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

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#### 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

#### **INTRODUCTION**

Mastitis is inflammation of the an 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).

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Bacteria are the most important causes of mastitis in dairy farms, especially in cows (Momtaz et al., 2012; Ruegg, 2017). The most microorganism causing important mastitis isStaphylococcus spp. and some in cases, Staphylococcus epidermidis (S. epidermidis), is responsible for antibiotic resistant mastitis in dairy herds (Britt-Marie, 2008:Pumipuntu *et al.*, 2019). It 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

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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, macrolidelincosamide-streptogramin Β, 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 bv 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  $\mu$ l 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 ul S. epidermidis DNA template, and 2.26 ul ddH<sub>2</sub>O. 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 AmpliTaq Gold (Applied Biosystems, Stockholm, Sweden), 0.2mМ 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  $\geq 80\%$ similarity were considered to belong to the same MLVA type, respectively.

tratA     F: TGGTCCGGAACAACACTTAT     268       ratB     R: CCGCCAATTCAGTTGTACAA     36       ratC     F: AGGCCCCAATCCAGAGAA     36       ratC     F: AAGGCCCCAATCCAGAGAA     47       ratC     R: CTGACCAATCCCAGAAGAA     47       ratC     F: AAAATCGATGGTAAAGGTTGGC     32       ratC     F: GAGGCCCAATCCCAGAAGCA     47       ratC     F: GAGGCCCAATAGGTAAAGGTTGGC     360       ratC     F: AGTGGGACAATAAACTCCCATA     360       ratC     F: AGTGGGACAATAAAGGTGTTAACAGA     360       ratC     F: AGGTGGACAATAAGAGTGTTAACAGAA     360       ratC     F: AGGTGGACAATAAGAGTGTTAACAGAA     360       ratC     F: AGGTGACAATAAGATGATCCCTATAGGT     360       ratC     F: AGGTAATACCATATGATAGATGTGTCCTGTT     360       ratC     F: AGGTAATACAATAAATTACCATAGGAA     360       ratC     F: AGGTAATACCATATGAATAGATGTGTCCTGTT     360       ratC     F: AAGTATATCATGAATAGATGTGTCCTGTT     360       ratC     F: AAGTATATCATGAATAGATGTGTCCTGTT     360       ratC     F: AAGTATATCATAAAATTATCCAATACCATA     370       ratC     F: AAGTATATCATATAAATTATCCATAAGGGC     323       ratC     F: AAGCGCCCAATCCTGCAAGCTTGAAT     320       ratC     F: CATCATCTTTTGAATACATAGATTTATCCAATACCACCGTTTA     360	ct (bp)	
outA     R: TCCACCGACAATAGAATAGGG     203       vatB     F: GCTGCCAATCCAGTTGTACA     136       retB     R: CGACCAATCCAGTGTACAA     467       retAGGCCCAATCCAGGAGAA     467       meeA     F: AAAGGTCTGTGGCAAAGGT     360       meeA     F: GTAGCGACAATAGGTAATAGT     360       blaZ     F: GTAGCGACAATAAGGTAGTC     360       blaZ     F: GTGGGGCAATAGGTAATAGT     360       meeA     F: GTGGGACAATAAGGTAGCTC     360       tetM     R: GCTGACCACTTTATACAGCA     360       tetM     R: GCTGGACCACTTTATACGCC     360       msrA     F: GGGACAATAAGGGGTATACAGAA     360       msrB     R: