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All communications should be addressed to:

The Editor-in-Chief
Philippine Journal of Veterinary Medicine
College of Veterinary Medicine
University of the Philippines Los Baños
Laguna, Philippines 4031
Telefax Nos. +63-49-536-2727, +63-49-536-2730
Email: pjvm1964@gmail.com, pjvm.uplb@up.edu.ph

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The Philippine Journal of Veterinary Medicine

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ORIGINAL ARTICLE

PREVALENCE, PHENOTYPIC, AND GENOTYPIC ASSESSMENT OF ANTIBIOTIC RESISTANCE, VIRULENCE MARKERS AND MOLECULAR TYPING OF *Staphylococcus epidermidis* STRAINS ISOLATED FROM BOVINE SUBCLINICAL MASTITIC MILK

Fatemeh Talebi, Hassan Momtaz, PhD*, Zahra Bamzadeh, PhD

Department of Microbiology, Shahrekord Branch,
Islamic Azad University PO. Box: 166, Shahrekord, Iran

ABSTRACT

Staphylococcus epidermidis **is one of the causative agents of bovine mastitis with an emergence of antibiotic resistance and virulence characters. One hundred and three mastitic milk samples were collected and confirmed through the use of the California mastitis test. Samples were cultured and *S. epidermidis* isolates were identified by Loop mediated isothermal amplification (LAMP) method. Molecular typing of *S. epidermidis* isolates was performed using the Multiple-locus variable-number tandem repeat analysis (MLVA). Eighteen out of 103 (17.47%) samples were positive for *S. epidermidis*. *S. epidermidis* isolates exhibited the uppermost prevalence of resistance toward penicillin (100%), tetracycline (83.33%), erythromycin (83.33%), and cefazolin (77.77%). ClfA (55.55%), agrI (50%), etA (33.33%), and agrIII (27.77%) were the most routinely identified virulence factors. TetM (88.88%), aacA-D (83.33%), tetK (77.77%), ermA (72.22%), msrA (55.55%), and ermC (55.55%) were the most routinely identified antibiotic resistance genes. A total of five separate loci (se1 to se5) were originated amongst 18 *S. epidermidis* isolates. Seventeen isolates were classified into one similar molecular cluster. The existing survey is an initial report of genetical characteristic of virulence factors and antibiotic resistance markers and MLVA-based typing of *S. epidermidis* bacteria isolated from bovine clinical mastitic milk. Similar genetical profile of *S. epidermidis* bacteria signifies the same sources of infections. The anthropogenic nature of S. epidermidis may display that infected milkers were the main source of udder infection.**

Keywords: *antibiotic resistance properties, mastitic milk, molecular typing, Staphylococcus epidermidis, virulence characters*

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INTRODUCTION

Mastitis is an inflammation of the mammary gland which is characterized by physical, chemical, and bacteriological changes in milk and pathologic changes in the glandular tissue (Ruegg, 2017). Mastitis caused severe decrease in the quality and amount of produced milk of dairy herds which is considered as an economic burden (Ruegg, 2017). Additionally, it has a serious zoonotic potential due to the distribution of bacteria and toxins through the milk (Momtaz *et al.,* 2012; Ruegg, 2017). Cows with clinical mastitis present some changes both in the udder and in the milk, whereas those with subclinical mastitis have no visible signs of infection and can be detected only by somatic cell count (SCC) with California mastitis test (CMT).

Bacteria are the most important causes of mastitis in dairy farms, especially in cows (Momtaz *et al.,* 2012; Ruegg, 2017). The most important microorganism causing mastitis is *Staphylococcus* spp. and in some cases, *Staphylococcus epidermidis* (*S. epidermidis*), is responsible for antibiotic resistant mastitis in dairy herds (Britt-Marie, 2008; Pumipuntu *et al.,* 2019). It is Gram-positive, non-spore forming, nonmotile, facultative anaerobic, and catalase-positive and coagulase-negative bacterium responsible for different types of clinical infections in both humans and animals (Britt-Marie, 2008; Pumipuntu *et al.,* 2019). *S. epidermidis* bacteria

***FOR CORRESPONDENCE:**
(e-mail: hamomtaz@yahoo.com)

usually harbored resistance against several types of antibiotic agents such as tetracyclines, aminoglycosides, cephalosporins, fluoroquinolones, penicillins, and macrolides (Schaefler, 1971; Klevens *et al.,* 2007; Duran *et al.,* 2012; Cabrera-Contreras *et al.,* 2013). Nowadays, resistant *S. epidermidis* has become a serious problem in both human and animal infections. Presence of certain antibiotic resistance genes is responsible for the occurrence of antibiotic resistance (Schaefler, 1971; Klevens *et al.,* 2007; Duran *et al.,* 2012; Cabrera-Contreras *et al.,* 2013). *MecA*, *aacA-D*, *tetK* and *tetM*, *ermA* and *ermC*, *msrA* and *msrB*, *linA* and *vatA*, *vatB,* and *vatC* antibiotic resistance genes are responsible for occurrence of resistance against methicillin, aminoglycosides, tetracyclines, macrolide–lincosamide-streptogramin B, macrolides, lincosamides, and streptogramin A groups of antibiotics, respectively (Schaefler, 1971; Klevens *et al.,* 2007; Duran *et al.,* 2012; Cabrera-Contreras *et al.,* 2013; Dehkordi *et al.,* 2017). Some potential virulence factors including toxic shock syndrome toxin-1 (TSST-1 encoded by *tst)*, exfoliative toxins A and B (*eta* and *etb*), clumping factor (*clfA*) and type I, II, and III of the accessory gene regulator (*agr*) are responsible for virulence characters of the *S. epidermidis* strains isolated from human clinical infections (Momtaz *et al.,* 2013).

