

## Staphylococcus aureus and Methicillin Resistant S. aureus in Commercial Soft Drink with Antimicrobial Resistance Test on Isolates in Ethiopia

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### Abstract

*Staphylococcus aureus* is common food contaminant and responsible for food poisoning. The aim of this study was to assess prevalence of *S. aureus* and Methicillin Resistant *S. aureus* (MRSA) in commercial soft drinks (CSDs) with antimicrobial resistance test on the isolates in two towns of Ethiopia. A total of 774 samples of ready-to-drink CSDs were randomly purchased from different public supply locations and analyzed for *S. aureus*. MRSA were screened phenotypically using Cefoxitin (FOX 30 µg) disk diffusion method. All of studied CSD products were within manufacturer shelf life. An overall 28 (3.6%) and 5 (0.6%) *S. aureus* and MRSA were observed, respectively. Similar prevalence of *S. aureus* ranging from 1.0-4.8% and 1.2-6.6% were observed by product category and by public supply location, respectively. Significantly higher (10.7%; OR=12, 95%OR CI: 6.1-23.7) *S. aureus* in carton box packed than in glass bottle canned (2.3%) and metal canned (2.4%) products were observed. *S. aureus* prevalence was higher in CSD products from Bangladesh (17.9%; OR=21.6, 95% OR CI: 10.3-45.6) and Portugal (8.9%; OR=9.8, 95% OR CI: 3.6-26.2) than from Ethiopia (1.3%; OR=1.3, 95% OR CI: 0.5-3.3). Prevalence of MRSA ranged from 0-6.7% within studied variable. High (64.3%) *S. aureus* were resistant to erythromycin followed by 32.2% to ampicillin. Equal, (21.4%) of *S. aureus* were resistant to streptomycin, amoxicillin and chloramphenicol. All of MRSA were resistant erythromycin. Equal, 4 (80.0%) MRSA were resistant to amoxicillin and chloramphenicol. No resistant *S. aureus* and MRSA were observed to gentamycin and trimethoprim-sulfamethoxazole. All MRSA isolates were also not resistant to ciprofloxacin. Eighteen *S. aureus* isolates were resistant to at least single to multiple of six drugs used in the study. Data indicated possible contamination of CSD products with *S. aureus* and MRSA including MDR strain during processing and post-process handling.

**Keywords:** Commercial soft drinks; *S. aureus*; Methicillin resistant; Antimicrobials resistance; Ethiopia

### Introduction

Processed commercial soft drinks are industry products canned and bottled beverage. They are either energy drink, juice or water ready-to-drink products getting popularity in food sectors [1]. They are produced and transported to a distance location to supply consumer under different handling conditions [2,3]. Risk of warmer weather condition and inadequate refrigeration were the principal causes of higher levels of contamination, increased diversity and change in microbial flora in the commercial product [2]. Range of spoilage microbial was reported from home-made tiger-nut beverages in Spain [3]. Abundant yeasts and fungi were isolated from yoghurts in Brazil [2]. The possible presences of some public health hazard microbial having most likely health threat in such processed products, including in the acidic products like juice, were also suggested, due to good acid-tolerance of such microbial [1-5]. Whether the products are produced by commercial or traditional methods, non-alcoholic beverages have risk of contamination with spoilage and public health pathogenic microbial [1-8].

Risk of contamination of bottled water and juices has also been reported. Presence microbial in and with contamination risk associated to traditional beverage in Ethiopia as also been reported by Ashanafi [4]. *Staphylococcus aureus* (*S. aureus*) are common contaminant food and responsible for food poisoning [9-13]. Again, there are also increasing public health impact of methicillin resistant *S. aureus* (MRSA) associated with food [14,15]. According to Karami et al. [16] phenotypic disk diffusion assays using cefoxitin had high sensitivity and specificity for detection of MRSA. However, investigation on occurrence of *S. aureus* and MRSA in commercial soft drink in Ethiopia was not yet assessed. The objective of this study was to assess the prevalence of *S. aureus* and MRSA in industrially produced commercial soft drink found on the markets of Ethiopia with antimicrobial resistance test on the isolates.

