

Isolation and Identification of Pathogenic Staphylococci and E. Coli From Raw Bovine Milk Collected From Milk Cooperative Centers in Hawassa, Southern Ethiopia

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**ISOLATION AND IDENTIFICATION OF PATHOGENIC *STAPHYLOCOCCI* AND *E. COLI*
FROM RAW BOVINE MILK COLLECTED FROM MILK COOPERATIVE CENTERS IN
HAWASSA, SOUTHERN ETHIOPIA**

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Abstract

Background: Pathogenic microorganisms commonly isolated from milk and milk products pose a serious threat to human health. *Escherichia coli* and *Staphylococci* are the major pathogens isolated from milk. The objective of this study was to isolate and identify pathogenic *Staphylococci* and *E. coli* from raw bovine milk collected from milk cooperative centers found in Hawassa City, Southern Ethiopia.

Result: The overall prevalence of pathogenic species of *Staphylococci* and *E. coli* was found to be 35.16 % and 8.59 % (n=384), respectively. From the total 384 raw milk samples examined, 1.56 % was found to be positive for both pathogenic species of *Staphylococci* and *E. coli*. The prevalence of pathogenic species of *Staphylococci* was found to be 33.33 %, 36.05 % and 39.21 % milk collected from Arsi Negele, Hawassa and Shashemene, respectively. Whereas, the prevalence of *E. coli* was found to be 9.68 %, 8.84 % and 3.92 %) milk collected from Arsi Negele, Hawassa and Shashemene, respectively. The study results showed a relative higher prevalence of pathogenic species of *Staphylococci* in raw milk collected from Shashemene than raw milk collected from Arsi Negele and Hawassa. However, the difference was statistically insignificant ($p > 0.05$).

Conclusion: Higher isolation rate of *E. coli* and pathogenic species of *Staphylococci* in raw milk samples collected from different milk cooperative centers in Hawassa could be associated to mastitis, poor udder preparation, poor milker's hygiene, poor milk handling practices, poor environmental sanitation and sanitation of milking equipment. Overall, this study showed that pathogenic species of *Staphylococcus* and *E. coli* are prevalent in raw milk produced and consumed in the area. Therefore, awareness should be created to producers and raw milk collecting cooperatives on strict preventive measures of raw milk contamination.

Key words: Arsi Negele, *E. coli*, Hawassa, pathogenic *Staphylococci*, Shashemene

Background

Milk provided for human beings should not contain any pathogenic microorganisms (Bertu *et al.*, 2010). However, microbes are isolated and identified from milk and milk products causing a critical health problem to human beings. Milk constitute a diverse biochemical compound as well as it has high water activity and nutritional value which serves as a favorable medium for growth and multiplication of microorganisms (Parekh and Subhash, 2008). Microbes which are commonly isolated from milk includes *Escherichia coli*, *Staphylococcus aureus*, *Salmonella Typhimurium*, *Listeria monocytogenes*, *Mycobacterium*, *Campylobacter*, *Leptospira*, *Clostridium*, *Pseudomonas aeruginosa* and *Proteus* species (Abeer *et al.*, 2012; LeJeune and Rajala, 2009). Milk could be contamination by microorganisms during the preharvest period by the infected lactating animals and during the post-harvest period during milking by milkers, milk handlers, unsanitary utensils and milking equipment's (Parekh and Subhash, 2008; LeJeune and Rajala, 2009).

Staphylococcus species found everywhere in the environment of dairy cattle. The major reservoir of *Staphylococci* is the infected mammary gland of lactating cows. At the time of milking these organisms transmitted from infected cow to healthy cow during milking through contaminated milker's hands and teat cup liners (Radostits *et al.*, 2007; Mayada and Fatma, 2013). *Escherichia coli* is a natural flora of the intestines of animals and humans. However, it is commonly recovered from food staffs which could affects the public health due to the possible presence of enteropathogenic and/or toxigenic strains. These enteropathogenic and toxigenic strains of *Escherichia coli* lead to critical gastrointestinal disorder. The presence of *E. coli* in milk and milk products is a reliable indicator of milk contamination by manure, soil and contaminated water (Quinn *et al*, 2002; Radostits *et al.*, 2007).