Despite the growing importance of *S. epidermidis* as a cause of infections, there is still limited information available regarding the epidemiology of *S. epidermidis* in humans and animals. Methods that may distinguish clinically significant strains from contaminant strains are lacking. For epidemiological studies, there is also a lack of easy-to-use, rapid typing methods with high reproducibility. Multiple-locus variable-number tandem repeat analysis (MLVA) has been successfully applied to genotyping of several bacterial species and indicated a high level of discrimination (Schouls *et al.,* 2009; Visca *et al.,* 2011; Chenal-Francisque *et al.,* 2013; Dahyot *et al.,* 2018). MLVA targets multiple genomic loci and relies on the detection of different copy numbers of short repeated sequences that are arranged in tandem arrays (Sobral *et al.,* 2012; Mirzaei *et al.,* 2019).

According to the high importance of *S. epidermidis* as a causative agent of bovine mastitis, the present research was done to study the prevalence rate, distribution of virulence factors and antibiotic resistance properties, and molecular typing of *S. epidermidis* strains isolated from bovine clinical mastitic milk samples.

MATERIALS AND METHODS

Ethical Statement

This study was ethically approved by the Council of Research of the Faculty of Basic Science, Shahrekord Branch, Islamic Azad University, Shahrekord, Iran (Consent Ref Number IR.IAU.SHK.REC.1399.008).

Samples

From June to October 2018, a total of 103 bovine subclinical mastitic milk samples were collected from dairy herds of different parts of Isfahan province, Iran. There were 110 bovine herds in these areas and 55 bovine herds from these regions were chosen. Mastitis was confirmed by the California mastitis test (CMT). The CMT results were scored as 0 (negative), trace, 1 (weak positive), 2 (distinct positive), and 3 (strong positive) based on gel formation. The CMT score of 0 was considered as negative while CMT scores of 1 to 3 were considered indicators of subclinical mastitis. Mastitic milk samples in a cooler with ice-packs were directly transferred to the Microbiology Research Center of the Islamic Azad University of Shahrekord.

S. epidermidis isolation

S. epidermidis was identified by conventional bacteriological tests. The sample was enriched in tryptic soy broth (TSB) (Merck, Germany), and grown on mannitol salt agar, then catalase, tube coagulase and urease tests, and carbohydrate fermentation were performed. *S. epidermidis* is catalase positive, coagulase negative, urease positive, unable to ferment D-mannitol and D-trehalose, and able to ferment D-mannose and D-maltose (Topley and Wilson, 2005; Tille, 2018).*Loop mediated isothermal amplification (LAMP)-PCR confirmation of S. epidermidis isolates S. epidermidis* isolates were sub-cultured on TSB media and further incubated for 48 hours at 37 °C. Genomic DNA was extracted from bacterial colonies using the DNA extraction kit (CinnaGen, Iran) according to manufacturer's instruction. Quantity and quality of extracted DNA were examined using the NanoDrop device (Thermo Scientific, Waltham, MA, USA) and agarose gel electrophoresis, respectively.

The LAMP reaction was carried out using the DNA thermostatic amplification kit (Guangzhou Deaou Bio-technology Co., Ltd, Guangdong, China) following the manufacturer's instructions. The visual LAMP detection was performed in a 10 µl reaction mixture containing

5 µl Reaction Buffer (2×), 0.2 µM each of F3/B3, 1.6 µM each of FIP/BIP, 0.4 µM each of FLP/BLP, 3.2 U *Bst* 2.0 WarmStart DNA polymerase, 0.5 µl *S. epidermidis* DNA template, and 2.26 µl ddH_2O. Set of primers designed by Miao et al. (2017) were used for this purpose. A total of 10 µl mineral oil was added to the top of reaction mixture, and 0.4 µl dye (SYBR Green® I, Guangzhou Deaou) was added on the inner wall of tube cap of each reaction (Liu *et al.,* 2016; Ye *et al.,* 2016; Ye *et al.,* 2017). The reaction tube was inoculated at 63°C for 20 to 40 minutes, and thereafter the reaction solution was mixed with SYBR Green® I by shaking. All reactions were run in triplicate, and the negative controls were performed using sterile water instead of a bacterial DNA template. The reaction was considered as positive if its color turned from orange to green under natural light, whereas for negative reactions, the solution retained the original orange color. To detect the rapidity of the LAMP assay, the LAMP products were analyzed at intervals of five minutes using agarose gel electrophoresis (2% agarose gel electrophoresis).

