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Full Length Research Paper

Antibiotic-resistance *Staphylococcus aureus* isolated from cow's milk in Hawassa area, South Ethiopia

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Quarter milk samples from cows were examined to determine the prevalence of *Staphylococcus aureus* (SA) and different antibiotic resistant pattern were determined in a cross-sectional study design. The aim of this study was to isolate *Staphylococcus aureus* from samples of cow's milk obtained from Hawassa area and to determine their antibiotic susceptibility patterns. A total of 160 milk (CCP1 to CCP5) samples were collected and screened for the presence of *S. aureus*. Gram staining, oxidase, catalase, DNase, haemolysis and coagulase tests were employed for bacterial identification. All the samples were contaminated with *S. aureus*. A total of 78 *S. aureus* isolates were obtained during this study. The levels of contamination with *S. aureus* were higher in milk obtained from CCP1, CCP2, CCP3, CCP4 and CCP5 at Hawassa area farms (18.0, 25.6, 27.0, 21.8 and 7.7%) respectively. A large percentage of the *S. aureus* isolates (25.6 and 27.0%) were from CCP2 and CCP3. All strains were resistant to penicillin G (PG), ampicillin (AP), amoxicillin-clavulanic acid (AC), ciprofloxacin (CIP), erythromycin (E), Ceftriaxone (CRO), trimethoprime-salfamethoxazole (TMP-SMZ) oxacilin (Ox) and vancomycin (V), 67.9, 70.9, 30.9, 0, 32.1, 23.1, 7.7, 60.3 and 38.5% respectively. The proportion of isolates resistant to CIP, TMP-SMZ, CRO, AC, E and V were low compared to AP, PG and Ox. *S. aureus* is normally resident in humans; therefore, the *S. aureus* present in the cow's milk may have resulted from transmission between the two species, emphasizing the need to improve sanitary conditions in the milking environment.

Key words: Penicillin G (PG), ampicillin (AP), amoxicillin-clavulanic acid (AC), ciprofloxacin (CIP), erythromycin (E), ceftriaxone (CRO), trimethoprime-salfamethoxazole (TMP-SMZ), oxacilin (Ox), vancomycin (V).

INTRODUCTION

Staphylococcus aureus is a ubiquitous human pathogen and a common cause of invasive and life threatening infections. It is the most common cause of community-associated cellulitis (Diekema et al., 2001; Brook and Frazier, 1995). and endocarditis (Hoen et al., 2002), and is a common cause of bacteremia (Diekema et al., 2001; Javaloyas et al., 2002; Weinstein et al., 1997). *S. aureus* strains were once nearly uniformly susceptible to semi-synthetic penicillinase-resistant β-lactams (e.g. methicillin, oxacillin), the most commonly used class of antibiotics for skin infection. These strains were termed 'methicillin resistant *Staphylococcus aureus,* or MRSA, a term that implied cross-resistance to all β-lactams including all penicillins and cephalosporins.

Staphylococci are normal inhabitants of the skin and mucous membranes of animals and humans. Pathogenic strains are usually coagulase-positive (Mahon and Larsen, 1995) and have been found to cause disease in their hosts throughout the world (Matsunaga et al., 1993; Larsen et al., 2000). Diseases in cattle caused by

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Staphylococcus aureus range from simple abscesses and mastitis to the more severe toxic shock syndrome (Matsunaga et al., 1993; Larsen et al., 2000; Onasanya et al., 2003). Milk is an excellent growth medium for a large number of micro-organisms, including *S. aureus* (Kalsoom et al., 2004). Bacterial contamination of milk usually occurs during the milking process and this depends on the sanitary condition of the environment, utensils used for milking and the milking personnel. It could also result from micro-organisms that enter the udder through the teat opening canal (Kalsoom et al., 2004).

