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Full Length Research Paper

Antimicrobial resistance profile of *Staphylococcus aureus* isolated from raw cow milk and fresh fruit juice in Mekelle, Tigray, Ethiopia

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This study was conducted to evaluate drug resistance profile of Staphylococcus aureus in raw milk, fresh fruit juice and dairy farms settings of Mekelle, Tigray, A cross-sectional study was conducted on the total 258 samples of raw cow milk and fresh fruit juice. Antimicrobial resistance status was also checked for identified S. aureus using various commonly used antimicrobial discs. The overall viable staphylococcal count mean and standard deviation of samples from milk shop, fruit juice and dairy milk were found to be 8.86±107, 7.2 × 107, 8.65±107 cfu/ml, 33.87±106, 6.68±106 and 22.0±106, respectively. Among the total 258 samples, 75 (29.07%) samples were found positive for S. aureus. Proportion of the isolation from milk shop, fruit juice and dairy milk samples were 20 (23.26%), 32 (37.21%) and 23 (26.74%), respectively. Antimicrobial test of the high resistance revealed vancomycin (100%), ampicillin (90.9%). ciprofloxacin (90.9%), ceftaroline (63.6%), penicillin-G (81.8%) and clindamycin (72.7%) whereas they are highly susceptible to some antibiotics like gentamicin (100%), streptomycin (81.8%), norfloxacin (63.6%), chloramphenicol (81.8%), sulfamethoxazole (96%), kanamycin (72.7%), polymixin B (72.7%), erythromycin (72.7%) and tetracycline (81.8); also, some S. aureus also showed multi-drug resistance pattern. The present study, we isolated and determined the drug Resistance profile of S. aruesu in Mekelle city, Northern Ethiopia alarmingly, the S. aureus isolates circulating in the raw cow milk, fresh fruit juice and dairy milk. High level of S. aureus isolation from personnel and equipment besides food samples reveals that the hygiene practice is substandard. Prudent drug use and improved hygienic practice is recommended in the raw cow milk of dairy farms and fresh fruit juice to safeguard the public from the risk of acquiring infections and multiple drug resistance (MDR) pathogenic S. aureus.

Key words: Antibiogram, bacterial load, multiple drug resistance (MDR), Mekelle, Milk, Staphylococcus aureus.

INTRODUCTION

Foods borne illness are public health problem in developed and developing countries. *Staphylococcus aureus* is among the most significant pathogens causing

a wide spectrum of diseases in both humans and animals. In humans, nosocomial and community acquired infections are common (Klotz et al., 2003). Pathogenic

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Author(s) agree that this article remain permanently open access under the terms of the <u>Creative Commons Attribution</u> <u>License 4.0 International License</u> strains are usuallycoagulase-positive and cause disease in their hosts throughout the world. *S. aureus* is one of the most significant food-borne pathogens (Le Loir et al., 2003). Raw unpasteurized milk may become contaminated with enterotoxigenic coagulase-positive *S. aureus* either through contact with the cow's udder during milking or by cross contamination during processing (Normanno et al., 2005, Ekici et al., 2004).

Food-borne pathogens are recognized as a major health hazard associated with street foods, the risk being dependent primarily on the type of food and the method of preparation and conservation (CDC, 2011; FAO/WHO, 2005). *S. aureus* is a gram positive, catalase and coagulase positive microorganism responsible for various foodborne outbreaks. Contamination of food with enterotoxigenic *S. aureus* causes staphylococcal enterotoxins (SEs) intoxication hence the associated symptoms like vomiting and diarrhea (Veras et al., 2008). In countries where food borne illness were investigated and documented, the relative importance of pathogens like *S. aureus*, Campylobacter, *Escherichia coli* and Salmonella species were recorded as a major cause (WHO, 2004).

The ability of these microorganisms to survive under adverse conditions and to grow in the presence of low levels of nutrients and at suboptimal temperatures and pH values presents a formidable challenge to the agricultural and food processing industries. The continued prominence of raw meats, eggs, dairy products, vegetable sprouts, fresh fruits and fruit juices as the principal vehicles of human foodborne diseases poses a major challenge to coordinate sectorial control efforts within each industry (Knife and Abera, 2007). Studies conducted in different parts of Ethiopia also showed the poor sanitary conditions of catering establishments and presence of pathogenic organisms (Knife and Abera 2007; Mekonnen et al., 2012).

