

## Freezing and thawing milk samples before culture to improve diagnosis of bovine staphylococcal mastitis

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### ABSTRACT

Diagnosis of staphylococcal mastitis cases may sometimes prove problematic due to the nature of the bacterium. Thus, in the present study, the effect of freezing and thawing bovine milk samples on the performance of standard cultures of staphylococcal mastitis was investigated to improve the diagnosis. Each of a total of 228 quarter milk samples from clinical and subclinical mastitis cases was plated on 7 % sheep blood agar directly, and after first being subjected to a process of freezing and thawing. The culture results from two methods were compared on the basis of *Staphylococcus* spp. positive udder counts and alteration in colony counts of the strains. In the first method, *Staphylococcus* spp. were isolated from 91 milk samples. In the second method, *Staphylococcus* spp. were isolated from an additional 11 milk samples that were negative in the first method. *Staphylococcus* spp. positive udder counts were found to be significantly different between the two methods. The changes in colony counts of the strains between the two methods were also found to be statistically significant in both clinical and subclinical mastitis cases, with the second method performing better. Consequently, these results indicate that a simple preculture step consisting of freezing and thawing milk samples has advantages for more sensitive diagnosis of staphylococcal mastitis in cattle milk.

**Key words:** cattle, freezing, mastitis, *Staphylococcus* spp.

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### Introduction

*Staphylococcus* spp. is one of the most prevalent etiological agents responsible for mastitis in cattle (McDONALDS, 1979; TENHAGEN et al., 2006; OLDE RIEKERINK et al., 2008). The mastitis cases caused by *S. aureus*, known as the most common pathogenic species, occur in both clinical (gangrenous) and subclinical forms, often progress to

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chronic form and respond poorly to antibiotic therapy (FOX and GAY, 1993). Therefore, accurate diagnosis and early initiation of therapy in staphylococcal mastitis cases are important, since the infection affects largely the continuity of profit in dairy enterprises (SEEGERS et al., 2003).

In routine diagnostic laboratories, the diagnosis of mastitis is performed by standard bacteriological culture methods. To increase the sensitivity of the culture methods, various applications have been included in standard culture methods in several studies, but controversial results have been obtained. Among them, increased volume of inoculum, pre-milking and post-milking sampling, longer incubation time, centrifugation of milk samples before culturing, freezing milk samples at -20 °C for different time periods, pre-enrichment of the samples before culturing have been the most tested applications (DINSMORE et al., 1992; THURMOND et al., 1989; SCHUKKEN et al., 1989; ZECCONI et al., 1997; GODDEN et al., 2002; SOL et al., 2002; HUBACKOVA and RYSANEK, 2007).

In this study, the effect of freezing cow milk samples at -20 °C for 24 hours and thawing at 37 °C on the diagnosis of staphylococcal mastitis cases was investigated.

#### **Materials and methods**

*Study area.* This study was conducted on the dairy cattle population in the Burdur province located in southwest Turkey. In the region, dairy cattle farms are concentrated and the region is one of the top-ranked places for cattle milk production in Turkey. According to Provincial Directorate of Agriculture in Burdur, the region had a total of 157 000 cattle in 2011.

The Burdur region is the crossing point of the Aegean, Central Anatolia and Mediterranean parts of Turkey. Burdur territories are located between 36-53 and 37-50 north latitude and 29-24 and 30-53 longitude. The area is 6 883 km<sup>2</sup> and mean altitude is approximately 1 000 m above sea level.

*Sampling.* By purposive sampling, a total of 228 milk samples, 99 of which were from clinical mastitis cases and 129 of which were from subclinical mastitis cases, were collected aseptically from 75 Holstein breed lactating cows on 36 farms. The clinically infected udders were diagnosed by clinical examination and subclinical infected udders were determined by the California Mastitis Test (CMT). Before taking the samples, the teats were sprayed with 70 % ethanol, the first few squirts of milk were discarded and approximately 5 mL milk samples were collected in sterile tubes. Then, the samples were transferred to the laboratory in a cooler.

*Laboratory diagnosis.* Within two hours of collection, 50 µL from each milk sample were spread onto 7 % sheep blood agar (Oxoid, England) plates after homogenization by vortexing. The plates were incubated at 37 °C in ambient air for 24 hours (Direct Inoculation Method). The remaining samples were frozen at -20 °C for 24 hours, thawed

at 37 °C, homogenized by vortexing and immediately 50 µL from each samples were cultured in the same manner as described above (Post-freezing Inoculation Method). The two culture plates were examined visually for presumptive *Staphylococcus* spp. colonies; suspect colonies with the same appearance in both plates for a sample were recorded as the same isolates, and the colonies were counted and recorded. The new isolates appearing in the post-freezing inoculation were also counted and recorded. Then, the colonies were subcultured to obtain pure culture of the presumptive *Staphylococcus* spp. strains and confirmation was performed by Gram staining, catalase, oxidase, oxidation and fermentation with glucose, and coagulase tests with lyophilised rabbit plasma (Merck) (clumping factor and tube agglutination tests) (WINN et al., 2006).