In Ethiopia, milk produced from smallholder farms is marketed and distributed without any form of pasteurization and quality control measures. According to different reports in Ethiopia, of the total milk production, 71 - 97 % of milk is consumed through an informal market without checking the quality measures (Tsehay, 2002). So far, there is no any study conducted on isolation and identification of pathogenic *Staphylococci* and *Escherichia coli* in dairy cooperative milk collection centers in Hawassa City. Therefore, the present study was planned to isolate and identify pathogenic

Staphylococci and *E. coli* from raw cow milk collected from dairy cooperative milk collection centers of Hawassa City.

Materials and Methods

Study Area

The study was conducted in and around Hawassa town. Hawassa is the capital city of Sidama Zone and SNNPRs, which is located in the northern part of SNNPRs and 275 km south of Addis Ababa with a total human population of 157,879. Geographically it lies between $7^{\circ}3'1.35''$ N latitude $38^{\circ}29'43.81''$ E longitude at an altitude of 1750 meters above sea level (m.a.s.l). The area annually receives an average of 800 - 1000 mm rain fall of which 67 % falls in long rainy season which extends from June to September with an average annual temperature of 22 °c and 51.8% mean relative humidity. The area is mainly covered by dry savanna and bush type of vegetation, the total livestock population of Sidama Zone (including Hawassa town) is estimated to constitute 1,721,341 cattle, 228,941 goats, 457,465 sheep, 57,643 horses, 54066 donkeys, 725,540 poultry and 44,492 beehives (CSA, 2008).

Sample source

The target populations were all raw cows' milk from individual dairy cooperative milk collection centers which are ready for human consumption in Hawassa town. The milk collection cooperative centers those collecting milk from Hawassa and surroundings, from Arsi Negele and Shashemene towns were included in this study.

Study Design

A cross - sectional bacteriological study was conducted from November 2016 to April 2017 to isolate and identify pathogenic *Staphylococci* and *E. coli* from raw cows' milk collected from milk cooperative centers in Hawassa town.

Sampling Method

Simple random sampling technique was conducted to select milk cooperative collection centers and to select milk containers to take appropriate raw milk samples.

Sample Size Determination

The desired sample size required for this study was determined depending on the expected prevalence of pathogenic staphylococci and *E. coli* in the study area and the desired absolute precision. The sample size was computed using the formula given in Thrusfield (2005) as follows.

$$N = \frac{1.96^2 \times P_{exp} (1 - P_{exp})}{d^2}$$

Where: N = required sample size; P_{exp} = expected prevalence; d = desired absolute precision

Since there was no previous study regarding the prevalence of pathogenic staphylococci and *E. coli* in the area, 50 % expected prevalence was used to determine the sample size. Using desired 95 % confidence interval, 5 % precision and 50 % expected prevalence, the necessary numbers of raw milk samples needed to isolate and identify pathogenic staphylococci and *E. coli* were 384.

Milk sample collection and transportation

Attempts were made to prevent contamination and cross contamination in the course of sample collection. Approximately 5 ml of raw milk samples were collected into sterile screw capped bottles. The milk samples then were held in an icebox with ice packs and transported to Microbiology Laboratory of School of Veterinary medicine, Hawassa University. All samples were clearly labeled with date of sampling, type of sample and with the name of milk collection cooperatives. In laboratory, raw milk samples were cultured immediately or stored at +4 °C for a maximum of 24 hour until they were inoculated onto a standard bacteriological media (NMC, 1999).

Bacteriological isolation and identification

Bacteriological examination was done according to the Quinn *et al.* (2002). A loop full of milk samples were streaked on to sterile blood agar plates and MacConkey agar plates using the quadrant

streaking method. Then the plates were incubated aerobically at 37°C for 24 to 48 hours and they were examined for growth, morphology, pigmentation and haemolytic characteristics of the colonies. For further identification the colonies suggested colonies of pathogenic staphylococci were subcultured onto sterile Trypticase Soya Agar (TSA) plates. All the suspected cultures of *Staphylococci* species were subjected to Gram's staining and examined using light microscope under oil immersion objective to appreciate their morphology, cell arrangement and the gram reactions of the isolate. So that the suggested colonies of *Staphylococci* species that showed gram positive cocci occurring in bunched, grape like irregular clusters were taken as presumptive colonies of *Staphylococci* species. All colonies those were considered as presumptive colonies of *Staphylococci* species were subjected to catalase test, oxidase, motility and OF tests. All isolates which were gram positives cocci arranged irregularly as bunch of grapes, did not show growth on MacConkey agar plates, catalase positives, oxidase negatives; non motile and facultative anaerobes were considered as isolates of *Staphylococci* species (Quinn *et al.*, 2002).