In addition to toxic effect of food borne microbial pathogens, antibiotic resistance remains a major challenge in human and animal health (Shekh et al., 2013). Food contamination with antibiotic-resistant bacteria can therefore be a major threat to public health, as the antibiotic resistance determinants can be transferred to other bacteria of human and animals, thereby has significant public health implications by increasing the number of food-borne illnesses and the potential for treatment failure (Adesiji et al., 2011). Studying antimicrobial resistance in humans and animals is important for detecting changing patterns of resistance, implementing control measures on the use of antimicrobial agents and preventing the spread of multidrug-resistant strains of bacteria. Up to now, many researchers have focused on the spread of resistant S. aureus in clinical settings (Ateba et al., 2010; Addo et al., 2011). However, limited number of investigations has been studied about the presence of antimicrobial resistance in food animals in Ethiopia (Mekonnen et al., 2005; Hundera et al., 2005). So far, there are no studies

conducted on the burden and drug sensitivity profile of *S. aureus* in Mekelle city, Northern Ethiopia. In this study, S. *aureus* was isolated and the drug Resistance profile was determined.

MATERIALS AND METHODS

Study area

The study was conducted from October 2016 to June 2017 in Mekelle city. Mekelle is the capital city of Tigray Regional State located at about 783 km away north of Addis Ababa, Capital city of the Federal Democratic Republic of Ethiopia at geographical coordination of 39°28` East longitude and 13°32` North latitude. The average altitude of the city is 2300 m.a.s.l. with a mean annual rainfall and average annual temperature of 629 mm and 22°C, respectively (TBA, 2017). The population of the city is 406,338 (195, 605 male and 210,733 female) (TBA, 2017). The city has seven sub-cities and 33 Kebelles where over 139 juice houses, 48 dairy farms and 123 milk shops (street vender or retailer shops) are inhabited. Besides, the city possesses an extensive public transport network and active urban-rural exchange of goods with about 30,000 micro and small enterprises (Bryant, 2017).

Study design

A cross-sectional survey was conducted from October 2016 to June 2017 on raw cow milk and fresh fruit juice samples collected from different sources of raw milk shops and dairy milk supply centers, and juice houses in Mekelle. Purposive sampling technique was employed.

Sampling technique and collection

A total of 258 food samples were collected among which 172 are milk samples (86 from milk shops, 86 from dairy farms) and the remaining 86 are fresh juice samples (from 86 juice houses) in the seven sub-cities of Mekelle city. Samples were collected according to standards described by Oyeleke and Manga (2008). After aseptic collection, samples were labeled and packed with sterile bottles and transported with an ice box to Mekelle University, College of Veterinary Medicine, Microbiology and Public health laboratories for bacterial isolation and characterization. Samples were processed immediately for bacterial identification to species level using culture media and then isolates were kept in refrigerator at 4°C until microbial characterization with regular sub-culturing described (Oyeleke and Manga, 2008).

Enumeration of total viable

One milliliter and gram of raw milk and fruit juice samples respectively were homogenized into 9 ml of serial peptone water/NSS and 10 g/1 g of each food item was weighed out and homogenized into 90 ml/9 ml of sterile distilled deionized water. Then, serial dilutions were prepared. From the 10-fold dilutions of the homogenates; 1 ml of 10-4, 10-5 and 10-6 dilutions were cultured in replicate on standard plate count agar (Hi Media, India), using the pour plate method. The plates were then incubated at 37°C for 24 - 48 h. At the end of the incubation period, colonies were counted using the illuminated colony counter. The counts for each plate were expressed as colony forming unit of the suspension (cfu/g) (Fawole and Oso, 2001). In order to determine the presence of *S. aureus*, 0.1 ml of samples from each dilution