CAAGTTATCCTGGCGCTGCTCA     360       msrB     F: GGCACAATAAGATTGTCCTCGTT     940       msrB     F: GGCACATAAGAGTGTCTCCTGTT     940       msrB     R: CAAGTTATATCATGAATAGATTGTCCTGTT     940       msrB     R: CAAGTTATATCATGAATAGATTGTCCTGTT     940       msrB     R: GCACACTTATATCATGAATAGATTGTCCTGTT     940       msrB     F: AAGTGATATCCATAGATAGATTGTCCTGTT     940       msrB     R: CACACTTATATCATGAATAGATTGTCCTGTT     940       msrB     F: AAGTGATACACATGAAGGGC     227       msrB     F: GGCGCCACCTTTCAATAGGTATTGTCCTGTT     247       msrD     F: AAGTGATACATCCATGATTACCATGA <td< td=""><td></td></td<>		
vatB     F: GCTGCGAATTCAGTTGTACA     136       R: CTGACCAATCCAGATCCAGAGAA     467       vatC     F: AAGGCCCCAATCAGGAGAA     467       meeA     F: AAAATCGATGGTAAAGGTTGGC     532       tetK     F: GTAGCGACAATAGGTAAGGTTGGC     532       tetK     F: GTAGCGACAATAGGTAATGGT     60       blaZ     F: AAGGATTTGCCTATGCTTC     360       tetM     F: AGTGGACGATAAGGTAATAGGT     90       fetM     F: GCTGACCACATAAGGTGTTACAGAA     158       msrA     F: GGCACAATAGAAGTGTTTAAAGG     90       msrB     F: TAGATATCATGAATAGATTGTCCTGTT     90       remmA     F: AAGGTATATCATAATTATCCACATA     27       aacA-D     F: TAATCCAAGAGCATTAACGAA     190       remmA     F: AAGGTGCAATACCCTTGTCAA     29       remmA     F: AAGGTGCAATACCCTTGTCAACCCTTGGA     29       remmA     F: AAGGTGCAATACCCTTGTAACGAATAAGGGC     29       remmA     F: AAGGTGCAATACCCTTGTCAACCCTTGTA     300       remmA     F: AATCGTCAATTCCTGCATGT     29       remmA     F: AATGGTCAATTCCTGCATGT     29       remmA     F: GGTGGCGGGGGTAGATCAGGTTGATA     300       remmA     F: GTGGCGCGGGGGTAGAGTTATTAACTGGAA     300       remmA     F: GTGGCGCGGGGGGTAGAGTTGATTATACTGG     300       rermC     F: ATGGCACTAGGCGGGGGGTAG		
bulb     R: CTGACCAATCCCACCATTTA     155       vatC     F: AAGGCCCCAATCCAGAAGAA     467       mecA     F: AAAATCGATGGTAAAGGTTGGC     532       tetK     F: GTAGCGACATAGCAGATTGCC     532       tetK     R: GTGTGGCACATAGGTAAAGGTTGCC     532       tetM     R: GTAGTGACAATAAGTAAGGTAGTGAATAGT     360       blaZ     F: AAGGCACTATGCCATTGCCTC     360       tetM     F: AGTGGACGATTACAGGA     158       msrA     F: GGCACAATAAGGTGTTAAAGG     940       r: AAGTTATATCATGAATAGATGGTGCTGTT     555       aacA-D     F: TATGATATCCATAATAGATTGCCTGTT     555       aacA-D     F: TAGTATACCATATAGATAGGCCCTCTGT     227       ermA     F: AAGGGGTAAACCCCTTGCAAT     190       ermA     F: AAGGGGTAAACCCCTTGCAAT     229       f: AAGTTATTCCAGAATCCATAGCAGGCATTACAGG     229       aacA-D     F: AAGCGGCTAAACCCCTTGGA     220       ermA     F: AAGGGGTAAACCCCTTGCAAT     233       ermA     F: AGTGGCAGGGGGGGGGGGGAGAGTGTATTAACTGG     223       isst-1     R: TTCCAATAACAGGTTGCAATTTCCAA     230       ermA     F: AGGGCGAATAACAGGGCTAAGTGGAAT     200       isst-1     R: TGCAGGGGGGGGGGGGGGGGGGAGATTAGAGGG     233       ermA     F: ATGCACATGGGGGGGAAGATTGAAGTGGAAT     200       isst-1     R: TGCAGGGG		