Antibiotic-resistant *S. aureus* isolates pose a severe challenge to both veterinary and health professions and dairy cattle producers because they have a negative impact on therapy (Sears and McCarthy, 2003; Brouillette and Malouin, 2005). The usage of antibiotics correlates with the emergence and maintenance of antibiotic-resistant traits within pathogenic strains (Shitandi and Sternesjö, 2004). These traits are coded for by particular genes that may be carried on the bacterial chromosome, plasmids, transposons or on gene cassettes that are incorporated into integrons (Rychlik et al., 2006), thus are easily transferred among isolates. Multiple antibiotic resistant *S. aureus* strains have been isolated from milk obtained from cattle, beef and human samples in many parts of the world (Shitandi and Sternesjö, 2004; Petinaki et al., 2001; Waage et al., 2002; Zhanel et al., 2005; Pesavento et al., 2007). The prevalence of antibiotic resistance usually varies between isolates from the different sampled stations and even between isolates from different herds on the same farm (Waage et al., 2002).

Determination of levels of *S. aureus* and an evaluation of the antibiotic-resistant phenotypes of the isolates could serve as a tool for determining the hygiene standards implemented during milking. Data on antibiotic resistance could also be used to characterize these opportunistic pathogens, which may further limit the risks associated with the consumption of contaminated milk and its products (Evenson et al., 1988). The aim of this study was to isolate *S. aureus* from milk obtained from farm level collected milk and further characterize these isolates using their susceptibility patterns to nine selected antibiotics.

METHODS AND MATERIALS

Area of the study and collection of samples

A cross-sectional study design was used to determine the bacteriological analysis of cow milk in Hawassa town. Cow's milk samples were collected from different farms in Hawassa area. This farm is situated in the Southern part of Ethiopia.

Samples of approximately 10 mL of fresh milk were collected from different critical control point (CCP1 to CCP5) milking containers and transferred into sterile sample collection bottles. The samples were immediately transported on ice to the Medical Microbiology Laboratory of the Hawassa University – Referral Hospital for analysis. Upon arrival in the Laboratory, samples were analyzed immediately.

Isolation of presumptive *S. aureus* from milk samples

Ten-fold serial dilutions were performed using 2% peptone water and aliquots of 100 µL from each dilution were spread plated onto mannitol salt agar (MSA) (Supplied by Oxoid Company). The plates were incubated aerobically at 37℃ for 18 to 24 h. Co nsequently, the 78 characteristic *S. aureus* colonies that were yellow in color from each MSA plate were further purified by sub-culturing onto MSA plates (supplied by Oxoid Company) and the plates were incubated aerobically at 37℃ for 18 to 24 h. These isolates were retained for further bacterial identification.

Bacterial identification

Gram staining was performed (Cruikshank et al., 1975) and Gram-positive cocci that occurred in clusters under the microscope were subjected to preliminary biochemical tests (the catalase and oxidase tests). The identities of the isolates were confirmed based on positive results for the DNase test, beta-haemolytic patterns on blood agar enriched with 5% (v/v) sheep blood and the coagulase slide test for *S. aureus* using the (PROLD Diagnostics, Canada). The slide agglutination test was performed according to the manufacturer's instructions. Briefly, cells from a pure colony were placed on the clean area of the slide using a sterile toothpick and a drop of the PROLD reagent was added. These were mixed using the toothpick and the isolates were identified based on the formation of agglutination. An isolates that formed agglutination were recorded as *S. aureus* and maintained at 4℃ in 30% glycerol for further characterization by antibiotic susceptibility testing.

Antibiotic susceptibility

Antibiotic susceptibility tests were performed on all *S. aureus* isolates, to determine their antibiotic-resistance profiles (Kirby et al., 1966). Fresh overnight cultures were prepared and used for antibiotic sensitivity tests. An aliquot (100 µL) from each isolate suspension was spread plated on Mueller Hinton agar (supplied by Oxoid Company). Susceptibilities of the isolates to a panel of nine different antibiotic discs (6 µm in diameter, Mast group LTD MERSEY SIDE, UK) were determined. The antibiotics tested were shown in Table 2 and were selected because large numbers of bacteria resistant to these had been documented in study performed in South Africa (Ateba and Bezuidenhout, 2008; Moneoang and Bezuidenhout, 2009). Antibiotic discs were gently pressed onto the inoculated Mueller Hinton agar to ensure intimate contact with the surface and the plates were incubated aerobically at 37℃ for 18 to 24 h (National Committee for Clinic al Laboratory Standards (NCCLS), 1999)]. Inhibition zone diameters were measured and values obtained from the National Committee on Clinical Laboratory Standards (National Committee for Clinical Laboratory Standards (NCCLS), 1999) (Table 2) were used to interpret the results obtained. *S. aureus* isolates were then classified as resistant, intermediate resistant or susceptible to a particular antibiotic. Multiple antibiotic resistant (MAR) phenotypes

Table 1. Details of the *S. aureus* isolates obtained from Hawassa area, South Ethiopia.

