

Isolation and Identification of *Staphylococcus* Species from Raw Bovine Milk in Debre Zeit, Ethiopia

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Abstract: In this study, investigation of the presence of *Staphylococcus* and determination of its prevalence and distribution, identification of *Staphylococcus* species and determination of their prevalence and distribution and characterization of the isolates in order to determine their ability in synthesizing coagulase from raw bovine milk samples were conducted from October 2008-April 2009 in Debre Zeit, Ethiopia. About 200 raw bovine milk samples consisting of 100 buckets milk of farms and 100 tanks milk of milk collection centers were analyzed. The identification results showed 33 and 46% prevalence of *Staphylococcus* in buckets milk and tanks milk, respectively with an overall prevalence of 39.5% (79/200). Comparison of the prevalence of *Staphylococcus* in raw bulk milk samples showed a relatively higher prevalence in tanks milk (46%) than buckets milk (33%). However, this difference was not statistically significant ($p > 0.05$). The 33 isolates identified as staphylococci from samples of buckets milk were tested for species assignment. They were grouped into *S. aureus* with 8 (8%) isolates, *S. intermedius* with 6 (6%) isolates, *S. hycius* with 6 (6%) isolates and Coagulase Negative Staphylococci (CNS) with 13 (13%) isolates. The 46 staphylococci isolates were grouped into *S. aureus* with 10 (10%) isolates, *S. intermedius* with 11 (11%) isolates, *S. hycius* with 6 (6%) isolates and CNS with 19 (19%) isolates. There was no significant difference ($p > 0.05$) among these proportion of isolates in both buckets and tanks milk. All the isolates were tested for the production of coagulase to determine their pathogenicity. Comparison of the prevalence of Coagulase Positive Staphylococci (CPS) showed a relatively higher CPS prevalence in tanks milk (27%) than buckets milk (20%). However, this difference was not statistically significant ($p > 0.05$). The high level of *Staphylococcus* isolate found raw milk samples in the present study represent a poor quality and public health risk to the consumer. Hence, raw milk intended for human consumption must be subjected to pasteurization or heat treatment at least equivalent to pasteurization temperature order to guarantee the quality of these highly popular products in Debre Zeit in order to decrease the risk of staphylococcal food poisoning.

Key words: Buckets milk, Debre Zeit, prevalence, *Staphylococcus*, tanks milk, Ethiopia

INTRODUCTION

Most foods contain viable bacteria unless thoroughly heated or made sterile. Otherwise, it serves as an important medium for transmission of pathogenic organisms to the consumers. Contamination of food products with pathogenic organisms may influence considerably their harmlessness, endanger the health of consumers and decrease shelf quality resulting in food-borne infections, intoxications and economic losses from food spoilage (Le Loir *et al.*, 2003). Staphylococcal Food Poisoning (SFP) is one of the most common Food Borne Disease (FBD) worldwide with high occurrence second to salmonellosis (Aycicek *et al.*, 2005). Staphylococcal food poisoning is often associated with the ingestion of

manually handled foods that contain one or more highly heat stable staphylococcal enterotoxins (Smith, 2007). The safety of milk with respect to FBD is of great concern around the world. This is especially true in developing countries like Ethiopia where production of milk often takes place under unsanitary conditions and the consumption of raw milk which is typically produced in small dairy farms under unsatisfactory hygienic conditions is a common practice (Wubete, 2004).

In spite of the aforementioned prevailing situation and the presence of a number of public health problems due to FBDs resulting from the consumption of different food items in Ethiopia there is paucity of well-documented information on the occurrence of *Staphylococcus* in raw bovine bulk milk. Therefore, this study was designed to

investigate the presence of *Staphylococcus* and identify *Staphylococcus* species and determine their prevalence and distribution in raw bovine milk as well as to characterize the isolates in order to determine their ability in synthesizing coagulase.