vatC     F: AAGGCCCCAATCCAGAAGAA     467       R: TCAACGTTCTTTGTCACAACC     F: AAAATCGATGGTAAGGTTGGC     532       meeA     F: AAAATCGATGGTAACGGATTGC     532       tetK     F: GTAGCGACATAAGGTATGGT     600       blaZ     F: AGAGATTAGCCTATGCTAC     600       blaZ     F: AGGGACGATAAAGCTGCTA     600       tetM     F: AGTGGACCAATAAAGAGTGTTTACAGC     600       msrA     F: GGCACAATAAGAGGTGTTTAAAGG     600       msrB     F: GGCACAATAAGAGGTGTTTAAAGG     600       msrB     F: GGCACAATAAGAGTGTTAAAGG     600       msrB     F: TATGATATCCATAATAATTATCCAGAA     600       msrB     F: TATGATATCCATAAAGAGTGTTTAAAGG     600       msrB     F: TATGATATCCATAATAATTATCCATAAGG     600       msrB     F: TAGGTAATCCATAGATAAGGTGTTTAAAGG     600       msrB     F: AAGGTTATATCATGAATAAGATGTGCCTGTT     600       msrB     F: AAGGTGATACCACCTCTGA     201       macA-D     F: AAGGGTAACCCCTTCGAATACGGGC     221       msrB     F: AAGGTGGTGGGGGGAGATGTATTACCATGA     200       msrB     F: AAGGGTGAACCCCTTCGAA     200       msrB     F: AAGGTGGTGCGGGGGGAGATGTATTACCATGA     200       msrB     F: GGTGGCTGGGGGGGGAGATGTATTTTTCGA     200       msrB     F: AGTGGGCGCGGGGGGGAGATGTATTTTTTTGGA     200 <td></td>		
burc     R: TCAAGGTTCTTTGTCACAACC     407       mecA     F: AAAATCGATGGTAAAGGTTGGC     532       tetK     F: GTAGCGACAATAGGTAATAGT     360       blaZ     F: AAGAGATTGCCTATGCTTC     360       blaZ     F: AAGAGATTGCCTATGCTTC     360       tetM     R: GCTTGACCACTTTTATCAGC     360       msrA     F: AGTGGAGCGATACGGCGGTTA     360       msrA     F: GGCCACATAAGGTGTTAAAGG     360       msrA     F: GGCACAATAAGGTGTTAAAAGG     360       msrA     F: TATGATATCCATAATAATGATGCCTGTT     360       msrA     F: TATGATATCCATAATAATGATGCTGTCTGTT     940       msrA     F: TATGATATCCATAAAAGGTGTTTAAAGG     360       msrA     F: TATGATATCCATAGAATAGATTGTCCTGTT     595       aaeA-D     F: TATGATATCCATAGAATAAGATTGTCCTGTT     595       aeermA     F: AAGCGGTAAACCCTTCGAACCATGA     227       ermA     F: GGTGGCTGGGGGGAAATTACCATGT     229       linA     R: GCTTCTTTTGAAATCCTGCAATGT     229       linA     F: CAGGCCAATACATACATGGTATTATCAA     360       ermC     F: AAGGCGGGGGAAGATGTATTACAGGTATT     323       ermA     F: CTAGTGCAGCTGGAGGTGAATGTATTACTGG     323       isst-1     R: TCCATCGCATATCATACATGTAAA     200       ermA     F: ATGCACAGGTGTAACACATGGTACATG     320       gg		
mecAF: AAAATCGATGGTAAAGGTTGGC532tetKF: GTAGCGACAATAGGTAATAGT360tetKF: GTAGCGACAATAAGCTCCTA360blaZF: AAGAGATTGCCCATGCTC360tetMF: AGTGGGAGCGATTACAGCA360tetMF: AGTGGGAGCGATTACAGCA360msrAF: GGCACAATAAGGTGTTAAAGG360msrBF: GGCACAATAAGGTGTTTAAAGG360aacA-DF: TATGATATCCATAATAGATTGTCCTGTT940acA-DF: TATGATATCCAAGAGGTGTTAAAGG360aacA-DF: TATGATATCCAAGAGCAATAAGGTCCTGTT360aacA-DF: AAGCGGTAAACCCCTCTGA390acach-DF: AGCGGCAAATCCCTTCTCCAAC190cermAF: AGCGGTAAACCCCTCTGA323test-IF: GGTGGCTGGGGGGGAGAATACGGTAGATA323test-IF: GGTGGCTGGGGGGAGAATCAGGTAGAT360agrIIF: ACGCCTATTAACAATCATGGAATAGCTCCAAT360agrIIIF: AGCACAAGATCAATTGTCAATT300rermAF: ACGCCAATAACATGGCAATAGCCCATGC375agrIIIF: ATGCACAAGGTACAATACATGGCCAATGC375agrIIIF: ATGCACAAGGTGCACATGC370rermAF: ATGCACAAGGTGCACATGC323rermAF: ATGCACAAGGTGCACATGC323rermAF: CAGGCAATAACATGGCCAATGC375rermAF: ATGCACATGGTGCACATGC375rermAF: ATGCACATGGTGCACATGC323rermAF: CTAGGCACATGCTGCAATGC375rermAF: CTAGGCACATGGTGCACATGC375rermAF: CTAGGCACATGGTGCACATGC376rermAF		