Sample source	HACCP level	No of sample collected	No of sample positive for *S. aureus*	Percentage of *S. aureus* isolates (level of contamination) with *S. aureus* per HACCP level (a)
Teat	CCP1	32	14	14 (17.9)
Bucket at farm level	CCP2	32	20	20 (25.7)
From storage containers at milk collection center	CCP3	32	21	21 (26.9)
From transportation container	CCP4	32	17	17 (21.8)
After cooling at the pasteurization plant	CCP5	32	6	6 (7.7)
			78	100

a Percentages were calculated from a total of 78 bacterial isolates studied using the biochemical identification method to determine those positive for *S. aureus.*

Table 2. Details of the antibiotics that were used in the study to test for antibiotic resistance.

Antibiotic	Abbreviation	Generally accepted antibiotic disc concentrations(µg)	Inhibition zone (mm)		
			R	I	S
Amoxicillin-Clavulanic Acid	AC	30	≤19	-	≥20
Erythromycin	E	15	≤ 13	14 to 22	≥ 23
Ciprofloxacin	CIP	5	≤ 15	16 to 20	≥ 21
Penicillin	PG	10	≤28	-	≥29
Ampicillin	AP	10	≤28	-	≥29
Vancomycin	VA	30	-	-	≥17
Trimethoprime-Salfamethoxazole	TMP-SMZ	25	≤10	11 to 15	≥16
Ceftriaxone	CRO	30	≤13	14 to 20	≥21
Oxacilin	Ox	1	≤10	11 to 12	≥13

were recorded for isolates showing resistance to three and more antibiotics (Rota et al., 1996).

RESULTS

Prevalence of *S. aureus* in tested milk samples

A total of 160 milk samples (32 per HACCP level) were analyzed and 78 were positive for *S. aureus* (Table 1), a total of 210 potential isolates were subcultured and further analyzed. However, only 78 isolates satisfied all the identification criteria and were used for subsequent analysis. These constituted a total of 78 *S. aureus* isolates (Table 1). *S. aureus* was obtained from each of the 32 different critical control point (CCP) samples taken per HACCP level, giving a prevalence of *S. aureus* of 48.75% for the 160 samples (Table 1). The results demonstrated the presence of *S. aureus* in all the milk samples, regardless of the farm setting. However, the levels of contamination with *S. aureus* were higher in milk samples obtained in this study area (Table 1).

Antibiotic susceptibility

All 78 *S. aureus* isolates were subjected to antibiotic susceptibility tests. Nine antimicrobial agents, from different antibiotic classes were used. Some were selected because some studies (Ateba and Bezuidenhout, 2008; Moneoang and Bezuidenhout, 2009) have shown that large numbers of bacteria isolated from South Africa were resistant to them. Antibiotics of veterinary and human health relevance were also considered. A summary of the percentage of *S. aureus* that were resistant to these antibiotics is provided in Table 3.

A large proportion of the isolates of this study area were resistant to Ampicillin,; Penicillin G; and Oxacilin, while a similarly large proportion of those from the transportation container were resistant to, Ampicillin, penicillin G, Erythromycin, Ceftriaxone, Amoxicillin-Clavulanic Acid and Oxacilin. There were no resistant groups for Ciprofloxacin antibiotics.

A general observation is the large percentage of Penicillin G, Ampicillin and Oxacillin resistant *S. aureus*

Table 3. Antibiotic-resistance profiles of *S. aureus* isolated from milk originating from Hawassa area, South Ethiopia (n=78).