Colonies those were considered as isolates of genus *Staphylococci* were further differentiated into pathogenic and opportunistic pathogenic species by tube coagulase test. The test was performed by adding a heavy colony of pure culture from Trypticase Soya Agar to a 0.5 ml of rabbit plasma. After mixing the suspension of bacteria with rabbit plasma by gentle rotation, the tubes were incubated at 37 °C aerobically for 4 - 24 hours and examined for any degree of clotting of plasma within the tube. Those isolates which were positive in tube coagulase test were considered as pathogenic species of *Staphylococci* (*S. aureus*, *S. intermedius* and most strains of *S. hyicus*). However, those isolates which were negative in tube coagulase test were considered as non pathogenic species of *Staphylococci* (mainly *S. saprophyticus* and *S. epidemics* and can be others) (Quinn *et al.*, 2002).

The pathogenic species of *Staphylococci* were differentiated by inoculating their colonies on Mannitol Salt Agar (MSA) plates. So that the colonies of pathogenic staphylococci were inoculated onto MSA plates and incubated at 37 °C aerobically for 24 - 48 hours. The plates were examined after 24 hours of incubation for the presence or absence of growth and if there was a colour change from red to yellowish in the medium due to bacterial growth. The presence of growth and colour change from red to yellow were regarded as confirmative identification of the salt tolerant staphylococci which ferment mannitol. Those colonies of pathogenic staphylococci which grew on MSA and

developed a yellowish discoloration of the medium were considered as *Staphylococci aureus* and 11 - 89 % stains of *S. intermedius*. While those colonies of pathogenic staphylococci which grew on MSA whereas colonies that develop without fermenting sugar mannitol were considered as *S. hyicus* (Quinn *et al.*, 2002).

Isolation of *E. coli* was done by inoculation of appropriate milk samples on to sterile blood agar plates and MacConkey agar plates. All the inoculated plates were incubated at 37 °C aerobically for 24 - 48 hours. Then colonies which were grayish, round, discrete haemolytic on blood agar plates and which grew on MacConkey agar plates by fermenting lactose (having pinkish colonies) were considered as presumptive colonies of *E. coli*. For primary identification of *Escherichia coli*; presence and absence of bacterial growth on MacConkey agar, colony characteristics (size, colour) and presence or absence of lactose fermentation on MacConkey agar plates, catalase, oxidase, motility and OF tests were noted and recorded. Those colonies which were round, discrete haemolytic and developed bright pink colour colonies on MacConkey agar plates; and those were positive in catalase test, negative in oxidase test, motile and facultative anaerobes were subcultured on Brilliant Green Agar (BGA) plates for their further characterization. Those isolates which grew on BGA plates and developed yellowish colonies were considered as the colonies of *E. coli* and were subjected for further identification by secondary biochemical tests (Quinn *et al.*, 2002)

Secondary biochemical tests such as indole test, methyl red test, Voges-Proskauer test and citrate utilization test(I/MR/VP/C) were performed as a presumptive identification of *Escherichia coli*. Those isolates were positive in indole test, positive in methyl red test, negative in Voges - Proskeur and citrate utilization tests were identified as the colonies of *E. coli*.

Data Management and Analysis

The data generated from this study were entered and managed in Microsoft Excel. All the data analysis was done using Statistical Package for Social Sciences (SPSS) software version 20. Descriptive statistics such as percentages and frequency distribution were used to describe the nature and the characteristics of data. The overall prevalence of pathogenic staphylococci and *E. coli* isolates was analyzed using percentages. The association of the risk factor with prevalence of pathogenic staphylococci and *E. coli* isolates was computed by Pearson's chi-square (χ^2) test. In all this analysis,

comparison having P - value less than 0.05($P < 0.05$) at 5 % level of significance were considered as statistically significant.