mecA     R: AGTTCTGCAGTACCGGATTTGC     502       tetK     F: GTAGCGACAATAGGTAATAGT     360       tetK     R: GTAGTGACAATAAACCTCCTA     360       blaZ     F: AAGAGATTTGCCTATGCTTC     360       tetM     R: GCTTGACCACTTTTATCAGC     360       tetM     F: AGTGGAGCGATTACAGAA     158       msrA     F: GGCACAATAAGAGTGTTAAAGG     940       msrB     F: TATGGATATCCATAATAATTATCCAATG     940       msrB     F: TATGGATATCCATAATAATTATCCAATG     395       aacA-D     R: GCCACACTAATAAGATTGTCCTGTT     595       aacA-D     R: GCCACACTATCATAACAGTAGGTGTCTGAA     227       ermA     F: AAGGGTAAACCCCTTGGA     190       ermA     R: TTCGCAATTCCTGCATGT     229       inA     F: GGTGGCTGGGGGGTAGATGTATTAACTGG     223       ermC     F: AATCGTCAATTCCTGCATGT     229       inA     F: GGTGGCTGGGGGGTAGATGTATTAACTGG     323       tsst-1     R: GTTCTTTTGAAATACATGGTATA     350       etA     F: CTAGGCACTAGCTTGATA     360       agrI     F: ATGCACATGGTGCCAATGC     211       etA     F: GGTGGCTGGGGGGTAGATGTATTAACTGG     323       f: eta     F: ATGCCACATGGTAGCATTAGATT     360       agrI     F: ATGCACATGGCCATAGCACCATGC     320       agrI     F: ATGCACATGGTGCACATGC		
tetK     F: GTAGCGACAATAGGTAATAGT     360       R: GTAGTGACAATAAACCTCCTA     360       blaZ     F: AAGAGATTTGCCTATGCTTC     360       R: GCTTGACCACTTTTATCAGC     360       tetM     F: AGTGGAGCGATTACAGAA     360       msrA     F: GGCACAATAAGAGTGTTTAAAGG     360       msrB     F: GGCACAATAAGAGTGTTTAAAGG     360       msrB     F: GGCACAATAAGAGTGTTTAAAGG     360       msrB     F: TATGGATATCCATAATAATTATCCAAGG     360       aacA-D     F: TATGGATATCCATAATAATTATCCAATC     360       aacA-D     F: TATGGATATCCATAATAATTATCCAATC     360       aacA-D     F: TATGGATATCCATAATAAGTTGTCCTGTT     360       aacA-D     F: TATGGATATCCATAATAAGTTGTCCTGTT     360       aacA-D     F: TATGGAAATCCAAGAGCAATAAGGGC     227       aacA-D     F: TATGGCACCACTACTAATACCACTGA     360       aacA-D     F: AAGCGGTAAACCCCTCTGAA     190       ermA     F: GCTGGCTGGGGGGTAGATGTATTATACCTGA     323       ermA     F: GGTGGCTGGGGGGTAGATTACATTAACTGG     323       inhA     F: GGTGGCTGGGGGGTAGATTCTTTTTTTTCGA     323       isst-1     F: ATGGCAACACTGGCTTTTTTTTTTTTTTTTTTTTTTTTT		
retkR: GTAGTGACAATAAACCTCCTA360blaZF: AAGAGATTTGCCTAGGTTC360ketMF: AGTGGAGCGATTACAGAA158retMR: CATATGTCCTGGCGTGTCTA158msrAF: GGCACAATAAGAGTGTTTAAAGG940msrBF: TATGATATCCATAGAATAGATTGTCCTGTT940msrBF: TATGATATCCATAGAATAGATTGTCCTGTT955aacA-DF: TAAGATAACCACTATCATAACAATGGCC227aacA-DF: AAGCGGTAAACCCCTCTGA190rermAF: GGTGGCTGGGGGGAGATCAATCCTGCAGT229inAF: GGTGGCTGGGGGGTAGATGTATTAACTGG223inAF: GGTGGCTGGGGGGAGATGATGTATTAACTGG223issi-1F: GTGGCATGGCACCAGCTTGATA350etAF: CTAGTGCATTGTAATACATGGTATTTAACTGG23issi-1F: ATGGCAGCATCAGCTTGATA119etBF: ACGGCATTATAACATGGTAATT200agrIIF: ATGCACAGTGGCAATGC575agrIIIF: ATGCACAGTGGCGCATGC323issi-1F: ATGCACAGTGGCGCATGC323issi-1F: ATGCACAGTGGCGCATGC320issi-1F: ATGCACAGTGGCGCATGC320issi-1F: ATGCACAGTGGCGCATGC320issi-1F: ATGCACAGTGGCGCATGC320issi-1F: ATGCACAGGGCCATGC320issi-1F: ATGCACAGGGCCATGC320issi-1F: ATGCACAGGGCCATGC320issi-1F: ATGCACAGGGCCATGC320issi-1F: ATGCACAGGGCCATGC320issi-1F: ATGCACAGGGCCATGC320issi-1F: ATGCACAGGGCCATGC320 </td <td></td>		