Sample source of milk	Antibiotic resistance (%)								
	PG	AP	AC	CIP	E	CRO	TMP	Ox	VA
Teat	64.3	64.3	14.3	0.0	21.4	14.3	7.1	57.1	57.1
Bucket at farm level	35.0	35.0	10.0	0.0	10.0	5.0	0.0	15.0	70.0
From storage containers at milk collection center	71.4	71.4	14.3	0.0	19.0	9.5	0.0	66.7	57.1
From transportation container	100	100	94.1	0.0	88.2	76.5	23.5	100	23.5
After cooling at the pasteurization plant	83.3	83.3	0.0	0.0	0.0	0.0	0.0	83.3	50.0

Note: Percentages were calculated by dividing the number confirmed as *S. aureus* resistant in a particular sample source by the total number of isolates tested.

Table 4. The predominant multiple antibiotic resistant phenotypes for *Staphylococcus aureus* isolated from cow's milk obtained from Hawassa area farms, South Ethiopia (n=78).

Phenotype	Number observed	Percentage
PG-AP-E	2	2.7
PG-AP-Ox	16	20.5
PG-AP-Ox-VA	6	7.7
PG-AP-TMX-Ox	1	1.3
PG-AP-AC-E-Ox-VA	3	3.8
PG-AP-AC-E-CRO-Ox	15	19.2
PG-AP-AC-E-CRO-Ox-VA	2	2.7
PG-AP-AC-E-TMX-Ox-VA	1	1.3
PG-AP-AC-E-TMX-Ox	2	2.7
PG-AP-AC-TMX-Ox-VA	1	1.3

Note: The percentage representations of the phenotypes were obtained by dividing the number of a particular phenotype by the total number of multiple antibiotic resistant isolates identified in a given area. VA, vancomycin; AP, ampicillin; PG, penicillin G; TMP-SMX, Trimethoprime-Salfamethoxazole; E, erythromycin; Ox, Oxacilin; CRO, Ceftriaxone; CIP, Ciprofloxacin; AC, Amoxicillin-Clavulanic Acid.

were isolated from the study area. These were also resistant to several other antibiotics. Therefore, one can easily conclude that these are Methicillin resistant *S. aureus* (MRSA).

Only a small proportion of the isolates from the teat, bucket and storage container were resistant to Trimethoprime-Salfamethoxazole; Erythromycin; Ceftriaxone and Amoxicillin-Clavulanic Acid. None of the isolates from bucket, storage container and freshly pasteurized milk were resistant to TMP-SMX. Furthermore, 23.5 to 70% of the isolates from the sample were resistant to Vancomycin, while 15 to 100% of those isolated was resistant to Oxacillin.

MAR phenotypes of *S. aureus*

MAR phenotypes were determined for *S. aureus* isolated from milk, obtained from all the HACCP levels (Table 4). The predominant MAR phenotypes for *S. aureus* isolated from this study area were PG-AP-AC-E-CRO-Ox and PG-AP-Ox in 19.2% and 20.5% of the isolates, respectively. Furthermore, MAR phenotypes PG-AP-AC-TMX-Ox-VA, PG-AP-AC-E-TMX-Ox-VA and PG-AP-TMX-Ox were obtained in 1.3% of the isolates. The MAR phenotypes PG-AP-AC-E-TMX-Ox, PG-AP-AC-E-CRO-Ox-VA and PG-AP-E were obtained in 1.3% of the isolates. Also PG-AP-AC-E-Ox-VA 3.8% and PG-AP-Ox-VA 7.7% were the MAR phenotypes for *S. aureus* isolated from this study area (Table 4).

It is thus evident that MAR *S. aureus* was isolated from all CCP level sampled. However, among the isolates from this study area, 62.8% of the isolates develop MAR. Among all MAR phenotypes of S. aureus, 42.9% of them were resistance to six different antibiotics and 6.1% were resistance to seven antibiotics. Fifty one percent (51%)

Table 5. Antibiotic-resistance profiles of *Staphylococcus aureus* from all CCP levels isolated from milk originating from Hawassa area, South Ethiopia *(n=78)*

Organism	Antibiotic resistance (%)								
	PG	AP	AC	CIP	E	CRO	TMP	Ox	VA
S. aureus	67.9	67.9	30.8	0.0	32.1	23.1	7.7	60.3	38.5

Percentages were calculated by dividing the number confirmed as *S. aureus* resistant in a particular sample source by the total number of isolates tested VA, vancomycin; AP, ampicillin; PG, penicillin G; TMP-SMX, Trimethoprime-Salfamethoxazole; E, erythromycin; Ox, Oxacilin; CRO, Ceftriaxone; CIP, Ciprofloxacin; AC, Amoxicillin-Clavulanic Acid.

of them are resistance to 3 or 4 antibiotics.