### Materials and Methods

#### Study design

A cross-sectional study was employed to assess the occurrences of *S. aureus* and MRSA in commercial soft drinks available on the Chiro and Dire Dawa towns markets with drug resistance test on the isolates in Ethiopia.

#### Study area location

Study was conducted in Chiro (9°05'-9°08'N, 40°52'E-40°87'E) and Dire Dawa (9°27'-9°49'N, 41°38'-42°19'E) towns located at about 350 km and 556 km distance from Addis Ababa, the Capital City, respectively in the eastern part of Ethiopia. The average annual temperature of Chiro with 33,670 population and Dire Dawa with 341,834 population was 20.2°C and 24.6°C, respectively [17,18].

#### Sample size calculation

Sample size was calculated using 50% expected prevalence of *Staphylococcus aureus* in commercial soft drinks within 95% Confidence Intervals (CI) at 5% desired precision, according to the Thrusfield formula [19].

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Received March 11, 2018; Accepted April 17, 2018; Published April 24, 2018

Citation: Hiko A (2018) *Staphylococcus aureus* and Methicillin Resistant *S. aureus* in Commercial Soft Drink with Antimicrobial Resistance Test on Isolates in Ethiopia. J Microb Biochem Technol 10: 40-45. doi: [10.4172/1948-5948.1000393](https://doi.org/10.4172/1948-5948.1000393)

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

Where, n: Required sample size; d=desired absolute precision;  $P_{exp}$ : Expected prevalence (50%)

The minimum of 384 samples for each town which becomes 768 for both, but 774 samples were collected and examined.

### Sampling and sample handling

Simple random sampling techniques were applied at public supply location in selected study towns. Whole volume of the sampled product, as it was canned, were randomly purchased. Information regarding expire date, product type, producing countries, source town, public supply locations and types of packaging material were immediately registered for each sample using specific identification number. Samples were immediately transported to Veterinary Microbiology Laboratory, College of Veterinary Medicine, Haramaya University on the day of sampling using ice box at +4°C for laboratory examination.

### Description of studied products and considered risk factors

The studied products were commercial soft drinks those packed into a volume of 125 ml to 1000 ml and available on the market. They were randomly purchased at points of public supply locations. As shown in Table 1, the available sampled products were described considering factors used for the study using expire date, product type, packaging material, source town, public supply locations and the producing county.

Studied risk factors		Total No. of samples	Frequency
Expire date	Within shelf life	774	100
	Expired	0	0
Product category*	Energy drink (pH=2.50-3.70)	195	25.2
	Juice drink (pH=3.3-4.19.)	373	48.2
	Bottled water (pH=7.0-7.2)	206	26.6
Packaging material	Carton box	84	10.9
	Glass bottle	259	33.5
	Metal can	246	31.8
	Plastic bottle	185	23.9
Public supply locations	Hotel	130	16.8
	Open Market	152	19.6
	Restaurants	166	21.4
	Street	156	20.2
	Supermarkets	170	22.0
Source town	Dire Dawa town	385	49.7
	Chiro town	389	50.3
Country of origin	Bangladesh	45	5.8
	Egypt	60	7.8
	Ethiopia	317	41.0
	Portugal	45	5.8
	Saud Arabia	49	6.3
	Thailand	120	15.5
	UAE Dubai	69	8.9
	Yemen	69	8.9
Total		774	100

\*Note: The pH is indicated in that different types of products having different pH were examined

**Table 1:** Description and proportional distribution the sampled commercial soft drink available on the studied towns by studied risk factors.