Antibiotic	Disc code	Deterroy (ve)	Zone diameter		
Antibiotic		Potency (µg)	S	М	R
Erythromycin	ERY	15	≥23	14-22	≤13
Penicillin-G	Р	10	≥29		≤28
Norflaxon	f	50	≥17	13-16	≤12
Sulphoxazole-trimethoprim	SXT-TMP	300	≥16	11-15	≤10
Streptomycin	S	10	≥15	12-14	≤11
Kanamycin	KAN	30	≥18	14-17	≤13
Chloramphenicol	CHL	30	≥18		≤18
Tetracycline	TE	30	≥22	19-21	≤19
Gentamicin	GM	10	≥18	-	≤18
Ampicillin	AMP	10	≥15	12-14	≤11
Ciprofloxacin	CIP	5	≥20		<u><</u> 20
Ciftriaxone	CRO	30	≥23	20-22	≤19
Vancomycin	VA	30	≥12	10-11	≤19
Clindamycin	CC	10	≥21	15-20	≤14
Polymixin B	Р	10	≥12	12-14	≤11

Table 1. Antibiotics used, their concentration and drug sensitivity interpretive zone of inhibition diameters

R= Resistant, I= intermediate, S = sensitive.

Source: CLSI (2008).

was introduced onto the Baird Parker agar (Oxorid). The plates were incubated at 37°C for 24 h. All the isolates were subjected to morphological and biochemical confirmation according to the prescribed method (Fawole and Oso, 2001).

Isolation and identification of S. aureus

About 10 ml of each raw cow milk and fresh fruit juice sample was suspended in 90 ml of Brain heart infusion broth supplemented with NaCl (6.5%) and incubated at 37°C for 24 h. A sterile loopful of the broth inoculum was streaked onto Baird Parker agar and incubated at 37°C for 24 h. Colonies that appeared black or greyish-black were Gram-stained and subjected to biochemical tests. Gram positive cocci that occurred singly and in pairs, tetrads, short chains and irregular grape-like clusters were suggestive of *S. aureus*. Conventional biochemical tests carried out for presumptive identification of *S. aureus* isolates included gram staining, catalase, coagulase, 5 % sheep blood agar, pigmentation (Mannitol salt agar) and DNase activity (McFaddin, 2000).

Antimicrobial susceptibility test

Antimicrobial susceptibility test, through Kirby diffusion test, was performed for all *S. aureus* isolates following the protocol of CLSI (2008). For susceptibility test, a pure culture of all *S. aureus* were taken and transferred to a tube containing 5 ml of sterile normal saline and mixed gently to make homogenous suspension which was adjusted to a turbidity equivalent to a 0.5 Mc Far land standard as measured by turbidity meter. The bacterial suspension was inoculated onto Muller–Hinton agar (Oxorid, UK) with the sterile swab to cover the whole surface of the agar. The inoculated plates were left at room temperature to dry. Before using the antimicrobial disks, they were kept at room temperature for 1 h and then

dispended on the surface of media. Following this, the plates were incubated aerobically at 37°C for 24 h (CLIS, 2012).

The diameters of the zone of inhibition around the disks were measured to the nearest millimeter using calibrated rulers, and the isolates were classified as susceptible, intermediate and resistant according to the interpretative accordance with the guidelines of (CLSI, 2008) as indicated in Table 1.

Data management and analysis

All data were checked against the standards and methods were used to perform the study. Data was entered in Microsoft Excel spreadsheet and analyzed using STATA Version 12. Descriptive statistics such as means, percentage and frequencies were computed to report desired outputs. The antimicrobial resistance test was analyzed using WHONET software version 5 statistical package

. (http://www.who.int/medicines/areas/rational_use/AMR_WHONET_SOFTWARE/en/). Analysis of Variance (ANOVA) was used to test the significant difference at p < 0.05.

RESULTS

Enumeration of total viable

The overall mean viable bacterial count recorded was 8.24×107 . The individual sample type mean viable count and standard deviation of milk shop, fruit juice and dairy milk is indicated in Table 2.

Isolation and Identification of organism

Among the total 258 raw cow milk and fruit juice samples

Sample type	Mean bacterial count	±SD	Minimum bacterial count	Maximum bacterial count
Milk shop	8.86 ± 10^7	33.87 ±10 ⁶	1.5 ± 10^7	1.25 ± 10^8
Fruit juice	7.2 ± 10^7	6.68 ± 10^{6}	6.37 ± 10^7	8.5 ± 10^7
Dairy milk	8.65 ± 10^7	22.0 ± 10^{6}	6.4 ± 10^7	1.23 ± 10^8
Total	8.24 ± 10^7	23.8 ± 10^{6}	1.5 ± 10^7	1.25 ± 10^8

Table 2. Total viable S. aureus count for different sample types.

