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Characterization of antimicrobial resistance in *Staphylococcus aureus* isolated from bovine mastitis in Central Ethiopia

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Staphylococcus aureus **is commonly associated with mastitis in dairy herds with potential public health implications. Overall, 303 samples were collected from September 2015 to July 2016 to characterize the phenotypic and genotypic pattern of drug resistance in *S. aureus* isolated from cases of clinical and sub-clinical bovine mastitis in Central Ethiopia. Milk samples were tested by using California Mastitis Test and positive samples were subjected for bacterial culture, disc diffusion test and polymerase chain reaction (PCR) to detect the presence of antimicrobial resistance. Based on California mastitis test (CMT) result and clinical examination, the prevalence of mastitis was 70.6%. *S. aureus* was isolated from 36.9% of CMT positive samples. The phenotypic determination of antimicrobial resistance showed that the isolates were most resistant to ampicillin (80%) followed by trimethoprim-sulfamethoxazole (23.3%), tetracycline (15%), streptomycin (10%) and gentamycin (3.3%) and equally to both erythromycin and chloramphenicol (1.6%). Characterization of the antimicrobial resistance gene was done by using PCR. Most of the isolates (56%) contained *bla*Z gene followed by *erm*B (33%), *erm*C (13.3%) and each *erm*A and *msr*A appeared only in 2% of the isolates. There was no isolate harboring the methicillin resistance *mec*A gene. Thirty six percent of the isolates contained more than one antibiotic resistance genes. The highest multidrug resistance (MDR) gene combination was observed by *bla*Z**erm*B (31.25%) genes and the least frequently occurred were *bla*Z **erm*A and *msr*A**erm*B (3.12%) each. This study showed that consumption of raw milk could be considered as a critical source of antibiotic resistant *S. aureus*.**

Key words: Bovine mastitis, *Staphylococcus aureus*, antimicrobial resistance, Ethiopia.

INTRODUCTION

Mastitis is one of the most important diseases in dairy significant economic losses to the dairy industry. *Staphylococcus aureus* causes one of the most common types of chronic and cows throughout the world and is responsible for subclinical mastitis in dairy animals.

The inappropriate use of antibiotics for medication and growth enhancers in farm animals contributes for emergence of antibiotic resistant organisms. It is more than two decades since the emergence of antimicrobial resistant in staphylococci(Lowy, 2003). Staphylococci are

the main etiological agents of mastitis in dairy cattle and cows are the second largest reservoir of *S. aureus* next to human nares and up to 75 million of cows can be infected by this bacterium form the world cattle population (Sakwinska et al., 2011; Somayyeh and Habib, 2014; Raney, 2009). The overall loss due to mastitis ranges from 31 to 749 kg in first lactation to losses between 117 and 860 kg in subsequent lactation (Hultgren and Svensson, 2009; Ostergaard and Grohn, 1999).

Although there is host range barrier among *S. aureus* lineage, some illustrates the potential hazard of animal origin *S. aureus* on human health which implies possible transmission of genotypes from one species to the other (Smith, 2015; Lowder et al., 2009). Despite the substantial economic impact and potential public health concern, the prevalence as well as the phenotypic and genotypic antibiotic resistance nature of *S. aureus* isolates are less studied in developing countries like Ethiopia. In these countries, scarce in veterinary services, shortage of variety of drugs and poor drug regulatory frameworks could lead to under dosage medication which may end up with development of antibiotic resistant organisms. On the other hand, low hygienic standards of housing and milking can disseminate mastitis causing pathogens including *S. aureus* among individual animals or farms (Marama et al., 2016).

In Ethiopia, prevalence rate ranging from 15.3 to 53.4% has been recorded from different parts of the country (Marama et al., 2016; Sori et al., 2011). However, there are few trends to detect antibiotic resistance genes and to correlate their association with the phenotypic resistance. Hence, this research intended to characterize phenotypic as well as genotypic antibiotic resistance of *S. aureus* isolated from bovine mastitis.

MATERIALS AND METHODS

Sample collection and preparation

A total of 303 lactating cows were screened for subclinical mastitis from September 2015 to July 2016 using California mastitis test (CMT). All the lactating cows were examined carefully and CMT screening procedures were done. Approximately, 2 ml of milk was taken from each teat into the four CMT paddle indentations. Then, equal amount of CMT reagent (COX, USA) was added and swirled gently for 15 s. The screening was done according to the procedure stated in Quinn et al. (1994). The CMT positive samples were kept in cold box and transported immediately to the National Agricultural Biotechnology Research Center Laboratory, Holetta, for further analysis.