MATERIALS AND METHODS

Origin of samples and description of milk collection centers: The raw bovine bulk milk samples were collected from Adaa-Liben district dairy and dairy product producer and marketing co-operative society (Liability limited). This cooperative in which the study was conducted has 860 members. The number of lactating cows present at the farms ranged from 1-5 with an average of 3 animals per farm. The estimated average milk yield was about 7.5 L/day/cow. The dairy owners after they milked their cows delivered the milk to the nearest Milk Collection Centers (MCCs) of their association. The MCCs after collecting the milk sell it to the local consumers either as raw or pasteurized.

There are 14 MCCs in Adaa-Liben District dairy and dairy product producer and marketing co-operative society. The randomly selected 100 farms that deliver their milk to the selected 7 MCCs and the randomly selected 7 MCCs were used as the sources of samples for this study. All the farms encountered in this study were smallholder dairy farms having 1-5 lactating dairy cows. All the milk collection centers were located along roadsides that could likely expose the milk to dust contamination created by moving vehicles.

Study type: A cross-sectional study was conducted from October 2008-April 2009. Bulk milk samples were taken from two critical points in milk handling (milking buckets of farms and storage tanks of the MCCs) which are supposed to be the major risk areas for the consumers as many people may share the pooled product.

Sample collection and transportation: Raw milk samples were collected from buckets of the farms and storage tanks at MCCs which are critical control points. Pooled raw bulk milk samples were collected from each critical point aseptically every 1 week for 7 months in the morning after thoroughly mixing the milk. Samples were aseptically collected and put into a sterile screw capped bottles and kept in an icebox containing ice packs and taken immediately to the laboratory of Microbiology at the Faculty of Veterinary Medicine, Addis Ababa University, Debre Zeit. Upon arrival, the samples were stored overnight in a refrigerator at 4°C until analyzed the next day.

Study methodology: Samples which were kept for overnight in a refrigerator at 4°C were thawed for 3-5 h at room temperature. About 25 mL of each raw milk sample was stirred separately into 225 mL of sterile Buffered Peptone Water (BPW) in a sterile stomacher bag. The pre-enriched samples were homogenized in a stomacher (Lab-Blender 400) for 2 min and incubated aerobically at 37°C for 24 h. Following this, 0.1 mL of the pre-enriched broth of the various dilutions were streaked (seeded) aseptically onto sterile Blood Agar Plates (BAP) enriched with 7% heparinized sheep blood and incubated at 37°C for 24-48 h under aerobic culture conditions. The plates were examined for the presence of *Staphylococcus* colonies. Isolates supposed to belong to *Staphylococcus* species on the basis of their morphological aspects (creamy, greyish, white or yellow colonies) and haemolytic pattern on the surface of BAP were collected. Presumed staphylococcal colonies were then sub-cultured on Nutrient Agar Plates (NAP) and incubated at 37°C for 24-48 h to get a pure culture (clone of cells derived from a single cell). The pure isolates in the NAP were preserved and maintained for biochemical differentiation tests and characterizing the isolates.

Isolation and identification of *Staphylococcus* species: Final identification of staphylococci organisms and species assignment were done based on gram staining, catalase test, sugar fermentation and coagulase test.

Gram's staining: All suspected cultures of *Staphylococcus* species were subjected to gram's stain and observed under a light microscope for gram's reaction, size, shape and cell arrangements. The gram-stained smears from typical colonies that showed gram-positive cocci occurring in bunched, grapelike irregular clusters were taken as presumptive *Staphylococcus* species.

Catalase test: Pure culture of the isolates were picked using a sterile loop from the agar slant and mixed with a drop of 3% H₂O₂ on a clean glass slide. If the organism was positive, bubbles of oxygen were liberated within a few seconds and the catalase negative isolates did not produce bubbles. The catalase positive cocci were considered as staphylococci.