### *Staphylococcus aureus* isolation and characterization

Using 1:10 ml enrichment procedure [5,20], each sample was enriched at proportion of 1 ml sample to 9 ml of Buffered Peptone Water (BPW) and incubated for 24 h at 37°C. A loop full of enrichment was streaked on 7% heparinized sheep blood based agar plate (Oxoid) and incubated at 37°C for 24 h under aerobic condition. Presumptive *Staphylococcus* colony was determined based on its opaqueness, whitish or creamy, grayish or yellowish colonies and beta hemolysis morphological characteristic [21]. Suspected colonies were further gram stained. Those with gram positive grape like colonies were transferred on mannitol salt agar and incubated at 37°C for 24 h. The characteristic colony were transferred on to nutrient agar and incubated at 37°C for 24 h. Biochemical test including catalase test, oxidase test, coagulase test by using rabbit plasma and DNase test to detection of DNase activity (for detection of deoxyribonuclease enzyme) were conducted to confirm *S. aureus* [22].

### Methicillin resistant *Staphylococcus aureus* (MRSA) identification

All isolates of *S. aureus* were tested using Kirby-Bauer disk diffusion method for Cefoxitin (FOX/30 µg) [23-25]. According to Karami et al. [16], phenotypic cefoxitin disk diffusion assays methods had high sensitivity and specificity for detection of MRSA. On the other hand, Methicillin was bane from market. Thus, MRSA was identified phenotypically by using cefoxitin disk diffusion method [24,25]. Overnight freshly grown *S. aureus* cultures were inoculated into 5 ml Brain Heart Infusion Broth (Merck, Germany). The inoculated broth was incubated for 4 h to approximately 10<sup>6</sup> CFU/ml at McFarland 0.5% level of turbidity. The bacterial lawn was evenly spread on Mueller Hinton agar (Oxoid, UK) using sterile cotton swabs. The culture was incubated 37°C for 24 h. Zone of bacterial growth inhibition was measured using caliper. The isolates were then classified susceptible, intermediate or resistant. Isolates with inhibition zone diameter of ≤ 21 mm was registered as methicillin resistant and determined as MRSA while those with ≥ 22 mm diameter of inhibition were reported as methicillin sensitive (none-MRSA) [24].

### Antimicrobial susceptibility test

Antimicrobial susceptibility test was done on all of 72 isolates by the Kirby-Bauer disk diffusion method [23,24]. The tested bacterium was taken from an overnight freshly grown culture and inoculated into 5 ml Brain Heart Infusion Broth (Merck, Germany). The inoculated broth was incubated for 4 h at 37°C to approximately 10<sup>6</sup> CFU/ml at McFarland 0.5% level of turbidity. With this culture, a bacterial lawn was spread on Mueller Hinton agar (Oxoid, UK). Antimicrobials against disk (Oxoid, UK) including amoxicillin (AML 30 µg), ampicillin (AMP 10 µg), chloramphenicol (C 30 µg), gentamycin (CN 30 µg), ciprofloxacin (CIP 10 µg), cefoxitine (FOX 30 µg), erythromycin (ET 15 µg) streptomycin (S 10 µg) and trimethoprim-sulfamethoxazole (SXT 1.25/23.75/µg) were mounted. The culture was incubated 37°C for 24 h. Result was interpreted using the diameter of zone of bacterial growth inhibition surrounding the disc [23,24].

### Data analysis

Data was entered into Microsoft Excel 2013© and calculated using Stata Ver. 11, SPSS 20 (IBM Corp., Armonk, NY) and WinPepi 11.35 (Abramson JH). The results were described in frequency distributions and percentages for which the 95% Confidence Interval (CI) Mid-prevalence exact were calculated. Odds ratio (OR) and 95% OR CI were also calculated determine the significance differences within studies factors.

## Results

As shown in Table 1, all of studied products are within manufacturer shelf life. The majorities (48.2%) are juice drink. Of the studied sample, relatively low (10.9%) were those packed in carton box? Most of them are of products of Ethiopia (40.0% followed by the Thailand (15.5%). However, the samples were evenly distributed at all public supply location and both study towns.