Antibiotic resistance profile

Patterns of antimicrobial resistance of the *S. epidermidis* strains were studied using the Kirby-bauer method. Simple disk diffusion technique on the Mueller–Hinton agar (Merck, Germany) medium was used for this purpose. Susceptibility of *S. epidermidis* isolates were tested against several types of antibiotic agents including penicillin (10 µg/disk), cefazolin (30 µg/disk), clindamycin (2 µg/disk), mupirocin (200 µg/disk), azithromycin (15 µg/disk), erythromycin (15 µg/disk), tetracycline (30 µg/disk), ciprofloxacin (5 µg/disk), trimethoprim-sulfamethoxazole (25 µg/disk), nitrofurantoin (300 µg/disk), and rifampin (5 µg/disk) (Oxoid, UK). Instructions of the Clinical and Laboratory Standards Institute were used for this purpose (CLSI, 2018). The plates containing the discs were allowed to stand for at least 30 minutes before incubated at 37°C for 24 hours. The diameter of the zone of inhibition produced by each antibiotic disc was measured and interpreted using the CLSI zone diameter interpretative standards. *S. epidermidis* ATCC 12228 was used as quality control organism in antimicrobial susceptibility determination.

Profile of virulence and antibiotic resistance genes

Table 1 represents the list of primers and PCR conditions used for the detection of virulence factors and antibiotic resistance genes (Haveri *et al.,* 2005; Momtaz and Hafezi, 2014). A programmable DNA thermo-cycler (Eppendorf Mastercycler 5330, Eppendorf-Nethel-Hinz GmbH, Hamburg, Germany) was used in all PCR reactions. Ten microliters of PCR product were exposed to electrophoresis in a 2% agarose gel in 1X TBE buffer at 80 V for 30 minutes, stained with SYBR Green. The UVI doc gel documentation systems (Uvitech, London, United Kingdom) was applied for analysis of images.

MLVA typing

MLVA typing of *S. epidermidis* strains was applied using the technique described previously (Johansson *et al.,* 2006). Initially, selected tandem repeat loci were investigated by PCR analysis of a panel of *S. epidermidis* isolates. Flanking primers were designed for each repeat locus based on the genome sequence of strain ATCC 12228. PCR mixtures of 50 µl contained 0.4 µM forward and reverse primers (CinnaGen, Iran), 0.05 U of Ampli*Taq* Gold (Applied Biosystems, Stockholm, Sweden), 0.2 mM each deoxynucleoside triphosphate, 1.5 mM MgCl2, and 60 ng of template DNA in PCR buffer II with bovine serum albumin. DNA amplification was carried out by initial denaturation at 95°C for 10 minutes and then cycling at 95°C for one minute, 55°C for one minute, and 72°C for one minute for 30 cycles, with a final incubation at 72°C for five minutes in a DNA thermo-cycler.

Data analysis

Statistical analysis was done using the SPSS 21.0 statistical software (SPSS Inc., Chicago, IL, USA). Chi-square test and Fisher's exact two-tailed test were used to assess any significant relationship between prevalence of *S. epidermidis* strains, virulence factors, and their antibiotic resistance properties. P value <0.05 was considered as statistical significant level.

MLVA electrophoretic patterns were analyzed either visually or by using the Bionumerics software (Applied Maths, Sint-Martems-Latem, Belgium). The BioNumerics analysis was performed using the Dice coefficient and the unweighted pair group method of averages (UPGMA) with a 1% tolerance limit and 1% optimization. Isolates that clustered with ≥80% similarity were considered to belong to the same MLVA type, respectively.

Table 1. Target genes, oligonucleotide primers, and PCR conditions used for detection of virulence factors and antibiotic resistance genes in the *S. epidermidis* strains isolated from bovine

Target gene	Primer sequence (5'-3')	PCR product (bp)
vatA	F: TGGTCCCGGAACAACATTTAT R: TCCACCGACAATAGAATAGGG	268
vatB	F: GCTGCGAATTCAGTTGTTACA R: CTGACCAATCCCACCATTTTA	136
vatC	F: AAGGCCCCAATCCAGAAGAA R: TCAACGTTCTTTGTCACAACC	467
mecA	F: AAAATCGATGGTAAAGGTTGGC R: AGTTCTGCAGTACCGGATTTGC	532
tetK	F: GTAGCGACAATAGGTAATAGT R: GTAGTGACAATAAACCTCCTA	360
blaZ	F: AAGAGATTTGCCTATGCTTC R: GCTTGACCACTTTTATCAGC	360
tetM	F: AGTGGAGCGATTACAGAA R: CATATGTCCTGGCGTGTCTA	158
msrA	F: GGCACAATAAGAGTGTTTAAAGG R: C AAGTTATATCATGAATAGATTGTCCTGTT	940
msrB	F: TATGATATCCATAATAATTATCCAATC R: AAGTTATATCATGAATAGATTGTCCTGTT	595
aacA-D	F: TAATCCAAGAGCAATAAGGGC R: GCCACACTATCATAACCACTA	227
ermA	F: AAGCGGTAAACCCCTCTGA R: TTCGCAAATCCCTTCTCAAC	190
ermC	F: AATCGTCAATTCCTGCATGT R: AATCGTCAATTCCTGCATGT	229
linA	F: GGTGGCTGGGGGGTAGATGTATTAACTGG R: GCTTCTTTTGAAATACATGGTATTTTTCGA	323
tsst-1	F: ATGGCAGCATCAGCTTGATA R: TTTCCAATAACCACCCGTTT	350
etA	F: CTAGTGCATTTGTTATTCAA R: TGCATTGACACCATAGTACT	119
etB	F: ACGGCTATATACATTCAATT R: TCCATCGATAATATACCTAA	200
agrI	F: ATGCACATGGTGCACATGC R: GTCACAAGTACTATAAGCTGCGAT	441
agrII	F: ATGCACATGGTGCACATGC R: TATTACTAATTGAAAAGTGGCCATAGC	575
agrIII	F: ATGCACATGGTGCACATGC R: GTAATGTAATAGCTTGTATAATAATACCCAG	323
coa	F: CGAGACCAAGATTCAACAAG R: AAAGAAAACCACTCACATCA	970
clfA	F: GGCTTCAGTGCTTGTAGG R: TTTTCAGGGTCAATATAAGC	980
X-region	F: CAAGCACCAAAAGAGGAA R: CACCAGGTTTAACGACAT	320
IgG binding region	F: CACCTGCTGCAAATGCTGCG R: GGCTTGTTGTTGTCTTCCTC	920