Mannitol salt agar: The colonies that were identified by Gram-staining and catalase test as *Staphylococcus* were streaked on Mannitol Salt Agar (MSA) plates and incubated at 37°C and examined after 24-48 h for growth and change in the colour of the medium. The presence of growth and change of pH in the media (red to yellow

colour) were regarded as confirmative identification of the salt tolerant staphylococci. Phenol red pH indicator detected the acidic metabolic product of mannitol. Fermentation of mannitol by *S. aureus* causes yellow discolouration of the medium.

Colonies that develop weak or delayed yellow colour after 24 h of incubation were taken as *S. intermedius* and colonies that failed to produce any change on the medium were considered as *S. hyicus* and CNS (Fig. 1).

Coagulase test: The tube coagulase test was performed in sterile tubes by adding 0.5 mL of selected isolates of *Staphylococcus* grown on Tryptone Soya Broth (TSB) at 37°C for 24 h to 0.5 mL of citrated rabbit plasma. After mixing by gentle rotation, the tubes were incubated at 37°C along with a negative control tube containing a mixture of 0.5 mL of sterile TSB and 0.5 mL of rabbit plasma. Clotting was evaluated at 30 min intervals for the first 4 h of the test and then after 24 h incubation. The reaction was considered positive if any degree of clotting from a loose clot to a solid clot that is immovable when the tube is inverted (tilted) was visible within the tube and no degree of clotting would be taken as negative (Fig. 2).

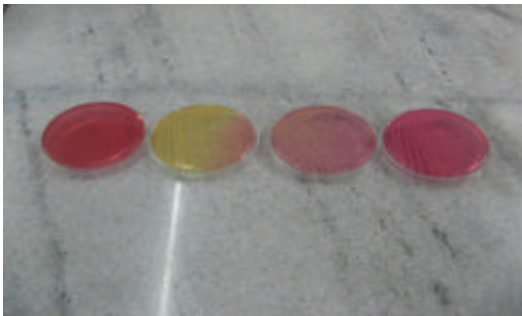


Fig. 1: Original mannitol salt agar, *S. aureus*, *S. intermedius* and *S. hyicus* and coagulase negative staphylococci (left to right)

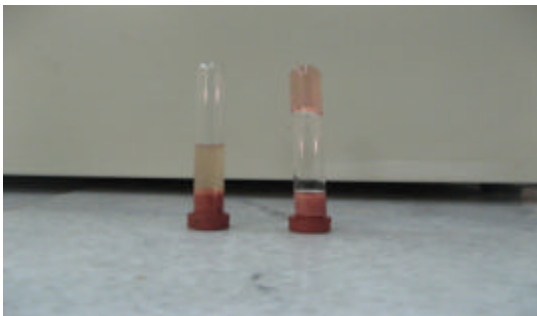


Fig. 2: Coagulase negative reaction showing no degree of clotting (left) and coagulase positive reaction showing a solid clot (right)

Purple agar base: The suspected culture was inoculated on Purple Agar Base (PAB) media plate with 1% of maltose and incubated at 37°C for 24-48 h to differentiate pathogenic staphylococci, particularly coagulase-positive isolates. The identification was based on the fact that *S. aureus* rapidly ferment maltose and the acid metabolic products cause the pH indicator (bromocresol purple) to change the medium and colonies to yellow. *S. intermedius* gives a weak or delayed reaction and *S. hyicus* did not ferment maltose but attacks the peptone in the medium producing an alkaline reaction (a deeper purple) around the colonies.

Data management and analysis: Prevalence of *Staphylococcus* and *Staphylococcus* species were computed as the number of each food items positive for *Staphylococcus* and *Staphylococcus* species divided by the sample sizes of food items examined.

The 95% Confidence Interval (CI) of a proportion was used to calculate the lower and upper limits of the proportion of *Staphylococcus* and *Staphylococcus* species in the samples examined.

The Pearson's χ^2 -test at a significance level of 5 and 95% CI was used to determine the differences of prevalence of *Staphylococcus*, *Staphylococcus* species and CPS between the bulk milk samples examined at the 2 critical control points. The difference was statistically significant if the $p < 0.05$.