### *Staphylococcus aureus* and MRSA prevalence

An overall 28 (3.6%) of sample was positive for *S. aureus*. Total prevalence of MRSA was 5 (0.6%). Prevalence of *S. aureus* ranged from 1.0-4.8% by product category and 1.2-6.6% by public supply locations with no significant differences. Significantly higher (10.7%, 95% CI: 5.7-19.1; OR=12, 95% OR CI: 6.1-23.7) in product packed in carton box than in those packed with glass bottle (2.3%) and metal can (2.4%) were observed. Considering countries of product origin, *S. aureus* was significantly higher in those from Bangladesh

(17.9%, 95% CI: 5.7-19.1; OR=21.6, 95% OR CI: 10.3-45.6) and Portugal (8.9%, 95% CI: 3.5-20.7; OR=9.8, 95% OR CI: 3.6-26.2) than from Ethiopia (1.3%, 95% CI: 0.5-3.2; OR=1.3, 95% OR CI: 0.5-3.3). With regards to MRSA within studied variable, 0-6.7% were observed (Table 2).

### Antimicrobial resistance pattern

As shown in Table 3, high (64.3%) of *S. aureus* were resistant to erythromycin followed by 32.2% to ampicillin. Equal proportions (21.4%) of *S. aureus* isolates were resistant to streptomycin, amoxicillin and chloramphenicol. Resistant isolates were not observed against gentamycin and trimethoprim-sulfamethoxazole.

Five isolates (17.9%) of the total were resistant to cefoxitine and identified as MRSA. All of MRSA were also resistant erythromycin. Equally 4 (80.0%) MRSA were resistant to amoxicillin and chloramphenicol. In contrast, no resistant MRSA were observed to gentamycin, trimethoprim-sulfamethoxazole and ciprofloxacin (Table 3).

Studied risk factors	No. of samples	<i>Staphylococcus aureus</i> positive				MRSA positive No. %	
		No. (%) positive	95% CI percentage	OR	95% OR CI		
Product category	Energy drink	195	8 (4.1)	2.1-7.9	4.1	2.1-8.6	1 (0.5)
	Juice drink	373	18 (4.8)	3.1-7.5	5.1	3.2-8.1	4 (1.2)
	Bottled water	206	2 (1.0)	0.0-3.5	1	0.3-3.6	0 (0)
Packaging material	Carton box	84	9 (10.7)	5.7-19.1	12	6.1-23.7	2 (2.4)
	Glass bottle	259	6 (2.3)	1.1-4.9	2.4	1.1-5.2	2 (0.8)
	Metal can	246	6 (2.4)	1.1-5.2	2.5	1.1-5.5	0 (0)
	Plastic bottle	185	7 (3.8)	1.8-7.6	3.9	1.9-8.2	1 (0.5)
Public locations supply	Hotel	130	2 (1.5)	0.2-5.4	1.3	0.4-5.8	0 (0)
	Open Market	152	10 (6.6)	3.6-11.7	7.0	3.8-13.2	2 (1.3)
	Restaurants	166	5 (3.0)	1.3-6.9	3.1	1.3-7.4	1 (0.6)
	Street	156	9 (5.8)	3.1-10.6	6.1	3.1-11.9	1 (0.6)
	Supermarkets	170	2 (1.2)	0.3-4.2	1.2	0.3-4.4	1 (0.6)
Source town	Dire Dawa	385	17 (4.4)	2.3-6.9	4.6	2.8-7.5	3 (0.8)
	Chiro town	389	11 (2.8)	1.6-5.0	3.0	1.6-5.3	2 (0.5)
Country of origin	Bangladesh	45	8 (17.8)	9.3-31.3	21.6	10.3-45.6	3 (6.7)
	Egypt	60	2 (3.3)	0.9-11.4	3.5	0.9-12.8	0 (0)
	Ethiopia	317	4 (1.3)	0.5-3.2	1.3	0.5-3.3	1 (0.3)
	Portugal	45	4 (8.9)	3.5-20.7	9.8	3.6-26.2	0 (0)
	Saud Arabia	49	4 (8.2)	3.2-19.2	8.9	3.3-23.8	1 (2.0)
	Thailand	120	3 (2.5)	0.9-7.1	2.6	0.9-7.6	0 (0)
	UAE Dubai*	69	2 (2.9)	0.8-9.9	3.0	0.8-11.1	0 (0)
	Yemen	69	1 (1.5)	0.3-7.8	1.5	0.6-8.4	0 (0)
Total	774	28 (3.6)	2.5-5.2	3.7	2.6-5.5	5 (0.6)	

Note: \*UAE: United Arab Emirate

**Table 2:** Prevalence of *Staphylococcus aureus* and MRSA in commercial soft drink purchased from the two towns by studied risk factors.