SD= standard deviation.

Table 3. Rate of isolation of S. aureus from raw cow milk and fruit juice samples.

Sample type	Number positive (%)	X ²	p-value
Milk shop (n=86)	20 (23.26)		
Fruit juice (n=86)	32 (37.21)	4 2007	0 1 1 1
Dairy milk (n=86)	23 (26.74)	4.3907	0.111
Overall (n=258)	75 (29.07)		

collected from different sources of Mekelle sub city, 75 (29.07%) samples were found positive for *S. aureus.* Proportion of the isolation from milk shop, fruit juice and dairy milk samples is described (Table 3). A statistically significant difference (χ 2=4.3987, P-value =0.111) was recorded among samples from the three sites (Table 3 and Figure 1).

Antimicrobial susceptibility profile of S. aureus

The antimicrobial resistance profile of the bacterial isolates from raw cow milk and fruit juice samples are summarized in Table 3. *S. aureus* showed high resistance to antibiotics like, vancomycin (100%), ampicillin (90.9%). ciprofloxacin (90.9%), ceftaroline (63.6%), pencillin-G (81.8%) and clindamycin (72.7%) whereas it was highly susceptible to some antibiotics like, gentamicin (100%), streptomycin (81.8%), norfloxacin (63.6%), chloramphenicol (81.8%), sulfamethoxazole (96%), kanamycin (72.7%), polymixin B (72.7%), erythromycin (72.7%) and tetracycline (81.8) (Table 4 and Figure 2).

The multi-drug resistance pattern of the bacterial *S. aureus* isolates is presented in Table 5. In general, antimicrobial susceptibility test revealed that gentamicin, streptomycin, norfloxacin, chloramphenicol, sulfamethoxazole, kanamycin, polymixin B, erythromycin, and tetracyclin were the antimicrobials indicated as active against *S. aureus* isolated from this study.

A total of 10 multiple drug resistance patterns were observed. The highest multiple drug resistance (MDR) noted was PEN, VAN, CIP and AMP (45.5%). The maximum multiple drug resistance registered was resistant to three and four antibiotics with the combination PEN, VAN, CIP and AMP.

DISCUSSION

The current finding indicates that samples from milk shop, fruit juice and dairy milk were found with a viable staphylococcal count load of 8.86×107 , 7.2×107 and 8.65×107 cfu/ml respectively with an overall mean viable bacterial count of 8.24×107 cfu/ml. The highest mean value of microbial load (8.86×107 cfu/ml) was found from milk shop samples.

The current study was found with higher viable staphylococcal count than previous reports such as viable bacterial count from raw cow milk and fresh fruit juice samples in 6.0×103 cfu/ml to 2.5×105 cfu/ml of (Lucky et al., 2016) in Bangladesh, 1.4×104 cfu/ ml (Tasnim, 2016) in Dhaka city; Staphylococcal count was from fresh vended fruit juices.

In the present study, out of 258 samples, 75 (29.07%) samples were found to be positive for S. aureus of which 20 (23.26%) were from milk shop, 32 (37.21%) from fruit juice and 23 (26.74%) from dairy milk. The result shows a high contamination rate, which might be attributed to poor hygeinic sanitation and handling improper. Statistically significant difference (P< 0.05) among the sample types in the prevalence of S. aureus was recorded. A similar report was also made by previous researchers (Abebe et al., 2013; Reta et al., 2016) that S. aureus was 15.5 and 24.2% in raw milk samples respectively in Ethiopia and in the other parts of the world which is contrary to this; different literatures revealed a very significant isolation rate of S. aureus from raw milk samples (Olatunji et al., 2009; Pourhassan and Taravat-Najafabadi, 2011; Mohanty et al., 2013; Sanaa et al., 2005).

The present overall isolation rate in raw cow milk products was 29.07% which seems to be higher than the findings of 6.25% by Thaker et al. (2013), 17% in Egypt (El-Gedawy et al., 2014), 18.2% in Turkey (Ekici et al.,



Figure 1. Map of the study area.