RESULTS

Prevalence and distribution of staphylococcus in raw milk: Out of the 200 bulk milk samples 39.5% (79/200) resulted contaminated with *Staphylococcus* species. The frequency of isolation of *Staphylococcus* varied between sources and sample type and ranged from 33-46%. The prevalence of *Staphylococcus* was 33% (33/100) and 46% (46/100) from buckets milk and tanks milk, respectively.

The results showed a relatively higher prevalence in tanks milk (46%) than buckets milk (33%). However, this difference was not statistically significant ($p > 0.05$) (Table 1). The 33 isolates identified as staphylococci from buckets milk were tested for species assignment using biochemical characteristics. They were grouped into *S. aureus* with 8 (8%) isolates, *S. intermedius* with 6 (6%) isolates, *S. hyicus* with 6 (6%) isolates and CNS with 13 (13%) isolates.

The 46 identified staphylococci from tanks milk were tested for species assignment using biochemical characteristics. They were grouped into *S. aureus* with 10 (10%) isolates, *S. intermedius* with 11 (11%) isolates, *S. hyicus* with 6 (6%) isolates and CNS with 19 (19%)

Table 1: Prevalence of *Staphylococcus* from raw milk samples

Type of sample	Examined	Prevalence (%)	95% CI	χ^2 -value	p-value
Buckets milk	100	33	24.56-42.69	3.536	0.060
Tanks milk	100	46	36.56-55.74	-	-

Table 2: Proportional distribution of *Staphylococcus* species from raw milk samples

Samples	Species	Prevalence (%)	95% CI	χ^2 -value	p-value
Buckets milk	<i>S. aureus</i>	8	4.11-15.00	0.244	0.621
Tanks milk		10	5.52-17.44		
Buckets milk	<i>S. intermedius</i>	6	2.78-12.48	1.607	0.205
Tanks milk		11	6.25-18.63		
Buckets milk	<i>S. hicus</i>	6	2.78-12.48	0.000	1.000
Tanks milk		6	2.78-12.48		
Buckets milk	CNS	13	7.76-20.98	1.339	0.247
Tanks milk		19	12.51-27.78		

Table 3: Proportional distribution of coagulase positive staphylococci species in raw milk

Type of sample	Examined	Prevalence (%)	95% CI	χ^2 -value	p-value
Buckets milk	100	20	13.34-28.88	1.363	0.243
Tanks milk	100	27	19.27-36.96	-	-

isolates. There was no significant difference ($p > 0.05$) among these proportion of isolates in both bulk buckets and tanks milk (Table 2).

Prevalence and distribution of coagulase positive staphylococci isolates: All isolates were characterized in order to determine their ability in synthesizing coagulase. Raw bulk milk samples yielded an overall prevalence of 23.5% (47/200) of CPS. Specifically the prevalence of CPS was 20% (20/200) and 27% (27/100) from buckets milk and tanks milk, respectively. The results showed a relatively high CPS prevalence in tanks milk than buckets milk. However, this difference was not statistically significant ($p > 0.05$) (Table 3).

DISCUSSION

The surveillance of food for microbial contamination is vital for the protection of public health and consumer interests. Production of safe food also has important economic implications in an increasingly competitive global market. *Staphylococcus* organisms can gain access to raw milk by direct excretion from udders having clinical and subclinical staphylococcal mastitis or by contamination from food handlers and pose public health risk to consumers (Yilma *et al.*, 2007). Different investigators have reported that *Staphylococcus* species isolated from dairy products of bovine are able to produce high levels of SEs. Smith (2007) reported that 54% of bovine mastitic milk isolates to be enterotoxigenic and Salandra *et al.* (2008) reported 55.9% enterotoxin producing *Staphylococcus* isolates from dairy products in Italy. The different rates of enterotoxin production found in these

reports could be explained by the different techniques used in these studies, differences in the origin of the isolates or by geographical differences. Based on the above information, out of 79 *Staphylococcus* species isolated and identified in this study some could be enterotoxigenic and raw milk sold in MCCs might be potential sources of food poisonings due to *Staphylococcus* to the public.