Bacterial isolation and identification

Bacteria were cultured and identified from CMT positive milk samples. The collected samples from each quarter were streaked on blood agar base plates enriched with 7% ovine blood. The inoculums were then incubated aerobically at 37°C for 24 h. After primary culture, identification of *S. aureus* was done by using microscopic and biochemical methods (Quinn et al., 2011; OIE, 2012).

Antimicrobial sensitivity test

Antimicrobial resistance patterns were determined by Kirby-Bauer disc diffusion method on Mueller-Hinton agar (Sigma-Aldrich, USA) (Kirby et al., 1966). The bacteria were inoculated on the plate at a rate of 5×10^5 Bacteria/ml after serial dilution determined by OD measurements according to CLSI recommendation. Antibiotic discs were placed and gently pressed by forceps on the bacterial culture spread on Mueller-Hinton agar (Sigma Aldrich, USA). The inhibition zone was measured after incubation of the plates at 37°C for 18 h under aerobic environment. The response of the isolates to each antimicrobial agent was evaluated by measuring the zone of inhibition categorized as sensitive, intermediate and resistant according to the standards recommended by CLSI (2007). The antimicrobials used in the experiment were ampicillin (10 µg), chloramphenicol (30 µg), gentamycin (10 µg), erythromycin (15 µg), tetracycline (30 µg), streptomycin (10 µg) and trimethoprim-sulfamethoxazole (1.25/23.75 µg).

Isolation of plasmid DNA

Plasmid DNA of the *Staphylococcus* isolates was performed by using Plasmid Midi Kit (QIAGEN). Single colony was taken from each isolate and inoculated into a separate 5 ml LB Broth and incubated over night at 37°C in an orbital shaker. Cells were harvested by centrifugation at speed of 6000 g for 15 min. The isolation procedures were performed according to the manufacturers' protocol. The concentration and the purity of the extract were measured by using a Nano-drop ND-1000 spectrophotometer (Thermoscientific, Wilmington, DE). The integrity of the plasmid DNA was assessed after electrophoresis in 1% agarose gel after mixing with gel loading dye (Thermoscientific, USA).

PCR

The genes involved in antimicrobial resistance (*mec*A, *bla*Z, *erm*A,*erm*B, *erm*C and *msr*A) were detected by PCR using the primers and cycle conditions described by Murakami et al. (1991) and Sawant et al. (2009) (Table 1). A single colony was picked and inoculated in to 10 ml of nutrient broth (Sigma Aldrich, USA) and incubated overnight at 37°C in shaker incubator at speed of 100 rpm/min. Bacterial plasmid DNA was extracted using kit (Biobasic, USA) from well grown broth cultures. The PCR reaction was prepared by mixing, reaction buffer (500 Mm KCl, 17.5 mM $MgCl_2$, 100 mM Tris-HCl, 0.1% TritonX-100) (Himedia, India),10m MdNTPs, 10 pmol of each primer, 1 U Taq DNA polymerase(Himedia, India) and Nuclease free water which was added up to 25 µl. PCR products were electrophoresed in 1.5% agarose gel after mixing with gel loading dye (Thermoscientific, USA) (0.5 µg/mL) and observed under UV illumination.

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Table 1. Genes involved and their oligonucleotide primers for the polymerase chain reactions.

Gene	Oligonucleotide sequences	Reference
*mec*A	F- CTTTGGAACGATGCCTAATCTCAT R- AAGAGATTTGCCTATGCTTC	Murakami et al. (1991)
*bla*Z	F- GCTTGACCACTTTTATCAGC R- ATCGGATCAGGAAAAGGACA	
*erm*A	R- CACGATATTCACGTTTTACCC F- AAGGGCATTTAACGACGAAA	
*erm*B	R- CTGTGGTATGGCGGGTAAGT F- TGAAATCGGCTCAGGAAAAG	Sawant et al. (2009)
*erm*C	F- TGAAATCGGCTCAGGAAAAG R- CAAACCCGTATTCCACGATT	
*msr*A	F- TGGTACTGGCAAAACCACAT R-AAACGTCACGCATGTCTTCA	

Table 2. Antimicrobial resistance rate (%) of *S. aureus* isolated from bovine mastitis.