DISCUSSION

In this study we describe the isolation and antibiotic susceptibility characterization of *S. aureus* from milk obtained from five different CCP level. Our results indicate that 40.6% of the samples were positive for *S. aureus* and that more of these bacteria were positively identified from milk obtained from Hawassa area, South Ethiopia. Several studies have been conducted in South Africa to evaluate the prevalence of *S. aureus* in milk (Shitandi and Sternesjö, 2004; Lee, 2003; Gündoğan et al., 2006). The results reported in our study were similarly high when compared to those studies (Shitandi and Sternesjö, 2004; Gündoğan et al., 2006). Although the prevalence of *S. aureus* has been reported to vary with the size and geographic region of the area sampled, a high proportion of these bacteria in milk relates to poor hygiene practices. Based on observations made during the collection of samples, we therefore report that improper hygiene and poor farm management practices contributed to the presence of *S. aureus* in the milk, especially in those from the Hawassa area. In this study area setting, milk was obtained from animals by farmers by washing their hands, the utensils and containers used. In certain cases, untreated groundwater was used to wash the containers that were used for milking. This may have contributed to the high level of *S. aureus*. Improving the hygienic conditions of the milking environment and utensils may reduce the prevalence of *S. aureus* in milk and prevent its transmission to humans.

A further objective of the study was to characterize and compare the antibiotic-resistance profiles of *S. aureus* isolated from the study area. The motivation for this was the fact that there were no clear studies conducted in the sample area. Furthermore, the presence of antibiotic-resistant *S. aureus* has been reported to negatively affect the treatment of its associated infections in humans and animals (Brouillette and Malouin, 2005; Petinaki et al., 2001; Moneoang and Bezuidenhout, 2009). We therefore believe that an investigation of the antibiotic-resistance profiles of these isolates may serve as a tool in assessing both the sanitary conditions employed during milking and the health risks that humans may encounter when infected by antibiotic-resistant strains.

PG, AP and Ox were the drugs to which a large proportion of the isolates were resistant (Table 5). As shown in Table 5, between 60.0 and 67.9% of the isolates from all CCP levels were resistant to these three drugs. Also VA, AC, E and CRO were resistance to *S. aureus* about 38.5, 32.1, 30.8 and 23.5% respectively. None of the *S. aureus* were resistance to CIP. However, very small proportions of *S. aureus* were resistance to TMP-SMX (7.7%). Contrary to our observations, a study reported that larger percentage (15.7 to 19%) of *S. aureus* isolated was resistant to TMP-SMX (Ateba et al., 2010). The finding that a large number of *S. aureus* were resistant to PG, AP and Ox is, however, a cause for concern and should be further investigated. It is thus our view that the results obtained in this study do not accurately reflect the usage of this antibiotic on farms. We cannot explain this phenomenon.

CIP resistance was almost non-existent in the all CCP levels of the isolates. Also no resistance *S. aureus* strain was isolated from immediate pasteurized milk for AC, E, CRO, TMP-SMX and CIP. Despite the fact that a significantly larger number of *S. aureus* isolated from immediate pasteurized milk was resistant to PG, AP, Ox and V, it was evident from our results that this antibiotic is not frequently used in animals by large-scale farmers. Contrarily to our observations, a study reported in South Africa, was 100% of the isolates from the two commercial farms were susceptible to vancomycin (Ateba et al., 2010). This drug is no longer used in veterinary medicine in many countries (Pace and Yang, 2006) including South Africa, which may account for the results reported here.