blaZF: AAGAGATTTGCCTATGCTTC360R: GCTTGACCACTTTATCAGC158tetMF: AGTGGAGCGATTACAGAAR: CATATGTCCTGGCGTGTCTA940msrAF: GGCACAATAAGAGTGTTTAAAGGmsrBF: TATGATATCATGAATAGATTGTCCTGTTP: TATGATATCCATGAATAGATTGTCCTGTT595aacA-DF: TAAGCGGTAAACCCCTCTGAermAF: GAGCGGTAAACCCCTCTGACPermAF: GAGCGGTAAACCCCTCTGATPermCF: GATGGCAGGGGGGGAGATGATTAACTGGR: GCTCTTTTGAAATACATGGTTGATA229linAF: GGTGGCTGGGGGGTAGATGTATTAACTGGR: GCTTCTTTTGAAATACATGGTTGATA360etAF: CTAGTGCAATCCCTGTGATAR: GCTCTTTTGAAATACATGGTTGATA360agrIIF: ATGGCAAGGCACCAAGTGCCATGCR: GTAATGTCAATGGGCAATGC575agrIIIF: ATGCACAGGTGGACATGGCCATAGCR: GTAATGTAAAGGTGGCCATAGC323coaF: ATGACACGGGGACAAGACTCCAAGCR: GTAATGACAATGGTGGCAATGC323agrIIIF: ATGCACAGTGGTGCAACGCR: GTAATGTAATAGATGGTGCAATGC323coaF: ATGACACAGGTGCAACGCR: GTAATGACAATGGTGCAACTGC323AcaF: ATGCACAGTGGCAATGCR: GTAATGTAATAGCTTGTATAATAAACTGGCCATAGCR: GTAATGTATAAAGCTGGCCATAGCR: GTAATGTATAAAGCTGGCCATAGCR: GTAATGTATAAAGCTGGACACAGCR: GTAATGTATAAAGCTGCACACGCR: GTAATGTATAAAGCTGCACACGCR: GTAATGTATAATACACACTGCAAGCR: GTAATGTATAATACATGGTGCAACAGCR: GTAATGTATAAAGCTGCACACGCR: GTAATGTATAAACACCACTGCAACAGCR: GTAATGTATAAAGCTGCAACAGC<		
blaZ     R: GCTTGACCACTTTATCAGC     360       tetM     F: AGTGGAGCGATTACAGAA     158       msrA     F: GGCACAATAAGAGTGTTTAAAGG     940       msrB     F: TATGATATCATGAATAGAGTGTTTAAAGG     940       msrB     F: TATGATATCCATAATAATATTATCCAATC     595       aacA-D     F: AAGTTATATCATGAATAGATTGTCCTGTT     595       aacA-D     F: AAGCGGTAAACCCCTCTGA     227       ermA     F: AAGCGGTAAACCCCTTCTCAAC     190       ermA     F: GGTGGCTGGGGGGGAGATTCCTGCATGT     229       inA     F: GGTGGCTGGGGGGGAGATGTATTAACTGG     223       isst-1     R: GCTTCTTTTGAAATACATGGTATTAACTGG     350       etA     F: CTAGTGCAGTGGGGGTAGATGTATTAACTGG     323       isst-1     R: GCTTCTTTTGAATACATGGTTATTAACTGG     323       etA     F: CTAGTGCAGTAGGTGTGTATA     350       etA     F: CTAGTGCAGTAGGTGTGTATA     350       etA     F: ATGCACATGGTGAATTACATGATA     200       agrII     R: GTCACAAGTACTATAAGCTGCCATGC     375       agrIII     F: ATGCACATGGTGCACATGC     375       coa     F: ATGCACATGGTGCACATGC     323       coa     F: ATGCACATGGTGCACATGC     323		
ItetM     F: AGTGGAGCGATTACAGAA     158       R: CATATGTCCTGGCGTGTCTA     940       msrA     F: GGCACAATAAGAGTGTTTAAAGG     940       msrB     F: TATGATATCCATAATAGAATGGATTGTCCTGTT     940       aacA-D     F: TAATCCAAGAGCAATAAGATTGTCCTGTT     940       aacA-D     F: TAATCCAAGAGCAATAAGGGC     227       aacA-D     F: AAGCGGTAAACCCCTCTGA     190       ermA     F: AAGCGGTAAACCCCTCTGAA     190       ermA     F: GGTGGCTGGGGGGTAGATGTATTAACTGG     229       finA     F: GGTGGCTGGGGGGTAGATGTATTAACTGG     323       tsst-1     F: ATGGCAGCATCAGCTTGATA     300       ettB     F: ACGGCTATATACATGGAATTCCTGCATAT     300       ettB     F: ACGGCTATATACATGGTGACATAC     300       agrII     F: ATGGACAGTATATACATGGTGCAATAGC     300       agrIII     F: ATGCACAAGGTGCAATAAGCGCCCATAGC     300       r: TATTACTAATTGAAAAGCGGCCAAGC     575       agrIII     F: ATGCACATGGTGCACATGC     300       r: GCAACATGTGATGATATAAAACAAGGGCCAATAGC     575       agrIII     F: ATGCACATGGTGCACATGC     300       r: GCAACATGGTGCACATGC     300     300       r: GTAATGTAAATAGGTTGTATTAAATAGCTGCAAGC     575       agrIII     F: ATGCACATGGTGCACATGC     300       r: CAAGAAACCACTGGTGCAAAGC     770       <		