*Statistical analysis.* A McNemar Test was used to determine if the difference in the number of *Staphylococcus* spp. positive udders diagnosed by the direct inoculation method and the post-freezing inoculation method was significant. In addition, the colony forming unit (cfu/mL) counts was statistically evaluated using the paired T-test between the two methods for clinical and subclinical cases separately, after logarithmic transformation ( $\log_{10}$ ) of cfu numbers (Minitab, Release 15). The level of significance was accepted as 0.01 in both statistical evaluations of the results in this study.

## Results

In the direct inoculation method, 91 udders (91/228, 39.91 %) were found to be infected by *Staphylococcus* spp. In the post-freezing inoculation method, a total of 101 milk samples (101/228, 44.29 %) were positive for *Staphylococcus* spp. Among the total positive milk samples, 11 of them yielded *Staphylococcus* spp. growth in the post-freezing inoculation method only, whereas there was only one milk sample that was determined as positive in the direct inoculation method, but that yielded no growth in the post-freezing method (Table 1). Statistically, the difference in the diagnosis rates of staphylococcal mastitis cases between the two methods was found to be significant ( $P = 0.006$ , McNemar test).

When the mean cfu/mL counts of 46 strains from clinical cases and 44 strains from subclinical cases (Table 2) were compared between the two methods, the differences were found to be statistically significant for both clinical and subclinical cases ( $P < 0.01$ , paired *t*-test).

Table 1. Comparison of the culture results of the milk samples (n = 228) inoculated by two methods

		Post-freezing inoculation method		
		<i>Staphylococcus</i> spp. positive milk samples (n)	<i>Staphylococcus</i> spp. negative milk samples (n)	Total (n)
Direct Inoculation Method	<i>Staphylococcus</i> spp. positive milk samples (n)	90	1	91
	<i>Staphylococcus</i> spp. negative milk samples (n)	11	126	137
	Total (n)	101	127	228

Table 2. Comparison of cfu/mL counts ( $\log_{10}$  cfu/mL) of *Staphylococcus* spp. strains from milk samples between two methods

Mastitis type	<i>Staphylococcus</i> spp. strains (n = 90)	Direct Inoculation - cfu/mL (Mean $\pm$ SE)	Post-freezing inoculation - cfu/mL (Mean $\pm$ SE)	Significance
Clinical	46	2.6185 $\pm$ 0.0854	2.7570 $\pm$ 0.0837	P<0.01
Subclinical	44	2.3928 $\pm$ 0.0640	2.6226 $\pm$ 0.0838	P<0.01

## Discussion

Even though bacteriological culture is accepted as the gold standard for mastitis diagnosis, some staphylococcal cases may be diagnosed as false-negative due to intermittent shedding of *Staphylococcus* spp. at low numbers in milk (VILLANUEVA et al., 1991). Until recently, to increase the sensitivity of bacteriological culture, one of the methods supplemented to standard cultures has been the freezing of mastitic milk samples for a period of time and then thawing them immediately before performing bacteriological culture. However, researchers have so far reported controversial results. According to GODDEN et al. (2002), the advantageous effect of pre-freezing milk samples before culturing was linked to the provision of a significant increase in mean cfu/mL for *S. aureus*. GODDEN et al. (2002) asserted that freezing and thawing disrupts the macrophages and neutrophils and this leads to the release of phagocytosed *S. aureus* cells. They also remarked that freezing and thawing disrupts bacterial clusters in the milk. VILLANUEVA et al. (1991) also showed a 1.48 times increase in the isolation percentage of *S. aureus* after freezing milk samples for 23 days. On the other hand, SCHUKKEN et al. (1989) showed that pre-freezing of milk samples for 4, 8 and 16 weeks increased the percentage of the