Antimicrobial disc	Sensitivity			Total (%)
	Sensitive (%)	Intermediate (%)	Resistant (%)	
Erythromycin	30 (54.5)	29 (43.9)	1 (1.6)	60 (100)
Chloramphenicol	55 (91.6)	4 (6.6)	1 (1.6)	60 (100)
Gentamycin	56 (93.3)	2 (3.3)	2 (3.3)	60 (100)
Ampicillin	10 (16.6)	2 (3.3)	48 (80)	60 (100)
Tetracycline	40 (66.6)	11 (18.3)	9 (15)	60 (100)
Streptomycin	45 (75)	9 (15)	6 (10)	60 (100)
Trimethoprim sulfamethoxazole	37 (61.6)	9 (15)	14 (23.3)	60 (100)

RESULTS

Prevalence of *S. aureus* and its antimicrobial resistance pattern by using disk diffusion method

Out of the 303 lactating cows, 214 (70.6%) of them were found positive by CMT for either of the four quarters. Among these 214 samples, 187 (87.4%) were bacterial culture positive in which 79 (36.9%) of the culture was identified as *S. aureus*. Antimicrobial resistance test was conducted for 60 of the 79 isolates. Antimicrobial resistance pattern of the *S. aureus* isolates is shown in Table 2. The isolates were resistant to ampicillin (80%) followed by trimethoprim-sulfamethoxazole (23.3%), tetracycline (15%), streptomycin (10%), gentamycin (3.3%) and equally to both erythromycin and chloramphenicol with the least resistance (1.6%).

Determination of antimicrobial resistance genes

Among all 45 isolates tested for the presence of antibiotic resistance gene, only 14 (35%) were found free from any of the anti-microbial resistance gene (Table 3). Isolates containing antibiotic resistance genes were observed after electrophoresis of the PCR product (Figure 1).

Out of the 45 studied isolates, most of them (55%) contain *bla*Z gene followed by *erm*B (33%), *erm*C (13.3%) and each *erm*A and *msr*A were detected in only 2% of the isolates. No isolate was detected harboring either *erm*A or *msr*A solely. The gene *mec*A was no detected at all (Table 3).

Assessment of multi-drugresistance (MDR) genes

Approximately 36% of the isolates contained more than one antibiotic resistance genes. The highest MDR gene combination was observed by *blaZ***erm*B (22.2%) genes and the least frequently occurred combinations were *bla*Z **erm*A and *msr*A**erm*B (2.2%) each. Combination of *bla*Z **erm*C accounts for 8.8%. The gene *bla*Z occurred in combination with all the *erm* genes (Table 4).

Among the 45 isolates in which the PCR test was

Table 3. Distribution of antimicrobial resistance genes among isolates (out of 45).

Gene	Solely appear (%)	In combination with other (%)	Total (%)
*bla*Z	10 (22.2)	15 (33.3)	25 (55)
*erm*A	0 (0.0)	1 (2.2)	1 (2.2)
*erm*B	4 (4.8)	11 (24.4)	15 (33.3)
*erm*C	2 (2.4)	4 (8.8)	6 (13.3)
*msr*A	0 (0.0)	1 (2.2)	1 (2.2)
*mec*A	0 (0.0)	0 (0.0)	0 (0.0)

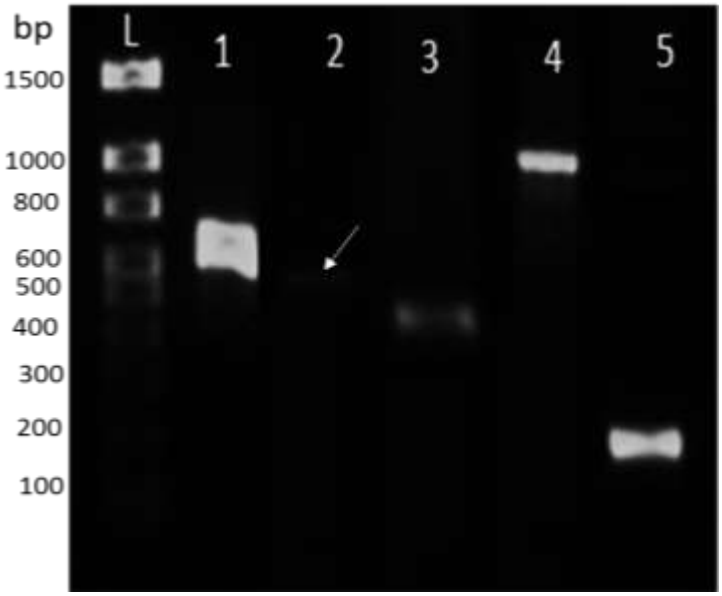

Figure 1. Agarose gel electrophoresis of representative isolates containing antibiotic resistance marker (1%). L. Ladder (100 bp -1.5 kb DNA ladder, Bio Basic), 1.*blaz* (517 bp), 2.*erm*A (486 bp), 3.*ermB* (423 bp), 4.*msr*A (1000 bp), 5.*ermC* (272 bp).