2004), 20.8% in Turkey (Aydin et al., 2011) and 21% in Iran (Ahmadi et al., 2009); were previously reported in milk samples collected from dairy farms. The present finding was much higher compared to the finding reported previously (Santana et al., 2010; Ekici et al., 2004) which found 17.39 and 18.18%, respectively; 4 (8%) of pasteurized milk samples, 9 (18%) of traditional butter samples and 12 (24%) of traditional cheese samples (Mirzaei et al., 2011) in Sarab whereas the new finding was much lower compared to the finding reported; 40% (Zakary et al., 2011), 22.5% (Hamid et al., 2017), 61.70% (Lingathurai and Vellathurai, 2011) and 44% (Mirzaei et al., 2011) from raw milk samples in Sarab.

The variation could be due to the reason that even when drawn under aseptic condition, milk always contains microorganisms which are derived from the milk ducts in the udder. In addition, contaminants coming from milking utensils, human handlers, unclean environmental conditions and poor udder preparation might expose raw milk to bacterial contamination.

Moreover, the present study showed the resistance of *S. aureus* to vancomycin (100%), ampicillin (90.9%), ciprofloxacin (90.9%), ceftaroline (63.6%), pencillin-G (81.8%) and clindamycin (72.7%) whereas was highly susceptible to some antibiotics like, gentamicin (100%), streptomycin (81.8%), norfloxacin (63.6%),

chloramphenicol (81.8%), sulfamethoxazole (96%), kanamycin (72.7%), polymixin B (72.7%), erythromycin (72.7%) and tetracycline (81.8) (Table 4). Similarly, the present investigation indicated that the resistance pattern of penicillin was found to be 93.1% by Melese et al. (2016) which is similar to the finding (87.2%) of Tariku et al. (2011) in Ethiopia, 80% in Sweden (Landin, 2006), 57% in Iran (Gooraninejad et al., 2007) and 50% in Finland (Myllys et al., 1998). This is in contrast to findings observed by (Adesiyun, 1994) who reported 23% of resistance to penicillin-G in West India.

The highest drug resistance recorded in the current study migh be due to high antimicrobial use in dairy farms and individual cows, to treat various diseases affecting the dairy sector. Similarly, several studies have indicated that *S. aureus* isolated from raw milk showed high resistance to vancomycin, ampicillin, ciprofloxacin, penicillin-G followed by ciprofloxacin and clindamycin thus indicating the safety of food products. However, few numbers of isolates exhibited resistance towards ampicillin (Thaker et al., 2013).

Antibiotic resistance development among the bacteria poses a problem of concern. Effectiveness of current treatments and ability to control infectious diseases in both animals and humans may become hazardous.

Different researchers reported antimicrobial resistant S.

Antibiotic	%R	%	%S	%R 95%C.I.
Penicillin G	81.8	0	18.2	47.7-96.8
Ampicillin	90.9	0	9.1	57.1-99.5
Cefoxitin	63.6	0	36.4	31.6-87.6
Gentamicin	0	0	100	0.0-32.1
Kanamycin	18.2	9.1	72.7	3.2-52.3
Streptomycin	18.2	0	81.8	3.2-52.3
Ciprofloxacin	90.9	0	9.1	57.1-99.5
Norfloxacin	0	0	100	0.0-32.1
Sulfamethoxazole	27.3	9.1	63.6	7.3-60.7
Clindamycin	72.7	0	27.3	39.3-92.7
Polymixin B	27.3	0	72.7	7.3-60.7
Erythromycin	9.1	18.2	72.7	0.5-42.9
Vancomycin	100	0	0	67.9-100
Chloramphenicol	0	18.2	81.8	0.0-32.1
Tetracycline	18.2	0	81.8	3.2-52.3

Table 4. Antimicrobial resistant of S. aureus isolated from raw cow milk and fruit juice of sample.

R= resistance, I= intermediate, S= susceptibility.