RESULTS

Prevalence of *S. epidermidis* strains in studied samples

A total of 103 bovine subclinical mastitic milk samples were analyzed for the prevalence, antibiotic resistance properties, and molecular characteristics of *S. epidermidis* strains. Table 2 signifies the prevalence of *S. epidermidis* strains amongst bovine mastitic milk samples. Eighteen out of 103 (17.47%) studied samples were contaminated with *S. epidermidis*. All culture positive bacteria were also confirmed using the LAMP-PCR technique (Figures 1 and 2).

Table 2. Prevalence of *S. epidermidis* strains amongst bovine subclinical mastitic milk.

Type of samples	No samples collected	No samples positive for *S. epidermidis* (%)
Bovine mastitic milk	103	18 (17.47)

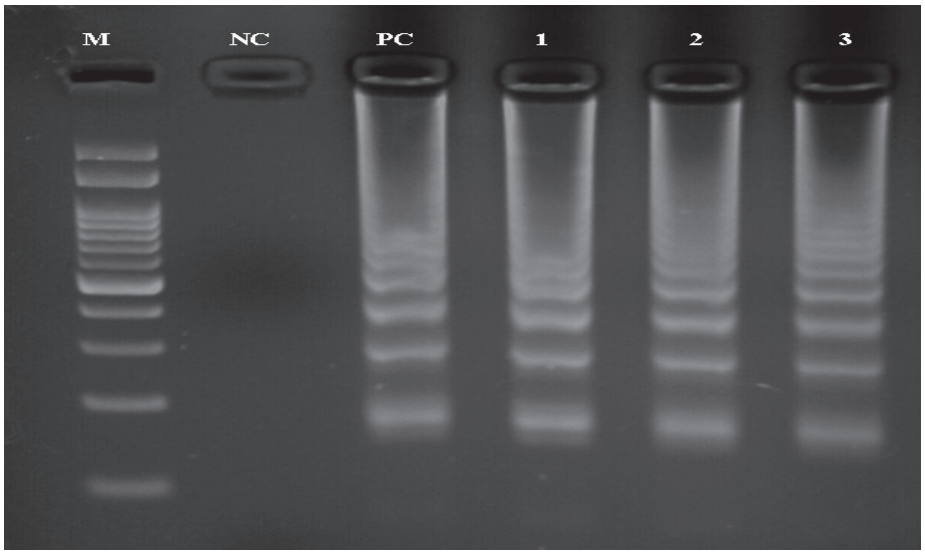

Figure 1. LAMP-PCR confirmation of culture positive *S. epidermidis* strains isolated from bovine subclinical mastitic milk. (Lane M= 100bp DNA Ladder, Lane NC= Negative control sample; Lane PC= Positive control sample; Lanes 1-3= Positive *S. epidermidis* strains).

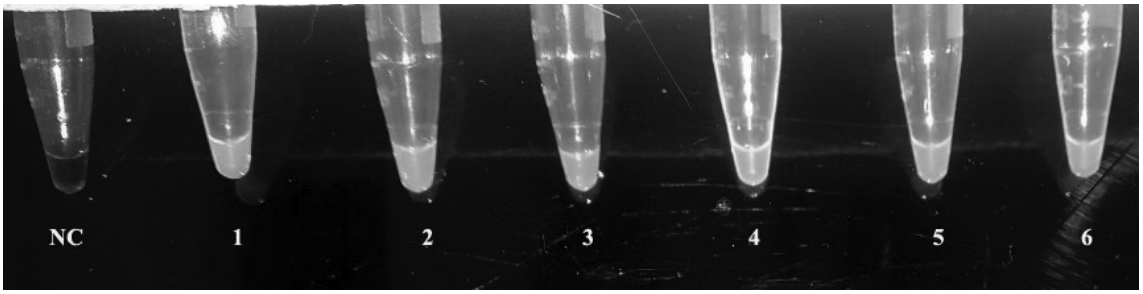

Figure 2. Specificity test of *S. epidermidis* by direct LAMP assay. Visual LAMP was carried out using DNA isolated from *S. epidermidis* (Lanes 1-6), and negative control (Lane NC). Each assay was carried out in triplicate.

Antibiotic resistance pattern of *S. epidermidis strains*

Table 3 signifies the antibiotic resistance pattern of *S. epidermidis* strains isolated from bovine subclinical mastitic milk. *S. epidermidis* isolates exhibited the highest prevalence of resistance against penicillin (100%), tetracycline (83.33%), erythromycin (83.33%), cefazolin (77.77%), and trimethoprim-sulfamethoxazole (72.22%) antibiotic agents. Prevalence of resistance against nitrofurantoin (22.22%), azithromycin (27.77%), clindamycin (33.33%), and mupirocin (33.33%) was lower than other tested antibiotic agents.