Antimicrobial agents		<i>Staphylococcus aureus</i> (n=28)*			MRSA (n=) R. No. %**
Name of drug	Abbreviation	S. No. (%)	I. No. (%)	R. No. (%)	
Erythromycin	ET	8 (28.6)	2 (7.1)	18 (64.3)	5 (100)
Ampicillin	AMP	17 (60.7)	2 (7.1)	9 (32.2)	2 (40.0)
Streptomycin	S	20 (71.4)	2 (7.1)	6 (21.4)	2 (40.0)
Amoxicillin	AML	18 (64.3)	4 (14.2)	6 (21.4)	4 (80.0)
Chloramphenicol	C	21 (75.0)	1 (4.6)	6 (21.4)	4 (80.0)
Cefoxitine	FOX	23 (82.1)	0	5 (17.9)	5 (100) <sup>†</sup>
Gentamycin	CN	24 (85.7)	0	4 (14.3)	0
SXT***	SXT	28 (100)	0	0	0
Ciprofloxacin	CIP	28 (100)	0	0	0

Note: \*S: Susceptible; I: Intermediate R: Resistant; \*\*No intermediate were observed; \*\*\*Trimethoprim-sulfamethoxazole; <sup>†</sup> re-tested for cefoxitine

**Table 3:** Antimicrobial susceptibility pattern of *Staphylococcus aureus* and the MRSA isolated from commercial soft drink products.

Out of the 28 tested *S. aureus*, 18 (46.3%) were resistant against one or more of the drug used in this study. As shown in Figure 1, seven of 28 tested *S. aureus* isolates were two drug resistant. Out of the 5 MRSA isolates, two each isolates were resistant to three and five drugs.

Out of 18 resistant *S. aureus* isolates, 2 (11.1%) were resistant erythromycin only as to single drug resistant. As high as six drugs, erythromycin, streptomycin, ampicillin, amoxicillin, chloramphenicol and cefoxitine resistant isolates were 2 (11.1%). With regards to resistant MRSA, two each isolates were resistant to combination three (amoxicillin, chloramphenicol and erythromycin) and five (erythromycin, streptomycin, ampicillin, amoxicillin, chloramphenicol) drugs (Table 4).

## Discussion

Food contamination with spoilage and/or public health risk pathogenic microbial could occur at any production and processing stage. The contaminate are responsible for quality deterioration, foodborne infections and intoxication, resulting in economic lose and health [5,26]. Although all of studied products are within manufacturer shelf life, the present 3.6% *S. aureus* positive commercial soft drink sample indicated the risk associated consumer infection in Ethiopia. The present finding was lower than the 34.15% *S. aureus* count positive samples soft drinks [27]. Staphylococcal count of 0 to  $2.3 \times 10^3$  CFU/ml

in juices from Dhaka City was also reported [28]. On the other hand, Aljaloud et al. [29] reported absence Staphylococcus in energy drink from Raid-Saud Arabia. Cutter also suggested the use of combination of rigid packaging materials made from metal, glass or plastic provides the heat as most effective inactivating microorganisms during product processing [30]. But, the presence of *S. aureus* at higher proportion (10.7%) in carton box packed products than in those packed with glass bottle (2.3%) and metal can (2.4%) could be either due to the number of sample (48.2% of studies samples are juice drink) or the presence of high nutrients level for microbial growth. Presence of high nutrients level for microbial growth and the less microbial hurdles properties of many modern soft drinks were suggested factors for microbial survival and multiplication in the products [1,26].