milk samples found positive for coagulase negative staphylococci, but they did not find any advantageous effect of pre-freezing milk samples for diagnosis of mastitis cases caused by *S. aureus*. Conversely, BEXIGA et al. (2011) stated that freezing milk samples at -20 °C for 24 hours was not helpful in diagnosing mastitis since they indicated that the number of Gram-positive cocci decreased in the milk samples after the pre-freezing step. Likewise, HUBACKOVA and RYSANEK (2007) reported only a slight increase in the *S. aureus* count in the milk after freezing the samples for 3, 7 and 21 days. In our study, 59 of 90 (65.55 %) strains showed an increase in colony counts following the post-freezing inoculation method. When the mean cfu/mL of the strains was compared between the two methods, post-freezing inoculation increased the mean cfu/mL of the strains in both clinical and subclinical cases, and both increases were statistically significant. Thus, our results support the studies suggesting that freezing and thawing milk samples disrupts the bacterial clusters and also the macrophages and neutrophils in milk, and consequently cause an increase in cfu/mL of *Staphylococcus* spp. strains cultured from milk (GODDEN et al., 2002). For 15 strains (15/90, 16.66 %), a decrease in *Staphylococcus* cfu counts was observed following the freezing/thawing treatment as compared to the direct culture. This decrease may be explained by the possible negative effect of freezing on the viability of the bacteria, as indicated in another study by BEXIGA et al. (2011).

In this study, according to post-freezing inoculation results, 11 udders were diagnosed with staphylococcal mastitis that were found negative for *Staphylococcus* spp. growth in the direct inoculation method. Thus, the positive udder rate for staphylococci increased from 39.91 % (91/228) to 44.29 % (101/228) and this increase was significant ( $P = 0.006$ ). The diagnosis of new cases proves that pre-freezing milk samples is helpful in diagnosing staphylococcal mastitis cases more accurately, as reported by VILLANUEVA et al. (1991) and GODDEN et al. (2002).

DINSMORE et al. (1992) stated that utilization of a higher inoculation volume increased the sensitivity of the culture method in diagnosing mastitis. The improvement in sensitivity was achieved when the inoculum was increased from 10 µL to 50 µL and 100 µL (DINSMORE et al., 1992). Thus, we used 50 µL inoculum from milk samples, to be able to diagnose the cases with low bacterial cell count and to increase the sensitivity of the culture method. Twenty-six udders (out of 91 udders) had less than 10 *Staphylococcus* spp. colonies in the direct inoculation method in our study. Thus, the use of 50 µL inoculum of milk samples may have prevented diagnosing them as false negative for staphylococci.

The eleven strains that were only isolated by the post-freezing inoculation method were from six subclinical and five clinical mastitis cases. Therefore, freezing milk samples improved the sensitivity of the culture method, for diagnosis of both clinical and subclinical staphylococcal mastitis cases in cattle.

As an overall finding, we conclude that it will be very beneficial for diagnostic laboratories to implement a pre-freezing and thawing step before culture for staphylococcal mastitis diagnosis from cow milk, which is especially important for samples from udders determined as culture negative by direct inoculation, and for the cases unresponsive to antibiotic therapy.

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**SAŽETAK**

Zbog osobina uzročnika dijagnostika stafilokoknog mastitisa ponekad može biti otežana. Radi poboljšanja dijagnostike u ovom je istraživanju istražen učinak smrzavanja i otapanja pretraživanih uzoraka mlijeka prije provođenja standardnog postupka uzgoja uzročnika. Svaki od 228 uzetih uzoraka mlijeka kod kliničkog i supkliničkog mastitisa bio je nacijepljen na krvni agar sa 7 % ovčje krvi izravno te nakon postupka smrzavanja i otapanja. Rezultati uzgoja obaju postupaka bili su uspoređeni na osnovi broja vimena pozitivnih na stafilokoke i broja poraslih bakterijskih kolonija. Kod izravnog nacjepljivanja *Staphylococcus* spp. bio je izdvojen iz 91 uzorka mlijeka. Nakon smrzavanja i otapanja *Staphylococcus* spp. bio je izdvojen iz još 11 uzoraka, koji su bili negativni kod izravnog nacjepljivanje bez prethodnoga smrzavanja i otapanja. Broj vimena pozitivnih na *Staphylococcus* spp. bio je statistički značajno različit između tih dvaju pretraživanih postupaka. Razlike u broju kolonija izolata bile su također statistički značajne između rabljenih postupaka kod dijagnosticiranja kliničkog i supkliničkog mastitisa. Boljim se pokazao postupak prethodnog smrzavanja i otapanja uzoraka mlijeka. Rezultati upućuju na zaključak da smrzavanje i otapanje uzoraka mlijeka prije nacjepljivanja na hranjivu podlogu ima prednosti s obzirom da se taj postupak pokazao osjetljivijim za dokazivanje stafilokoka kod pojave mastitisa u mliječnih krava.

**Ključne riječi:** govedo, mastitis, mlijeko, smrzavanje, *Staphylococcus* spp.

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