Profile of antibiotic resistance genes of *S. epidermidis strains*

Table 4 signifies the distribution of antibiotic resistance genes amongst the *S. epidermidis* strains isolated from bovine subclinical mastitic milk. *TetM* (88.88%), *aacA-D* (83.33%), *tetK* (77.77%), *ermA* (72.22%), *msrA* (55.55%), and *ermC* (55.55%) were the most routinely identified antibiotic resistance genes amongst the *S. epidermidis* strains isolated from bovine subclinical mastitic milk. Reversely, *vatC (16.66%), vatA (22.22%),* and *vatB (33.33%)* had the lowest distribution amongst studied antibiotic resistance genes. Statistically significant differences were obtained between the prevalence of *msrA* and *msrB* ($P < 0.05$), *vatB and vatC* ($P < 0.05$), and *ermA* and *ermC* ($P < 0.05$) antibiotic resistance genes.

Table 3. Antibiotic resistance pattern of *S. epidermidis* strains isolated from bovine subclinical mastitic milk.

Samples (No positive)	No samples resistance against each antibiotic agent (%)										
	P10*	CZ30	CIP5	CC2	AZM15	E15	MUP200	RA5	TE30	SXT25	F300
Mastitic milk (18)	18 (100)	14 (77.77)	12 (66.66)	6 (33.33)	5 (27.77)	15 (83.33)	6 (33.33)	9 (50)	15 (83.33)	13 (72.22)	4 (22.22)

*P10: penicillin (10 µg/disk), CZ30: cefazolin (30 µg/disk), CIP5: ciprofloxacin (5 µg/disk), CC2: clindamycin (2 µg/disk); AZM15: azithromycin (15 µg/disk), E15: erythromycin (15 µg/disk), MUP200: mupirocin (30 µg/disk), RA5: rifampin (5 µg/disk), TE30: tetracycline (30 µg/disk), SXT25: trimethoprim-sulfamethoxazole (25 µg/disk), and F300: nitrofurantoin (300 µg/disk) antibiotic agents.

Table 4. Distribution of antibiotic resistance genes amongst the *S. epidermidis* strains isolated from bovine subclinical mastitic milk.

Samples (No positive)	No samples positive for each gene (%)											
	mecA	*msrA*	*msrB*	*AacA-D*	*tetK*	*tetM*	*vatA*	*vatB*	*vatC*	*ermA*	*ermC*	*linA*
Mastitic milk (18)	9 (50)	10 (55.55)	8 (44.44)	15 (83.33)	14 (77.77)	16 (88.88)	4(22.22)	6 (33.33)	3 (16.66)	13(72.22)	10 (55.55)	9 (50)

Profile of virulence factors of *S. epidermidis strains*

Table 5 signifies the distribution of virulence factors amongst the *S. epidermidis* strains isolated from bovine subclinical mastitic milk. There were no positive results for *coa, X-region, and IgG binding region* virulence factors. *ClfA* (55.55%), *agrI* (50%), *etA* (33.33%), and *agrIII* (27.77%) were the most routinely identified virulence factors. Statistically significant differences were obtained between the prevalence of *eta and etb* ($P < 0.05$), and *agrI and agrII* ($P < 0.05$) virulence factors.

MLVA typing of *S. epidermidis* strains

All 18 *S. epidermidis* isolates were subjected to MLVA molecular typing. Figure 3 signifies the MLVA typing pattern of *S. epidermidis* strains isolated from bovine subclinical mastitic milk samples. A total of five separate loci (se1 to se5) were found amongst 18 *S. epidermidis* isolates (strains 19 to 36). *S. epidermidis* strain 22 did not have any similarities with other isolates. Other 17 isolates were classified in one profile with 99.6 to 100% similarities.

Table 5. Distribution of virulence factors amongst the *S. epidermidis* strains isolated from bovine subclinical mastitic milk.

Samples (No positive)	Virulence factors (%)									
	coa	*clfA*	*X-region*	*IgG binding region*	*tsst-1*	*etA*	*etB*	*agrI*	*agrII*	*agrIII*
Mastitic milk (18)	-	10 (55.55)	-	-	3 (16.66)	6 (33.33)	4 (22.22)	9 (50)	5 (27.77)	7 (38.88)

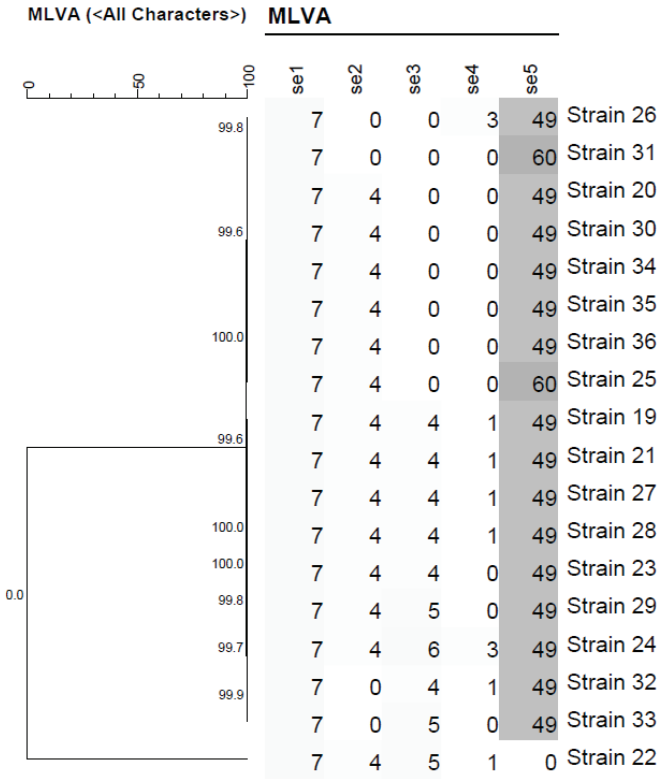

Figure 3. MLVA typing pattern of *S. epidermidis* strains isolated from bovine subclinical mastitic milk