letM     R: CATATGTCCTGGCGTGTCTA     138       msrA     F: GGCACAATAAGAGTGTTTAAAGG     940       msrB     F: TATGATATCCATGAATAGATTGTCCTGTT     940       aacA-D     F: TATGATATCCATAATAGATTGTCCTGTT     595       aacA-D     F: TAATCCAAGAGCAATAAGATTGTCCTGTT     595       aacA-D     F: AAGCTGTATATCATGAAAGAGTGTCCTGTT     595       aacA-D     R: GCCACACTATCATAACCACTA     227       aacA-D     F: AAGCGGTAAACCCCTCTGA     190       ermA     F: AAGCGGTAAACCCCTCTGAA     190       ermC     F: AATCGTCAATTCCTGCAGTGT     229       linA     F: GGTGGCTGGGGGGGAGAGTGTATTAACTGG     323       tsst-1     F: ATGCACCACGCATCAGCTTGATA     350       etA     F: CTAGTGCAATTCGTAACCCCGTTT     119       etA     F: ATGCACAGGTGCACATGGCTGACATG     119       etA     F: ATGCACAGGTGCACATGC     441       ggrII     F: ATGCACAGGTGCACATGC     575       aggrIII     F: ATGCACATGGTGCACATGC     323       coa     F: ATGCACATGGTGCACATGC     323       coa     F: ATGCACATGGTGCACATGC     323       etB     F: ACGCCATGGTGCACATGC     320       ggrIII     F: ATGCACATGGTGCACATGC     323       f: GTAATGTAATTAATTGAAAAGTGGCCATAGC     575       ggrIII     F: ATGCACATGGTGCACATGC     3		
msrAF: GGCACAATAAGAGTGTTTAAAGG940R: C AAGTTATATCATGAATAGATTGTCCTGTT595msrBF: TATGATATCCATAATAATATATCCAATC595aacA-DF: TAATCCAAGAGCAATAAGGGC227aacA-DF: AAGCGGTAAACCCTCTGA190ermAF: AAGCGGTAAACCCTTCCAAC190ermAF: GGTGGCTGGGGGGGAGAGTGTATTAACTGG229innAF: GGTGGCTGGGGGGGAGAGTGTATTAACTGG323innAF: CGTGGCTGGGGGGAGAGTGTATTAACTGG320innAF: CAGGCACACAGCTTGATA350innAF: CAGGGCAGCACCAGCTTGGTAT350innAF: CAGGGCATGAGCATCAGCTTGATA360innAF: CAGGGCATGAGCATCAGCTTGATA360innAF: ATGCACATGGCATTGTATTCAA360innAF: ATGCACTGGCACATGGCTTGATA360innAF: ATGCACTGGCACTTGGTATATCAATT360innAF: ATGCACTGGCACTTGGTATATACATGGA370innAF: ATGCACTGGCACTTGGTATATACATGGAATT360innAF: ATGCACATGGCACATGGCACATGC370innAF: ATGCACATGGTGCACATGC370innAF: ATGCACATGGTGCACATGC <td< td=""><td></td></td<>		
msrAR: C AAGTTATATCATGAATAGATTGTCCTGTT940msrBF: TATGATATCATGAATAGATTGTCCTGTT595aacA-DF: TAATCCAAGAGCAATAAGGGC227aacA-DF: TAATCCAAGAGCAATAAGGGC227ermAF: AGCGGTAAACCCTTCTGA190ermAF: AAGCGGTAAACCCTTCTGAAC190ermAF: GGTGGCTGGGGGGGGGGGGGGGGTAGATGCTGTGT229linAF: GGTGGCTGGGGGGGGGGGGGGGGTAGATGTATAACTGG323tsst-1F: ATGGCAGCATCAGCTTGATA350etAF: CTAGTGCATTGTCATAGCACCGTTT350etAF: CTAGTGCATTGTATTCAA119tsst-1F: ACGGCTATAACCACCGTTT350etAF: CTAGTGCATTGTCATAGAATT200agr/IF: ATGCACTGGTGCACATGC371agr/IIF: ATGCACATGGTGCACATGC375agr/IIF: ATGCACATGGTGCACATGC375coaF: ATGCACATGGTGCACATGC323coaF: CGAGACCAAGATTCAACAGC370restrictF: ATGCACATGGTGCACATGC375agr/IIF: ATGCACATGGTGCACATGC323restrictF: ATGCACATGGTGCACATGC375agr/IIF: ATGCACATGGTGCACATGC323restrictF: ATGCACATGGTGCACATGC323restrictF: ATGCACATGGTGCACATGC323restrictF: ATGCACATGGTGCACATGC323restrictF: ATGCACATGGTGCACATGC323restrictF: ATGCACATGGTGCACATGC323restrictF: ATGCACATGGTGCACATGC323restrictF: ATGCACATGGTGCACATGC323restrictF: ATGCACAT		