Although high number of sampled product from Ethiopia (40.0%) and Thailand (15.5%), present finding indicated those imported from Bangladesh (17.8%), Portugal (8.9%) and Saud-Arabia (8.2%) are highly contaminate with *S. aureus* than those manufactured in Ethiopia (1.3%). The present finding indicated the presence of *S. aureus* in products from Saud-Arabia (8.2%) as the counter zero reports of Aljaloud et al in energy drink [29]. This could be either due to differences in the types of studied product, and multiplication of the few microbial present in product during packaging, while distance transportation of the products to Ethiopia [30]. Akond et al. [31] also indicated the posing

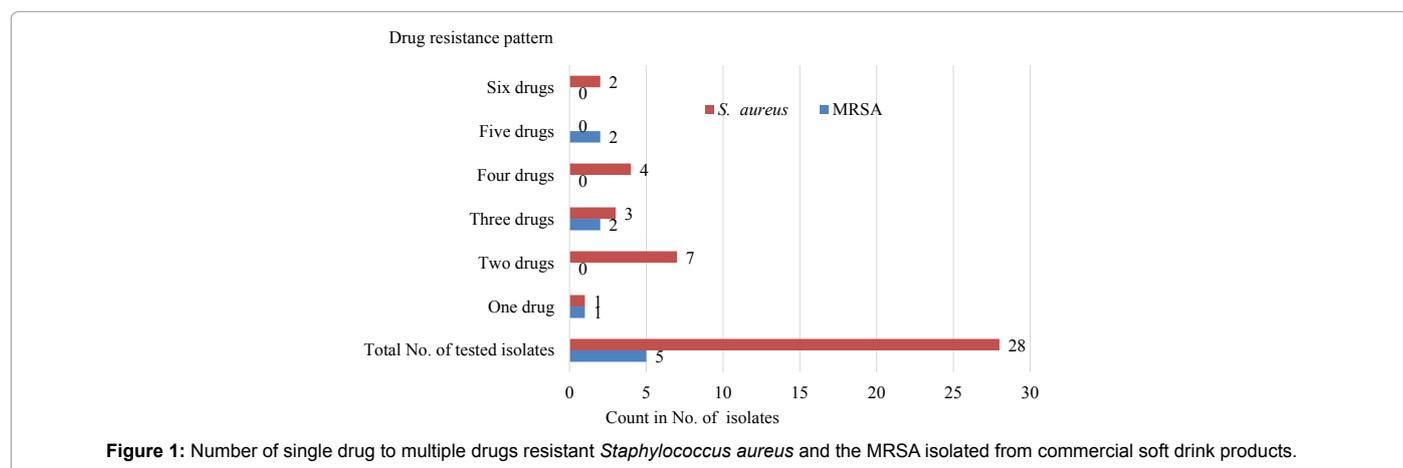


Figure 1: Number of single drug to multiple drugs resistant *Staphylococcus aureus* and the MRSA isolated from commercial soft drink products.

Isolates	Resistance pattern ** and the type of drug/s combination	No. (%)	
<i>S. aureus</i> (n=18)*	Single drug	Erythromycin	2 (11.1)
	Two drugs	Ampicillin, erythromycin	2 (11.1)
		Chloramphenicol, erythromycin	1 (5.5)
		Gentamycin, erythromycin	3 (16.7)
		Erythromycin, cefoxitine cefoxitine	1 (5.5)
	Three drugs	Ampicillin, amoxicillin, erythromycin	1 (5.5)
		Ampicillin, erythromycin, streptomycin	2 (11.1)
	Four drugs	Ampicillin, chloramphenicol, erythromycin, streptomycin	1 (5.5)
		Amoxicillin, chloramphenicol, erythromycin, cefoxitine	2 (11.1)
		Ampicillin, gentamycin, erythromycin, streptomycin	1 (5.5)
Six drugs	Erythromycin, streptomycin, ampicillin, amoxicillin, chloramphenicol, cefoxitine	2 (11.1)	
	Total	18 (100)	
MARS (n=5)	Single drug	Erythromycin	1 (20.00)
	Three drugs	Amoxicillin, chloramphenicol, erythromycin	2 (40.0)
	Five drugs	Erythromycin, streptomycin, ampicillin, amoxicillin, chloramphenicol	2 (40.0)
		Total	5 (100)