The products specific prevalence of *Staphylococcus* was found to be 33 and 46% from buckets milk and tanks milk samples, respectively. Comparing the prevalence of *Staphylococcus* in the raw bulk milk samples in the present study the high prevalence of *Staphylococcus* was seen in tanks milk than buckets milk. This could be attributed to the cumulative effects of milk contamination at different critical points. Additionally, handling of milk in different plastic containers and the use of sieves may cause contamination of milk.

Plastic containers have characteristics that make them unsuitable for milk handling. Plastic containers scratch easily and provide hiding places for bacteria during cleaning and sanitization and plastic containers are poor conductor of heat and hence will hinder effective sanitization by heat (Soomro *et al.*, 2003). Also, the number of personnel working at MCCs were higher which might have contributed to milk contamination.

Staphylococcus aureus was detected in 8% of the buckets milk and 10% tanks milk samples. The findings of the present study revealed a lower prevalence rate than 75% in 220 bovine bulk milk reported by Jorgensen *et al.* (2005), 68% (15/22) by Le Loir *et al.* (2003), 61.3% (49/80) by Hein *et al.* (2005) and 40% (32/81) in Bendahou *et al.* (2008).

Staphylococcus intermedius was detected in 6 and 11% in buckets milk and tanks milk samples, respectively. The prevalence of *S. intermedius* in the present research was >2% of 81 milk and milk product samples by Bendahou *et al.* (2008). *Staphylococcus hicus* was isolated in 6 and 6% of buckets milk and tanks milk, respectively. Lamprell *et al.* (2004) recorded slightly lower findings of 4% in 1036 samples. Coagulase-negative *Staphylococcus* species were detected in 13 and 19% from the milking buckets of the farms and raw bovine milk from the tanks of the MCCs samples, respectively.

CONCLUSION

The results showed that CNS species more frequently occurred in raw milk but the findings of the present study was lower than the investigation of Tsegmed (2006) who reported CNS in 54% of raw milk of cattle by Mongolia and Lamprell of 29% in 1036 samples. The high number of CNS isolated in the current study may be due the fact that CNS are a part of the normal teat skin flora and mucosa of

humans and animals, some species are also found free-living in the environment. Therefore, they are common cause of contamination of milk and milk products. In addition, unpasteurized milk may contain CNS if the cow suffers from mastitis of CNS (Kaloreu *et al.*, 2007). Coagulase production was described as one of the most reliable criteria for the identification of pathogenic *Staphylococcus* species. Staphylococci producing coagulase are usually pathogenic (Quinn *et al.*, 2002). In the present study, CPS were identified in 20 and 27% in buckets milk of farms and tanks milk of MCCs samples, respectively. The CPS isolated in this study comprised of 59.5% (47/79) of the total *Staphylococcus* isolates. The high number of CPS in this study showed the risk of consuming raw milk in the study area.

ACKNOWLEDGMENTS

The research incorporated in this research was undertaken using the research grant allocated by Faculty of Veterinary Medicine, Addis Ababa University. Researcher is grateful to the university in particular and government of Ethiopia, in general, for providing the research fund. Researcher is highly indebted to academic advisors, Prof. M. Pal and Dr. Moses N. Kyule for their technical advice, provision of materials and daily supervision throughout the research, time devotion to review the manuscript and encouragement, for their invaluable comments and suggestion on the editorial and scientific aspect of this study. Researcher wishes to extend my profound gratitude to Adaa-Liben District dairy and dairy products producers and marketing co-operative, dairy owners and milk collection centers workers in Debre Zeit who participated in this exercise. Without the cooperation and enthusiasm shown by them, the study would not have been possible.

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