently used for the treatment of mastitis. However, the frequent use of intramammary infusions was associated with increased risk of drug residues in milk (Allison, 1985; Booth and Harding, 1986; McEwen et al., 1991; Mitchell et al., 1998). Allison (1985) reported that 92% of drug residues in milk were caused by the use of intramammary infusions.

The SCC was reduced in cured quarters with drugs, but uncured quarters resulted in little change in SCC (Timms and Schultz, 1984; Seymour et al., 1989; Greene et al., 1991). Milk samples collected from the uncured quarters can cause higher false-positive outcomes because of high levels of natural inhibitors, compared with those of the cured quarters. Cullor et al. (1994) reported that the rates of false-positive outcome for the Delvotest assay were 3.7 and 11.1% in the milk samples with low

Table 3. Somatic cell count of negative, false-positive, and positive samples.

Samples	Somatic cell count ($\times 1000$ cells/mL) ¹	
	Before treatment	After treatment
Negative samples ² (n = 49)	4.932 $\pm$ 3.157	2.447 $\pm$ 1.907
False-positive samples ³ (n = 21)	5.821 $\pm$ 3.587	5.368 $\pm$ 2.246
Positive samples ⁴ (n = 3)	6.049 $\pm$ 4.313	4.752 $\pm$ 1.074

¹Average $\pm$ SD.²Samples exhibited color-type D before heat treatment at 82°C for 5 min.³Samples exhibited color-type D after heat treatment at 82°C for 5 min.⁴Samples exhibited positive results in Charm and LacTek tests.

SCC ($<600 \times 10^3$ cells/mL) and high SCC ($>600 \times 10^3$ cells/mL) obtained at 21 d after the first drug treatment, respectively. In addition, Van Eenennaam et al. (1993) found that the milk with positive results for the Delvotest assay (average 5.616×10^3 cells/mL at 9 or 11 milking and 5.211×10^3 cells/mL at 21 d after drug treatment) had a significantly higher SCC than the milk with negative results (average 1.891×10^3 cells/mL at 9 or 11 milking and 1.781×10^3 cells/mL at 21 d after drug treatment). Moreover, the fact that high SCC was associated with a rate of false-positive outcome for the Delvotest assay has been identified in several studies (Carlsson et al., 1989; Sischo and Bruns, 1993; Cullor et al., 1994; Kang and Kondo, 2001). Our study also identified a similar result that the SCC in false-positive samples was higher than that in negative samples (Table 3). However, Hillerton et al. (1999) suggested that there is no obvious correlation between SCC in individual quarter milk from midlactation cows and false positive of Delvotest assay by natural inhibitors.

The persistence of drug residues in milk beyond recommended withdrawal times is related to several factors, such as treatment with more than one drug by multiple routes (Oliver et al., 1990), milk production of cow (Mercer et al., 1970; Booth and Harding, 1986), type of vehicle used in drug formulation (Mercer et al., 1970), and extended dosage or excessive dosage of drugs (McEwen et al., 1992). Mercer et al. (1970) also reported that persistently high SCC was related with the prolonged excretion of drug. In our study, the prolonged excretion was identified in 3 samples, which were positive by LacTek and Charm tests. However, the prolonged excretion rates (4%) of our study were lower than those (17 to 21%) of previous studies using milk samples taken from cows treated by several methods such as intrauterine, intramuscular, and intramammary administration, exclusive of cows treated with more than one drug by multiple routes (Seymour et al., 1988a; 1988b; Oliver et al., 1990). These differences may be due to false-positive results that can occur in the test using the microbial growth inhibition assays, but previous studies (Seymour et al., 1988a; 1988b; Oli-

ver et al., 1990) did not investigate whether the positive results were caused by drug residues or false positive results.

On the other hand, Gibbons-Burgener et al. (2001) reported that the Delvotest assay might not be useful for the detection of drug residues in individual milk samples from cows treated for mastitis because of the low positive reliability. The Delvotest assay may be applicable to individual milk samples, but in North America, it is labeled for use on bulk tank, or other commingled milk samples rather than individual cow milk samples.

The method of heat treatment to inactivate natural inhibitors can be used to prove a false-positive result in the microbial growth inhibition assays. Kosikowski and O'Leary (1963) determined that heat treatment of milk at 82°C for 5 min removed all natural inhibitor activity from 11 milk samples that exhibited false-positive results on disc assay, resulting in negative readings when retested. Oliver et al. (1984) found that 4 samples from cows exposed to dry cow therapy were positive by the Delvotest P assay, but only one was confirmed positive by disc assay after heat treatment. The heat treatment eliminated false-positive results, but had no effect on positive results (Kang and Kondo, 2001). In our study, 21 samples exhibiting false-positive results were negative when retested following heat treatment at 82°C for 5 min. But, 3 samples containing drugs were positive after heat treatment at 82°C for 5 min. Our study revealed that the positive results in the milk samples were often caused by false-positive results when drug residues were identified by the Delvotest SP assay, one of the microbial growth inhibition assays.

Moreover, the test-retest system that the same test was repeated on samples with positive results resulted in a 9% decrease in true positives and in an 81% decrease of in false positives (Cullor, 1995). This system has been recommended as a method to decrease false-positive results, but requires a lot of time and can be expensive. On the contrary, testing milk samples after heat treatment is a fast, simple, and inexpensive method. Therefore, we suggest that heat treatment be-

fore screening tests is an effective means to reduce false-positive results in the milk samples.

CONCLUSIONS

Seventy-three milk samples over withdrawal times after the last intramammary infusion were prepared from treated quarters of cows and tested using the Delvotest SP assay. There were 24, 20, and 12 positive samples at the reading times of 150, 165, and 180 h, respectively. All 24 positive milk samples were heated at 82°C for 5 min and retested to determine if the positive results were caused by drug residues or natural inhibitors. The fact that 21 samples were false-positive results and 3 samples were true positive results was identified after a heat treatment of 82°C for 5 min. These results suggest that the positive results in the milk samples over withdrawal times were often caused by false-positive results, and that heat treatment before screening tests is an effective means to reduce false-positive results in the milk samples.

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