msrBF: TATGATATCCATAATAATTATCCAATC595R: AAGTTATATCATGAATAGATTGTCCTGTT217aacA-DF: TAATCCAAGAGCAATAAGGGCR: GCCACCACTATCATAACCACTA217ermAF: AAGCGGTAAACCCCTCTGAR: TTCGCAAATCCTTCCAAC190ermCF: AATCGTCAATTCCTGCATGTR: GCTTCTTTTGAAATACAGGGAATAACGGG229linAF: GGTGGCTGGGGGGGTAGATGTATTAACTGGR: GCTTCTTTTGAAATACATGGTATTAACTGG323tsst-1F: ATGGCAGCATCAGCTTGATAR: TTCCAATAACCACCGTTT300etAF: CTAGTGCATTGTATTCAAR: TGCATTGACACCATAGTACT119etBF: ACGGCTATATACATGGTGCACATGCAggrIIF: ATGCACAGGTGCACATGCR: TATTACTAATTGAAAAGTGGCCATAGC323aggrIIIF: ATGCACATGGTGCACATGCR: GTAATGTAATAGCTTGTATAATAATAACCAGG323cagrIIIF: ATGCACATGGTGCACATGCR: GTAATGTAATAGCTTGTATAAAAGTGGCCATAGC323cagrIIIF: ATGCACATGGTGCACATGCR: GTAATGTAATAGCTTGTATAATAATAACCACGCoaF: CGAGACCAAGATTCAACAGR: GTAATGTAATAGCTTGTATAATAATAACCAGGR: GTAATGTAATAGCTTGTATAATAATACCCAGR: AAAGAAAACCACTCACATGACoaF: CGAGACCAAGATTCAACAAGR: AAAGAAAACCACTCACATGAR: AAAGAAAACCACTCACATGAR: AAAGAAAACCACTCACATGAR: AAAGAAAACCACTCACATGAR: AAGAAAACCACTCACATGAR: AAGGAAACCACTCACATGAR: AAAGAAAACCACTCACATGAR: AAGGAAACCACTCACATGAR: AAGGAAAACCACTCACATGAR: AAGGAAACCACTCACATGAR: AAGGAAAACCACTCACATGAR:		
msrBR: AAGTTATATCATGAATAGATTGTCCTGTT595aacA-DF: TAATCCAAGAGCAATAAGGGC227R: GCCACACTATCATAACCACTA227ermAF: AAGCGGTAAACCCCTCTGA190ermCF: AATCGTCAATTCCTGCATGT229R: AATCGTCCAATTCCTGCATGT229linAF: GGTGGCTGGGGGGGTAGATGTATTAACTGG323tsst-1F: ATGGCAGCATCAGCTTGATA350etAF: CTAGTGCATTGTTATTCAA119etAF: CTAGTGCATTGTTATTCAA119etAF: ATGCACATGATACCACCGTTT200agrIIF: ATGCACAGGTGCACATGC441R: GTCACAAGTACTATAAGCTGCGCAT441GagrIIIF: ATGCACATGGTGCACATGC575agrIIIF: ATGCACATGGTGCACATGC323coaF: ATGCACATGGTGCACATGC323f: ATGCACATGGTGCACATGC323griiiF: ATGCACATGGTGCACATGCgriiiF: ATGCACATGGTGCACATGCgriiiF: ATGCACATGGTGCACATGCgriiiF: GGAGCCAAGATTCAACAAGgriiiF: GGAGCCAAGATTCAACAAGgriiiF: GGAGCCAAGATCAACAAGGgriiiF: GGAGCCAAGATTGCAACAGG </td <td></td>		
aacA-DF: TAATCCAAGAGCAATAAGGGC R: GCCACACTATCATAACCACTA227ermAF: AAGCGGTAAACCCCTCTGA R: TTCGCAAATCCCTTCTCAAC190ermCF: AATCGTCAATTCCTGCATGT R: GCTTCTTTTGAAATACATGGTATTAACTGG R: GCTTCTTTTGAAATACATGGTATTAACTGG R: GCTTCTTTTGAAATACATGGTATTTACTGG R: GCTTCTTTTGAAATACATGGTATTTTCCAA AATCGCAGCATCAGCTTGATA R: TTTCCCAATAACCACCCGTTT323tsst-1F: ATGGCAGCATCAGCTTGATA R: TTTCCCAATAACCACCCGTTT350etAF: CTAGTGCATTGTTATTCAA R: TGCATTGACACCATAGTACT119etAF: ACGGCTATATACATCGATAT R: TCCATGATAATATACATACATGC200agrIIF: ATGCACATGGTGCACATGC R: ATTACTAATTGAAAAGTGGCCATAGC323agrIIIF: ATGCACATGGTGCACATGC R: GTAATGTAATAGCTTGTATAATAATACCCAG R: GTAATGTAATAGCTTGTATAATAATACCCAG R: AAAGAAAACCACTCACATGA R: AAAGAAAACCACTCACATGA323acaF: CGAGACCAAGATTCAACAAG R: AAAGAAAACCACTCACATGA R: AAAGAAAACCACTCACATCA323		
aacA-DR: GCCACACTATCATAACCACTA227ermAF: AAGCGGTAAACCCCTCTGA190R: TTCGCAAATCCCTTCTCAAC190ermCF: AATCGTCAATTCCTGCATGT229linAF: GCTGGCTGGGGGGGGAGATGTATTAACTGG323rsst-1F: ATGGCAGCATCAGCTTGATA350etAF: CTAGTGCATTGTAATCCAGCTTGATA350etAF: CTAGTGCATTGGTACACCCGTTT119etAF: ACGGCTATATACATGGTGATATTAACTAG200agrIF: ATGCACAGGTGCACATGC411R: GTCACAAGTACTATAAGCTGCGGAT575agrIIIF: ATGCACATGGTGCACATGC323coaF: ATGCACATGGTGCACATGC323r: GTAATGTAATAGCTTGATAATAATAATACCCAG320r: AAGGAAAACCACTGGTGCACATGC323r: AAGGACAAGGTGCACATGC323r: ATGCACATGGTGCACATGC323r: GTAATGTAATAGCTTGTATAATAATAACCCAG323r: GTAATGTAATAGCTTGTATAATAACATGC323r: GTAATGTAATAGCTTGTATAATAACCCAG323r: GTAATGTAATAGCTTGTATAATAGCTGC323r: GTAATGTAATAGCTTGTATAATAACCCAG323r: GTAATGTAATAGCTTGTATAATAACCCAG323r: GTAATGTAATAGCTTGTATAATAACCCAG323r: GTAATGTAATAGCTTGTATAATAACCACAG323r: GTAATGTAATAGCTTGTATAATAACCACAG323r: GTAATGTAATAGCTTGTATAATAACCACAG323r: GTAATGTAATAGCTTGTATAATAACCACAG323r: GTAATGTAATAGCTTGTATAATAATAATACCCAG323r: GTAATGTAATAGCTTGTATAATAACCACCAGAGATTCAACAAG323r: GTAATGTAATAGCTTGTAATAAGCACCACAGAGATCAACAAG323 <td <="" gtaatgtaatgaacacaccactcacatga<="" r:="" td=""><td></td></td>	<td></td>	