Note: \*the 18 *S. aureus* are including MRSA isolates; \*\*no five drug resistant *S. aureus*, No two drugs and four drugs resistant MRSA isolate

Table 4: Distribution of single drug to multiple drugs resistant *Staphylococcus aureus* and the MRSA isolated from the studied commercial soft drink products.

substantial public health risks of commercial soft drinks in Bangladesh. The relatively higher and similar *S. aureus* prevalence in products from open market shop (6.6%), and street (5.8%) than in those from hotels, restaurant and supermarkets could be due to differences in handling of the products where the former's sale products under ambient environmental temperature but the latter use refrigeration. Moreira et al. [3] also suggested the contribution of warmer weather and inadequate refrigeration for high levels of contamination as well as diversity and flora of microbial in the commercial product. Moreover, handling the products under uncontrolled temperature could increase multiplication of agent and also responsible for deterioration of the product quality. Juvonen et al. [1] suggested microbiological spoilage of commercial products leads to the deterioration of the sensory quality and changes in the product. Isolation of 5 (0.6%) MRSA strain from commercial soft drinks in present study indicates the consumer risk significances of acquiring infection from such beverage. The risk associated with MRSA infection from food and hospital infection were also suggested [14,15].

Observing a range of (14.3%- 64.3%) resistant *S. aureus* isolates to most of the studied drugs showed the challenge with health risk of public acquiring resistant pathogen. Similar suggestions were given by Livermore [32], Zapun et al. [33], Wayne [34] and Divah et al. [35]. The present 32.2% *S. aureus* resistant to ampicillin was lower than the 42.9% [36]. The present 21.4% *S. aureus* isolates resistant to amoxicillin were lower than 48% and 62% reported in isolates from mastitis cow [37,38]. The present 21.4% *S. aureus* isolates were resistant to chloramphenicol were differed from the zero report of Dibah et al. [35]. Resistant isolates were not observed against gentamycin and trimethoprim-sulfamethoxazole.

Five (17.9%) of the total *S. aureus* isolates were resistant to cefoxitin and identified as MRSA. All of MRSA were also resistant erythromycin, equally 4 (80.0%) were resistant to amoxicillin and chloramphenicol. The present resistant profile of all MRSA to erythromycin was similar with the 84.2% reported by Dibah et al. [35]. The present 46.3% *Staphylococcus aureus* and the 5 MRSA isolates resistant to one or more drugs showed the possible risk of acquiring MDR isolates with these beverage [36-38]. The presence of such MRSA isolates were also reported where 28% were resistant to trimethoprim-sulfamethoxazole and 35% were resistant to sulfonamides [39,40]. MDR *Staphylococci* posing a growing problem for human health particularly MRSA, the virulent strains [32-34,41]. However, no resistant MRSA against gentamycin, trimethoprim-sulfamethoxazole and ciprofloxacin were observed in this study. Thus, regardless of other factors, these drugs are of choice.

## Conclusion

Staphylococcal contamination of the food product could originate either during the production process, from raw materials, factory environment, packaging equipment and lack of hygiene. The contaminate could be further multiply in the product while poor handling and transportation condition and becoming responsible for product spoilage and consumer health risk. Thus, quality control attention with good handling shall be made while processing, transportation and supply to consumers. The already methicillin resistant *S. aureus* strain showed resistance to one or more six drugs as MDR in this study. Under the present finding, gentamycin, trimethoprim-sulfamethoxazole and ciprofloxacin were drugs of choice for treatment of staphylococcal infection.

## Acknowledgement

Thematic research area leadership of Dr. Abebaw Adgo was appreciated.

## Funding

Haramaya University Research Grant (HURG) No. HURG-2014-03-04.

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