DISCUSSION

Bovine mastitis is an economically significant disease of the dairy industry worldwide. Although numerous pathogens are listed, *Staphylococcus* spp. and particularly, coaguase-negative species such as *S. epidermidis*, is considered as imperative causative agent of subclinical mastitic milk in bovine herds. In keeping with this, bovine clinical mastitis caused by antibiotic-resistant *S. epidermidis* strains has higher implications due to the tendency to replicate chronically and more complex treatments (Pumipuntu *et al.,* 2019).

The current survey was conducted in order to assess the prevalence rate, phenotypic and genotypic patterns of antibiotic resistance, distribution of virulence factors, and MLVA typing of *S. epidermidis* strains isolated from bovine clinical mastitic milk samples. In total, 17.47% of studied samples were contaminated with *S. epidermidis* strains. *S. epidermidis* is well adapted to the human host and may prevail in large numbers in the skin microflora while it is absent or rare in the bovine microflora (Britt-Marie, 2008). Thus, one possible explanation for considerable prevalence of *S. epidermidis* in mastitic milk samples is possible transmission of *S. epidermidis* from milkers to cows. Otherwise, as dairy cows are not a natural host for *S. epidermidis*, authors suggest a human source of these udder infections. High prevalence of *S. epidermidis* was described in different human clinical infections (Nguyen *et al.,* 2017; Méric *et al.,* 2018). Coagulase-negative staphylococci (CoNS) were known as major human skin resident organisms and nowadays they are considered as one of the most important agents of

frequent nosocomial infections. A Systematic Review and Meta-Analysis conducted in Tehran showed that the frequency of MRSE infections was 73.9% [95% confidence interval (95% CI) 1.4 - 83.4] among culture-positive cases of *S. epidermidis* in different parts of Iran (Razavi *et al.,* 2018).

MLVA-based molecular typing of studied strains revealed similar genetic groups between 17 isolates. This finding revealed that all strains had similar genetic types which showed their same source of infection. This may show that all of these strains were transmitted from infected milkers and staffs of the milking halls to udder tissues. Thorberg *et al.* (2006) found *S. epidermidis* isolates with same pulsed-field gel electrophoresis (PFGE) types in samples from milk and milker's skin, which indicate that *S. epidermidis* may be transmitted from milkers to cows. Hosseinzadeh and Saei (2014) reported that 11 out of 158 (6.96%) bovine mastitic milk samples were contaminated with *S. epidermidis* strains which was much lower than our findings. Studies on prevalence of coagulase-negative *Staphylococcus* species isolated from bovine mastitic milk show a wide variation with regard to species isolated most frequently. More recently, the predominant coagulase-negative *Staphylococcus* species isolated from bovine intramammary infections are *S. haemolyticus*, *S. chromogenes*, *S. epidermidis*, *S. simulans*, and *S. xylosus* (Piessens *et al.,* 2011; Waller *et al.,* 2011; Ajitkumar *et al.,* 2013; Tremblay *et al.,* 2013). In a study from the UK in the late 1970s, 1.7% of clinical mastitis cases were reported to be due to *S. epidermidis* (Pearson and Mackie, 1979). Oliveira *et al.* (2006) stated that 37.50% of *S. epidermidis* isolates from mastitic milk samples had the ability to produce biofilm. Bentolhoda *et al.* (2016) stated that *S. epidermidis* (62.50%) and *S. chromogenes* (25.00%) were the most prevalent staphylococcal species isolated from bovine mastitic milk in Iran. The findings were also similar with those reported by Contreras *et al.* (2007) that introduced *S. epidermidis, S. chromogenes* and *S. xylosus* among the most commonly isolated coagulase-negative *Staphylococcus* species in subclinical intramammary infections in small ruminants. In another study in Turkey (Ergün *et al.,* 2009), *S. epidermidis* (35.70%) and *S. xylosus* (10.20%) were the most prevalent staphylococcal species isolated from subclinical mastitis in ewes. Pilipčincová *et al.* (2010) in Slovakia reported *S. epidermidis* (36.30%) as the most common coagulase-negative staphylococci isolated from subclinical mastitis in sheep.