Small proportions (67.8%) of the isolates from this study area were resistant to the beta-lactam penicillin G, when compared to the isolates from the communal farms, in which 69 to 98% were resistant (Ateba et al., 2010). The antibiotic resistance obtained for *S. aureus* isolated from does not correlate with the extensive usage of penicillin on dairy cattle farms in Hawassa town. The ampicillin-resistance pattern for isolates from these all

levels of CCP were similar to the penicillin G resistance pattern, thus a smaller proportion of the isolates were resistant to this drug when compared to the isolates from the South Africa. Ampicillin is also commonly used on dairy cattle farms in the study area. Resistance to penicillin G is thus used as a marker to assess the susceptibility of *S. aureus* isolates against other beta-lactam antibiotics (Waage et al., 2002; Pace and Yang, 2006). This correlates with our findings but suggests that further studies need to be conducted to determine the exact inter-relation between these drugs in the expression of antibiotic-resistant phenotypes on dairy cattle farms in Ethiopia. The methicillin-resistance pattern had a similar trend to that of several of the previously discussed antibiotics. This is because larger numbers of Oxacillin-resistant isolates were obtained from the milk at this study area.

What is of concern is the large number of Oxacilin (60.3%) that were isolated from the Study area; this is an indicator of MRSA. High levels of MRSA have been identified in patients in the United States and some European countries (Mark et al., 2003). In these countries, 44.4, 34.7, 41.8 and 32.4% of isolates from patients in the United States, France, Italy and Spain, respectively, were resistant to methicillin. These levels, however, are lower than those in our study. Methicillin is not used on dairy cattle farms in the South Ethiopia. However, methicillin resistance could be explained by the inter-relationship between beta-lactam resistance and resistance to this drug. In MRSA, methicillin resistance is conferred by the penicillin binding protein (PBP) 2a that is encoded for by the *mec*A gene (Gündoğan et al., 2005). PBP2a does not readily bind the beta-lactam moiety. However, in MRSA that are exposed to beta-lactam antibiotics, this PBP2a would contribute to the resistance by providing transpeptidase activity to the native PBPs during cell wall synthesis. In our study, the resistant phenotype PG-AP-Ox was frequently identified usually with the addition of one or more antibiotics (Table 4). It is thus recommended that future studies should confirm the presence of the *mec*A gene in observed MRSA due to these β-lactamase antibiotics.

It has also been documented that MRSA isolates that are resistant to beta-lactam antibiotics may develop induced resistance to vancomycin (Gündoğan et al., 2005). All the isolates from the study area were resistant to Oxacillin were also resistant to penicillin G and Ampicilin. In addition, all PG, AP, and AC antibiotic resistance *S. aureus* were also resistance to Ox antibiotics. Moreover, 26.5% of the Oxacillin resistances *S. aureus* were also resistance to vancomycine. We therefore suggest that this may account for the identification of vancomycin-resistant isolates in the study because vancomycin is not used in either veterinary or human medicine in the area (Pace and Yang, 2006). Moreover, beta-lactam-induced vancomycin-resistant MRSA are not easily detected by conventional antibiotic susceptibility tests (Hideaki et al., 2004), and this may also account for the very low proportion of vancomycin-resistant *S. aureus* observed.

The MAR phenotypes (Table 4) obtained in the study correlated with the percentage of antibiotic resistance. Although the development of resistance to a particular antibiotic depends on the level of exposure to the anti-microbials; (Rychlik et al., 2006), there are many other factors that are involved. We are therefore suggesting that molecular methods be used to characterize these isolates for the presence of antibiotic-resistance determinants, which may provide data to support our conclusions.

S. aureus is normally resident in humans; therefore the *S. aureus* present in the cow's milk may have resulted from transmission from humans, which raises questions regarding the hygiene practices followed.

CONCLUSION AND RECOMMENDATION

S. aureus was isolated from all milk samples obtained from Southern Ethiopia. A large proportion of the isolates obtained were resistant to three or more antibiotics. These were also resistant to Vancomycine. This was particularly the case in the public setting and is cause for concern. The high level of MAR *S. aureus* and the implycations thereof warrant further investigation. One of the aspects that need to be investigated is the cause of the observed resistance phenotypes. Furthermore, impacts and dynamics of genetic antibiotic determinants should also be investigated using molecular methods.

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