RESULTS

Overall Isolation rate of Pathogenic Staphylococci and *E. coli*

A total of 384 raw milk samples were collected and bacteriologically investigated to determine the isolation rate of pathogenic staphylococci and *E. coli*. Out of the total 384 raw milk samples examined, 186 (48.44 %), 147(38.28 %) and 51(13.28 %) origin were found from Arsi Negele, Hawassa and Shashemene, respectively as shown in Table 1 below. Out of the total 384 raw milk samples examined 162(42.18 %) raw milk samples were found to be positive. Out of 162 (42.18 %) positive raw milk samples 78(20. 31 %), 62(16.14 %), 22(5.72 %) were found to be from raw dairy milk samples collected from selected raw milk cooperative collection centers in Hawassa which primary origin were found from Arsi Negele, Hawassa and Shashemene towns respectively as shown in Table 1 below.

Table 1: Overall isolation rate of pathogenic staphylococci and *E. coli* in raw milk samples of study area.

Origins of raw milk samples	Total examined (n=384)	No of positives	Isolation rate (%)	Results		
				Positive for only pathogenic staphylococci	Positive for only <i>E. coli</i>	Positive for both pathogenic staphylococci and <i>E. coli</i>
Arsi Negele	186	78	41.93	60	16	2
Hawassa	147	62	42.17	49	9	4
Shashemene	51	22	43.13	20	2	0
Total	384	162	42.18	129	27	6

Association of the Overall Isolation rate of Staphylococci and *E. coli* Isolates in Relation to Raw Milk Samples Origin

The isolation rate of pathogenic staphylococci and *E. coli* was found to be different in raw milk samples collected from three towns. It was observed that out of the total 186 raw milk samples collected from Arsi Negele by milk cooperatives found in Hawassa, 33.33 % (62/186) and 9.68 % (18/186) were found to be positives for pathogenic staphylococci and *E. coli*, respectively. Whereas from the total of 147 raw milk samples collected from selected raw milk collection cooperatives found in Hawassa town which origin was Hawassa itself, 36.05 % (53/147) and 8.84 % (13/147) were found to be positive for pathogenic staphylococci and *E. coli* respectively. Finally, from the total of 51 raw milk samples collected from selected milk collection cooperatives found in Hawassa town which origin was Shashemene, 39.21 % (20/51) and 3.92 % (2/51) were found to be positive for pathogenic staphylococci and *E. coli*, respectively. The result showed a relative higher isolation rate of pathogenic staphylococci isolates in raw milk samples collected from selected raw milk collection cooperatives found in Hawassa town which origin was found from Shashemene (39.21%) than from raw milk samples collected from selected raw milk collection cooperatives in Hawassa which origin was found in Arsi Negele town (33.33%) and Hawassa town (36.05%). But the difference was statistically insignificant ($P = 0.62$; $\chi^2 = 4.46$) as shown in Table 2 below.

Table 2: The isolation rate of pathogenic staphylococci and *E. coli* isolates in raw milk samples and its association in relation to raw milk samples sources.

Origins of raw milk samples	Pathogenic staphylococci		<i>Escherichia coli</i>		Chi-square value	P -value		
	<u>No of</u> <u>positives</u>	Prevalence (%)	<u>No of</u> <u>positives</u>	Prevalence (%)				
Arxi Negele	62	33.33	18	9.68	4.46	0.62		
Hawassa	53	36.05	13	8.84				
Shashemene	20	39.21	2	3.92				