This research showed that *S. epidermidis* strains isolated from bovine mastitic milk samples exhibited high prevalence of resistance against commonly used antibiotic agents. Highest prevalence of resistance was obtained against penicillin, tetracycline, erythromycin, cefazolin, and trimethoprim-sulfamethoxazole antibiotic agents. Unauthorized and illegal prescription of antibiotics is the main reason for high prevalence of antibiotic resistance. Additionally, higher prevalence of resistance against human-based antibiotic agents revealed that the *S. epidermidis* strains may recover from infected milkers who were sources of resistant *S. epidermidis* strains. Similar to these findings, high prevalence of resistance of *S. epidermidis* strains against penicillin, tetracycline, erythromycin, cefazolin, and trimethoprim-sulfamethoxazole antibiotic agents was reported by Eladli *et al.* (2019), Ma *et al.* (2011), and Bentolhoda *et al.* (2016). Results also revealed that the phenotypic pattern of antibiotic resistance was also confirmed by the genotypic pattern. Otherwise, high prevalence of *tetM*, *aacA-D*, *tetK*, *ermA*, *msrA,*and *ermC* which encode resistance against tetracyclines, aminoglycosides, tetracyclines, erythromycin, macrolides, and erythromycin antibiotics, respectively was also described in the survey. Prevalence of antibiotic resistance in *Staphylococcus epidermis* strains in human and animal samples has been cited in previous studies. Chabi and Momtaz (2019) reported that *S. epidermidis* strains isolated from human clinical infections harbored the highest prevalence of resistance against penicillin (95.65%), tetracycline (91.30%), erythromycin (82.60%), cefazolin (78.26%), and trimethoprim-sulfamethoxazole (73.91%) antibiotic agents. Reversely, *S. epidermidis* strains harbored the lowest prevalence of resistance against nitrofurantoin (34.78%) and mupirocin (50%) antibiotic agents. In this study, the prevalence of resistance against ciprofloxacin, clindamycin, azithromycin, and rifampin antibiotic agents were 69.56%, 65.21%, 60.86%, and 60.86%, respectively. Antók *et al.* (2020) reported that of the Staphylococcal strains isolated from bovine mastitis in Rwanda, a high number of the isolates was resistant to penicillin (45.3%) and tetracycline (39.1%). Twenty-three isolates were resistant to clindamycin, ten to erythromycin, and six isolates were resistant to trimethoprim-sulfamethoxazole.

Mekonnen *et al.* (2018) stated that the prevalence of resistance of *S. aureus* bacteria isolated from bovine mastitic milk in Ethiopia toward penicillin, ampicillin, tetracycline,

clindamycin, erythromycin, trimethoprim sulfamethoxazole, and rifampicin antibiotic agents were 86%, 86%, 54%, 4%, 4%, 1%, and 1%, respectively. Maalik *et al.* (2019) reported that the prevalence of resistance of *S. aureus* bacteria isolated from bovine mastitic milk in Pakistan toward augmentin, ampicillin, cefoxitin, clindamycin, chloramphenicol, ciprofloxacin, erythromycin, fosfomycin, gentamicin, kanamycin, oxacillin, ofloxacin, penicillin, rifampicin, tetracycline, teicoplanin, trimethoprim, and vancomycin antibiotic agents were 92.30%, 84.61%, 92.30%, 92.30%, 84.61%, 83.33%, 84.61%,100%, 92.30%, 100%, 100%, 84.61%, 100%, 84.61%, 84.61%, 83.33%, 100%, and 83.33%, respectively.

One of the most imperative mechanisms involving resistance against clindamycin is modulated by methylase enzyme which is often encoded by *ermA* and *ermC* genes (Zelazny *et al.,* 2005). Additionally, majority of isolates carried two tetracyclines, two erythromycins, one macrolide, and several streptogramin resistance determinants revealed a great diffusion of these types of resistance. Presence of *tetK* gene on small multicopy plasmids and *tetM* on conjugative transposons contribute to the spread of these determinants (Johler *et al.,* 2011). Some of the *S. epidermidis* strains harbored *ermC* gene. This gene is often located on small multicopy plasmids which are present in many different staphylococcal species (Johler *et al.,* 2011). The *ermA* gene is usually carried by transposons which could explain its high prevalence amongst the *S. epidermidis* strains. Resistance to aminoglycosides which is encoded by the *aacA-D* gene is more prevalent. It is because this gene is usually more diffused in staphylococci of human origin (Abdolmaleki *et al.,* 2019). Phenotypic pattern of antibiotic resistance of staphylococci isolates of other surveys were also confirmed by the genotypic pattern (Zelazny *et al.,* 2005; Dehkordi *et al.,* 2017). Eksi (2017) revealed the higher prevalence of *ermA* than *ermC* antibiotic resistance genes amongst the clindamycin, erythromycin, and telithromycin-resistant and also higher prevalence of *tetM* than *tetK* antibiotic resistance genes amongst the tetracycline-resistant MRSA strains. Similar to the findingsof this study, Duran *et al.* (2012) and Adwan *et al.* (2014) reported the high distribution of *mecA*, *ermA*, *ermB*, *ermC*, *tetK*, *tetM*, *msrA,* and *blaZ* antibiotic resistance encoding-genes in the *Staphylococcus* strains isolated from human clinical infections which may show the similar patterns of antibiotic resistance genes between human and animal clinical samples. Chabi and Momtaz (2019) reported that *aacA-D* (69.56%), *tetK* (56.52%), *mecA* (45.65%), *msrA* (39.13%), and *tetM* (39.13%) were most commonly detected antibiotic resistance genes amongst the *S. epidermidis* strains isolated from human clinical infections. Eladli et al. (2019) reported that antibiotic-resistant *S. epidermidis* strains were routinely isolated from patients, healthy students, and also pasteurized milk in the Riyadh Region. Lower prevalence of specific antibiotic resistance genes in some resistant *S. aureus* bacteria may be due to the fact that phenotypic resistance may be caused by point mutations rather than gene acquisition. Furthermore, except for the general resistance mechanisms, other factors such as biofilm formation may be the main resistance mechanism. Mechanisms of resistance to antibiotics are so complex that the presence or absence of a certain resistance gene does not certainly indicate that the particular isolate is resistant or sensitive to the corresponding antimicrobial agent. Thus, further researches are required to find additional information about the exact role of antibiotic resistance genes.