Table 4. Occurrence of multiple drug resistance genes among isolates (out of 45).

MDR	Genes frequency (%)
*bla*Z***erm*B	10 (22.2)
*bla*Z***erm*C	4 (8.8)
*bla*Z***erm*A	1 (2.2)
*erm*B***msr*A	1 (2.2)
subtotal	16 (35.5)

performed, 16 (35.5%) of them showed resistance on the disc diffusion test containing the respective resistance gene. High association between ampicillin resistance and the presence of *bla*Z gene has been observed. Sixty-eight percent of the isolates harboring the *bla*Z gene were found resistant to ampicillin.

DISCUSSION

S. aureus causes one of the most common types of chronic mastitis in dairy animals worldwide. In this study, the prevalence of bovine mastitis was found in 70.6%. This prevalence of mastitis is comparable with previous

reports from Ethiopia who reported a prevalence rate of 71.0 and 75.2% in dairy farms located at Holetta and Jimma towns, respectively (Sori et al., 2011; Mekibib et al., 2009). But, higher result was found in Holetta, Bahir-dar and Gondar towns of Ethiopia (Marama et al., 2016; Bitew et al., 2010; Moges et al., 2011). The difference on the prevalence of mastitis among the studies could be due to difference in management system, milking practices, productivity, breed of the animals and location (Marama et al., 2016; Sori et al., 2011).

The prevalence of *S. aureus* was found at 36.9% which is higher than the finding of Marama et al. (2016) and lower than Sori et al. (2011). In Kenya, equivalent prevalence rate (30.6%) of *S. aureus* was reported by Shitandi and Sternesjo (2004). However, prevalence rate of 25.5, 23.3 and 10% was reported from China, Iran and Finland, respectively (Wang et al., 2008; Bahraminia et al., 2017; Pitkalla et al., 2004). The reason for the variability may be due to lactation stage of the cow, age of the cow and milking method (Kivaria et al., 2007; Ergn et al., 2009). In this study, 85% of the *S. aureus* isolates showed resistance to at least one antimicrobial drug. This is similar with the previous finding from Jimma and central Ethiopia with overall antibiotic resistance rates of 92 and 97.5%, respectively (Sori et al., 2011; Mekibib et al., 2009). However, in Brazil relatively higher susceptibility to antimicrobials (49.1%) was reported by Rabello et al. (2005). This variation might occur due to difference in milking practice, purpose of use of the antibiotics and inappropriate therapeutic treatment by nonprofessionals.

Considerable resistance to beta-lactam antibiotic by *S. aureus* appears with serious threat to the world (Lowy, 2003). In the current study, higher resistance to ampicillin (80%) was observed. This is comparable with the previous reports from Gondar (81.5%) and Italy (88%) (Moges et al., 2011; Virdis et al, 2010), but higher than other reports from Hawassa (67.9%) (Teshome et al., 2016). However, Daka et al. (2016) reported lower resistance rate (7.7%) from Hawassa, Ethiopia.

Tetracycline and its derivatives are the most extensively used antibiotics in Ethiopia for treatment of animal diseases. So that, certain level of resistance to this drug was expected. The present finding on resistance against tetracycline was 15% which is nearly half of the figure observed at Gondar and Holetta with the resistance rate of 29.6 and 33.3%, respectively (Moges et al., 2011;Marama et al., 2016). The most effective drugs in this experiment were gentamycin followed by erythromycin and chloramphenicol. These drugs are not first choice for treatment of mastitis in most part of the country hence the chance to develop resistance by *S. aureus* will be minimum. In contrast, there are evidences of erythromycin resistance development in some parts of the country (Moges et al., 2011; Teshome et al 2016).

Multiple drug resistance is the ability of an organism to resist and grow against two or more antimicrobials. In this study, the isolates had shown inverse relation between the multi-drug resistance nature and the number of antimicrobials applied. This observation match with the finding of Sori et al. (2011) who demonstrate MDR pattern of 25, 10.45 and 7% for two, three and four types of drugs, respectively. However, it differs from Teshome et al. (2016) who record MDR of 34.8% of the isolates for three and 8.7% for two antimicrobials. The difference observed in the pattern of MDR could be explained by group of drugs with similar chemical structure and mechanism of action may exhibit cross-resistance by the bacteria despite the number of drugs involved (Pechere, 2001).