DISCUSSION

A total of 384 raw cows' milk samples was collected from selected milk cooperative centers in Hawassa town. The result of the current study showed that the overall isolation rate of pathogenic Staphylococci was found to be 35.16 % which was comparable to the findings of Mekuria *et al.* (2014), Abera *et al.* (2013) and Addis *et al.* (2011) who reported 35.20 % in Hawassa, 42.14 % in Adama and 39.50 % in Debre Zeit, respectively. However, the prevalence of pathogenic staphylococci in this study was relatively lower than the report of Kundu *et al.* (2013), Mekibib *et al.* (2010), Demme and Abegaz (2015); and Takle and Birhe (2015) who reported 61.10 % in Khartoum state, 47.10 % in Holeta town, 53.94 % in Addis Ababa; and 46.10 % in Sidama Zone, respectively. On contrary, the prevalence of pathogenic staphylococci in this study (35.16%) was higher than the reports of Etifu (2012); study conducted in Alagae state dairy farm (29.00 %); Abera *et al* (2012); study conducted in Shashemene (28.10 %) and Zerihun *et al.* (2013); study conducted in and around Addis Ababa (28.8%). This difference in prevalence of pathogenic staphylococci in raw cows' milk might be arise from the differences in farm management and husbandry practices, breed, environmental conditions, milk handling and/or differences in study methodology and sensitivity of tests employed by the investigators. The high prevalence of staphylococci might be partly explained by presence of this agent on the skin and mucus membranes of various parts of the animal body (Quinn *et al.*, 2002) and their contagious nature, especially *S. aureus* (Radostits *et al.*, 2007).

The overall prevalence of *Escherichia coli* was found to be 8.59 %. The present finding was closely related with the works of Etifu (2012), Abera *et al* (2012), Mekuria *et al* (2014) and Rajeev and Amit (2010) who reported the prevalence of *E. coli* in raw cows' milk; 9.40 % in Alagae state dairy farm, 10.60 % in Shashemene, 8.20 % in Hawassa and 8.15 % in Pantnagar, respectively. In contrast, the present finding was relatively higher than the report of Takle and Birhe (2015), Mekibib *et al.* (2010) and Dieser *et al.* (2014) who reported 3.80 % in Sidama Zone, 4.57 % in Holeta town and 2.10% in Argentinean, respectively. However, the current finding was lower than the work of Soomro *et al* (2002) in Tandajom, Asmahan and Warda (2011) in Khartoum, Worku *et al* (2012) in Borona pastoral community and Demme and Abegaz (2015) in Addis Ababa who reported the prevalence of *E. coli* in raw cows' milk 51.66%, 37.00 %, 12.91 % and 18.60 % respectively. Such difference in prevalence might be arise from differences in farm management and husbandry practices, breed, environmental

conditions, milk handling and transportation and/or differences in study methodology and sensitivity of tests employed by the investigators. The higher prevalence of *Escherichia coli* in raw milk is presumably due to the fact that *E. coli* is the commonest environmental contaminant, which is closely associated with hygiene (Radostits *et al.*, 2007). Moreover, the existence of high numbers of *E. coli* in milk also indicates the relatively poor quality of milk, related with substandard hygiene of the farm management, poor milk collection and poor transportation system to the market (FAO, 1990).

CONCLUSION

The current study showed that the higher prevalence of pathogenic species of *Staphylococci* and *E. coli* in raw milk produced and distributed in the study area. This indicated that raw milk contamination by pathogenic species of *Staphylococci* and *E. coli* found to be the major problem to reduce the quality of raw milk in the area. The higher isolation rate of *E. coli* and pathogenic species of *Staphylococci* in raw milk taken from randomly selected cooperative centers found in Hawassa could be associated with poor udder preparation before milking, milkers' hygienic condition, poor milk handling and transportation practices, poor environmental sanitation and poor sanitation of milking equipment. Overall, this study showed that pathogenic species of *Staphylococcus* and *E. coli* are prevalent in raw milk produced and consumed in the area; and the majority of raw dairy milk produced and consumed in the area was poor in quality.

Abbreviations

SNNPRs: Southern Nation Nationalities Peoples Regional State; NMC: National Mastitis Council; MSA: Mannitol Salt Agar.

DECLARATIONS

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Availability of data and materials

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

Authors' contributions

Alemayehu Gebeyehu Yenealem: Contributed to conception of the research idea, designing and data collection, data analysis, interpretation of data, writing and editing of the manuscript.

Ethics approval and consent to participate

Ethical approval and consent for this study was obtained from Hawassa University College of Computational sciences and Agriculture Minutes of Animal Research Ethics and Review committee (Reference AREC001/2017). Verbal consent was also obtained from the milk cooperation managers to take samples from their shop and for further research use of the samples.

Consent for publication

Not applicable.

Competing interests

The author declares that there is no competing interest.

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