Another section of the current survey focused on the detection of putative virulence factors in the *S. epidermidis* strains isolated from bovine clinical mastitic milk samples. Results showed that *clfA*, *agrI*, *etA,* and *agrIII* had the highest prevalence amongst studied genes. Targeted factors are mainly associated with pathogenicity of *S. epidermidis* strains in clinical samples. However, there were no previously published work about the detection of virulence factors in *S. epidermidis* strains isolated from bovine clinical mastitic milk samples. Eftekhar *et al.* (2017) reported that the frequency of the spa, *fnbB*, *fnbA*, *clfB*, *clfA*, *can*, *bbp*, *ebp*, *etb*, *eta*, *pvl*, and *tst* virulence genes amongst the *S. aureus* strains isolated from hospitalized patients was 100%, 75.70%, 74.30%, 78.60%, 71.40%, 24.30%, 0%, 58.60%, 2.90%, 7.10%, 21.40%, and 51.40%, respectively. Prevalence of *tst* gene was 16.66% amongst studied *S. epidermidis* strains which was comparable with those reported from Sweden (22.00%) (Nowrouzian *et al.,* 2019), Colombia (10.00%) (Jiménez *et al.,* 2011), Malaysia (0.50%) (Lim *et al.,* 2012), and Iran (11.60%) (Alfatemi *et al.,* 2014). *Eta* and *etb* virulence genes were detected in 33.33% and 22.22% of *S. epidermidis* strains which were relatively higher than those reported from Iran (Alfatemi *et al.,* 2014), Colombia (Jiménez *et al.,* 2011), and Malaysia (Lim *et al.,* 2012) and were comparatively lower that those reported from Czech (Sila *et al.,* 2009) and Turkey (Demir *et al.,* 2011). Ghasemian *et al.* (2015) reported the high prevalence of the *clfA*

(100%) and *clfB* genes (100%) which were similar to the findings of the present survey and those conducted in Brazil (Almeida *et al.,* 2013) and China (Zhang *et al.,* 2018). Prevalence of *agrI*, *agrII* and *agrIII* virulence genes amongst the *S. epidermidis* strains were 50%, 27.77%, and 38.88%, respectively. *Agr* virulence gene was also predominant amongst the *S. epidermidis* strains isolated from clinical samples recovered from China (Zhang *et al.,* 2018) and USA (Cheung *et al.,* 2011). The accessory gene regulator (*agr*) locus influences the expression of many virulence genes in the *S. epidermidis*. Four allelic groups of *agr*, which generally inhibit the regulatory activity of each other, have been identified within the species. Interference in virulence gene expression caused by different *agr* groups has been suggested to be a mechanism for isolating bacterial populations and a fundamental basis for subdividing the species (Gomes-Fernandes *et al.,* 2017). It encodes a two-component signal transduction system that leads to down-regulation of surface proteins and up-regulation of secreted proteins during vitro growth. A role for *agr* in virulence has been demonstrated by the attenuated virulence of *agr* mutants in different animal infection models (Gomes-Fernandes *et al.,* 2017).

In conclusion, considerable prevalence of *S. epidermidis* strains was found in bovine subclinical mastitic milk samples. Furthermore, high prevalence of resistance against penicillin, tetracycline, erythromycin, cefazolin, and trimethoprim-sulfamethoxazole antibiotic agents which was accompanied with considerable distribution of *tetM*, *aacA-D*, *tetK*, *ermA*, *msrA,* and *ermC* antibiotic resistance genes were important findings of the present research. Additionally, boost prevalence of *clfA*, *agrI*, *etA,* and *agrIII* virulence factors were also found in *S. epidermidis* strains. Resistant and virulent *S. epidermidis* strains had similar molecular types which may show their similar genetic characters. Additionally, this may pose a similar source of infection of bacterial strains. The phenotypic pattern of antibiotic resistance was also confirmed by genotypic profile. Higher prevalence of resistance against human-based antibiotics may show that milkers and staff of the milking halls were sources of resistant and virulent *S. epidermidis* strains. Data gained from this study emphasized the need of the comprehensive research on other aspects of the *S. epidermidis* strains isolated from bovine subclinical mastitic milk samples, particularly, their antibiotic resistance pattern against other important antibiotic agents, prevalence of other important antibiotic resistance genes and virulence factors, comparison of different typing methods, and also the typing pattern of *S. epidermidis* strains isolated from different sources.

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STATEMENT ON COMPETING INTEREST

The authors declare that they have no competing interests.

AUTHOR'S CONTRIBUTION

HM and FT carried out the molecular genetic studies, participated in the primers equence alignment and drafted the manuscript. FT and ZB carried out the sampling and culture method. HM and ZB participated in the design of the survey, performed the statistical analysis and writing the manuscript. All authors read and approved the final manuscript.

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