The two main mechanisms of macrolide resistance are drug-efflux membrane pumps and modification of the drug target site in the ribosome. The third mechanism of resistance is drug inactivation (Bailey et al., 2008; Ojo et al., 2006; Jensen et al., 1999; Lina et al., 1999). It has been known that, isolates harbor *erm* genes (erythromycin resistance rRNA methylase) code for the protein called methyl transferase which induces N6 - dimethylation of an adenine residue of 23S rRNA. This process produces conformational changes in the phosphate site of the rRNA and prevents the macrolide binding at the peptidyl transferase center, hence the protein production will proceed and the bacteria will survive (Kot et al., 2012; Westh et al., 1995). Expression of the three related factors; *erm*A, *erm*B, and *erm*C, are responsible to make the bacteria resistance to macrolides and other related antibiotic groups (Ojo et al., 2006; Westh et al., 1995).

In the present work, among the three erythromycin resistance determinants, *erm*B (33.3%) resistance gene was the most frequently identified followed by *erm*C (13.3%) and *erm*A (2.2%). Bahraminia et al. (2017) reported similar pattern of *erm* genes distribution while studying bovine mastitis caused by *S. aureus*. A work done to determine types of *S. aureus* linages affecting human showed that *erm*A as the most frequently found resistance gene (Bahraminia et al., 2017; Westh et al., 1995) although there are some findings supporting higher prevalence of *erm*C among the three determinants (Ross et al., 1990). Lina et al. (1999) correlate the distribution of erm genes with methicillin resistance and susceptibility in coagulase positive or negative staphylococci species.

The *erm*A gene found more common in methicillin resistant *S. aureus* stains (57.6%) compared to the sensitive ones (5.6%) (Westh et al., 1995).

The contribution of each of the *erm* determinants (*erm*A, *erm*B and *erm*C) towards phenotypic macrolide resistance is essential. Westh et al. (1995) determined that *erm*A and *erm*C are responsible for erythromycin resistance in more than 98% of *S. aureus* strains. In another report *erm*C was the dominant *erm* gene in *S. aureus* and was responsible for erythromycin resistance in 72% of the strains in the years 1983 to 1988 (Duran et al., 2012).

In the current study, despite the high prevalence of *erm*B gene, we have observed only a single erythromycin

resistance isolate (1.6%). This finding agrees with Westh et al. (1995) which reported that *erm*B is less responsible for phenotypic macrolide resistance. However, we have identified a single isolate harboring this gene together with *erm* genes (2.2%). The present finding showed similar results of previous works by Lina et al. (1999) and Ross et al. (1990) which identify prevalence of 3.3 and 3.6%, respectively for similar combination.

Staphylococcal resistance to penicillin is attained by the gene *bla*Z (β- lactamase). It encodes for extracellular enzyme β-lactamase which hydrolyze the β-lactam ring rendering the β-lactam inactive (Lowy, 2003). In the present study, the gene was widely spread among the isolates (80%) which are in line with the report by Duran et al. (2012). According to Lowy (2003), more than 90% of staphylococcal isolates produce penicillinase (β-lactamase enzyme). This indicates the wide distribution of the resistance gene globally through spread of resistance strains. Although *bla*Z is the primary key player for penicillin resistance among staphylococcus isolates, it is not the sole factor. In this study, not all isolates which show penicillin resistance by disk diffusion test harbor *bla*Zgene. This finding agrees with previous studies by Yang et al. (2015) and Gao et al. (2012) who identified staphylococcus and streptococcus isolates that showed resistance to penicillin but not carrying *bla*Zgene. Point mutation rather than gene acquisition could be another factor for only phenotypic resistance. Biofilm production and multi-drug efflux development are also other possible protection mechanism of the bacteria (Katayama et al., 2005; Wielders et al., 2002).

In this study, no isolate containing *mec*A gene was found. Similarly, another study mentioned that *mec*A is rarely found in many *Staphylococcus* isolates originated from animal infections (Ross et al., 1990). The limited distribution of *Staphylococcus* chromosomal cassette mec (SCCmec) which carries *mec*A gene by nature may be considered for its rare occurrence.

The occurrence of *S. aureus* resistance to antimicrobial agents is growing in alarming rate. So, the community should be aware by responsible bodies about the risk of consuming raw dairy products. The veterinary service delivery should be improved in order to avoid subjective treatment of animals by non-professionals. Besides, further studies should be conducted to obtain full figure of phenotypic as well as genotypic antimicrobial resistance pattern.

ABBREVIATIONS

CMT, California mastitis test; **OD,** optical density; **PCR,** polymerase chain reaction; **MDR,** multiple drug resistance.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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