Figure 2. Antibiotic sensitivity pattern of S. aureus isolated from different samples.

aureus isolates of raw milk in their previous studies from Ethiopia. Reports from other researchers had also indicated S. aureus isolates resistance to vancomycin (100%), ampicillin (90.9%), ciprofloxacin (90.9%). ceftaroline (63.6%), penicillin-G (81.8%) and clindamycin (72.7%). Similar result was most frequently observed for penicillin (100%), ampicillin (100%), followed by erythromycin (95.7%) (Wang et al., 2014) in China, penicillin (100%), ampicillin (96%), amoxicillin (92%), and trimethoprim- sulphamethoxazole (88%) (Beyene, 2016) in Ethiopia, whereas the percent were found higher compared to what was reported by Hamid et al. (2017) of 94.4, 83.3 and 50% resistance for penicillin, ampicillin and ceftriaxone, respectively of *S. aureus* isolates with particular emphasis on penicillin G. The present observation agrees with preliminary finding conducted by Reta et al. (2016) of 93.1%. This is in accordance with the findings of Tariku et al. (2011) who reported resistance of *S. aureus* to chloramphenicol (16%), vancomycin (3%) it lower with compare the present finding while as (Khakpoor et al., 2011) in other study reported that all isolate of *S. aureus* from mastitis cattle were resistant to penicillin and 83.3% resistance to ampicillin in this study is much higher than that of 41.44%

Number of AMR	MDR profile	% Isolates
1	VAN CIP	9.1
	VAN CIP AMP	9.1
2	PEN VAN AMP	9.1
1	PEN VAN CIP AMP	45.5
	PEN VAN CIP AMP STR	9.1
2	CHL PEN VAN CIP AMP	9.1
1	CHLPEN VAN CIP AMP STR	9.1

 Table 5. Multi-drug resistance of S. aureus isolated from raw cow milk and fruit
 Juices sample.

VAN= Vacomycin; PEN= penicillin G; AMP= ampicillin; STR= Streptomycin; CHL = chloramphenicol; CIP = ciprofloxacin.

as reported by (Mubarack et al., 2012) and lower as compared to 100% resistance reported by (Khakpoor et al., 2011). The current study was highly susceptible to some antibiotics like, gentamicin (100%), streptomycin (81.8%), norfloxacin (63.6%), chloramphenicol (81.8%), sulfamethoxazole (96%), kanamycin (72.7%), polymixin B (72.7%), erythromycin (72.7%) and tetracycline (81.8); similar results were found for chloramphenicol. streptomycin and gentamycin (100, 94, and 90%), respectively by Beyene (2016) in Ethiopia but disagree with the observation made by Kassaify et al. (2013) in the case of streptomycin (95%), but it disagree with the observation made by Tariku et al. (2011) in the case of tetracycline (0%) and clindamycin (4%) in dairy farms in Jimma town, cefoxitin (100%) and clindamycin (each 100%) (Wang et al., 2014) in China. The probable explanation could be that S. aureus strains have the capacity to change their resistance behavior to the exposed antimicrobials.

The emergence degree of resistance to many drugs represents public health hazard due to the fact that food borne outbreaks would be difficult to treat and this pool of MDR S. aureus in food supply represents a reservoir for communicable resistant genes. The reason for the existence of antimicrobial resistant salmonella isolates could be due to the indiscriminate use of antimicrobials, self-medication, administration of sub therapeutic dose of antimicrobials to livestock for prophylactic purpose (Szyfers and Acha, 2001) and limited updating of the long used drug groups. Hence, due to the relatively limited access and high price to get the newly developed cephalosporin and quinolone drugs, the reports of prevalence of antimicrobial-resistant to relatively lowpriced and regularly available antibiotics are alarming for a low-income society living in most developing countries, like Ethiopia.

Conclusion

The current study showed insights into the magnitude and incidence of *S. aureus* from raw cow milk and fresh fruit juice samples. The study revealed the development of antibiotic resistant against *S. aureus* which could pose serious threat for consumers as well as for health professionals in the study area. Hence, attention should be given to proper handling of the food items, and using recent antibiotics in the treatment of diseases both in humans and animals.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

Abbreviations: AMP, Ampicillin; ANOVA, analysis of variance; CHL, Chloramphenicol; CIP, Ciprofloxacin; ERY, Erythromycin; I, Intermediate; MDR, multiple drug resistance; NSS, normal saline solution; R, resistant; S, Sensitive; SD, standard deviation, O, Observation; STR, Streptomycin; TCY, Tetracycline.

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