ermAF: AAGCGGTAAACCCCTCTGA R: TTCGCAAATCCCTTCTCAAC190ermCF: AATCGTCAATTCCTGCATGT R: AATCGTCAATTCCTGCATGT229linAF: GGTGGCTGGGGGGGTAGATGTATTAACTGG R: GCTTCTTTTGAAATACATGGTAATTAACTGG R: TTTCCAATAACATGGTATTTTTCGA R: TTTCCAATAACCACCGTTT323etAF: ATGGCAGCATCAGCTTGATA R: TTTCCAATAACCACCGTTT320etAF: CTAGTGCATTGTTATTCAA R: TGCATTGACACCATAGTACT R: TCCATCGATAATAACATCAATT R: TCCATCGATAATAACCACCAATGC R: TCCATCGATAATAACATGCACATGC R: ATGCACATGGTGCACATGC300agrIIF: ATGCACAAGTACTATAAGCTGCGCAATGC R: TATTACTAATTGAAAAGTGGCCCATAGC R: ATGCACATGGTGCACATGC R: GTAATGTAATAGCTTGTATAATAATACCCAG R: AAGGAAAACCACTCACATGA R: AAAGAAAACCACTCACATGA R: AAAGAAAACCACTCACATGA R: AAAGAAAACCACTCACATGA R: AAAGAAAACCACTCACATGA R: AAAGAAAACCACTCACATGA R: AAAGAAAACCACTCACATCA R: AAAGAAAACCACTCACATGA R: GGCTTCAGTGCTGTAGGC R: AAAGAAAACCACTCACATGA R: AAAGAAAACCACTCACATGA R: AAAGAAAACCACTCACATGA R: GGCTTCAGTGCTGTAGG R: AAAGAAAACCACTCACATGA R: GGCTTCAGTGCTGTAGGC R: GGCTTCAGTGCTGTAGGC R: GGCTTCAGTGCTGTAGGC R: GGCTTCAGTGCTGTAGGC R: GGCTCAGTGCTGTGTAGGC R: GGCTCAGTGCTGTGTAGGC R: GGCTCAGTGCTGTGTAGGC R: GGCTCAGTGCTGTGTAGGC R: GGCTCAGGCTTGTAGGC R: GGCTCAGTGCTGTGTAGGC <br< td=""><td></td></br<>		
ermAR: TTCGCAAATCCCTTCTCAAC190ermCF: AATCGTCAATTCCTGCATGT R: AATCGTCAATTCCTGCATGT229linAF: GGTGGCTGGGGGGGGAGATGTATTAACTGG R: GCTTCTTTTGAAATACATGGTATTTAACTGG R: GCTTCTTTTGAAATACATGGTATTTTTCGA323tsst-1F: ATGGCAGCATCAGCTTGATA R: TTTCCAATAACCACCGGTTT350etAF: CTAGTGCATTGTATATCAA R: TGCATTGACACCATAGTACT119etAF: ATGCACTGACACCATAGTACT R: TCCATCGATAATATACATTCAATT R: TCCATCGATAATATACCTAA200agrIIF: ATGCACATGGTGCACATGC R: GTCACAAGTACTATGAAAGTGGCCCATAGC323coaF: ATGCACATGGTGCACATGC R: GTAATGTAATAGCTTGTATAATAATACCAGG R: GTAATGTAATAGCTTGTATAATAATACCAGG R: GTAATGTAATAGCTTGTATAATAATACCAGG323coaF: ATGCACATGGTGCACATGC R: GTAATGTAATAGCTTGTATAATAATACCCAG R: GTAATGTAATAGCTTGTATAATAATACCCAG R: AAAGAAAACCACTCACATGG R: AAAGAAAACCACTCACATGA300		
ermCF: AATCGTCAATTCCTGCATGT R: AATCGTCGATTCCTGCATGT229IinAF: GGTGGCTGGGGGGGGGGGGGAGATGTATTAACTGG R: GCTTCTTTTGAAATACATGGTATTTAACTGG R: TTTCCAATAACAGGTGGATTGATA R: TTTCCAATAACCACCCGTTT333tsst-1F: ATGGCAGCATCAGCTTGATA R: TTTCCAATAACCACCCGTTT350etAF: CTAGTGCATTGTATTCAA R: TGCATTGACACCATAGTACT119etBF: ACGGCTATATACATTCAATT R: TCCATCGATAATATACCTAA200agrIIF: ATGCACATGGTGCACATGC R: TATTACTAATTGAAAAGTGGCCATAGC313cagrIIIF: ATGCACATGGTGCACATGC R: GTAATGTAATAGCTTGTATAATAATACCCAG R: GTAATGTAATAGCTTGTATAATAATACCCAG R: GTAATGTAATAGCTTGTATAATAATACCCAG R: AAAGAAAACCACTCACATCA F: GGCTTCAGTGCTTGTAGG323		
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tsst-1     R: TTTCCAATAACCACCCGTTT     350       etA     F: CTAGTGCATTTGTTATTCAA     119       R: TGCATTGACACCATAGTACT     119       etB     F: ACGGCTATATACATTCAATT     200       agrI     R: TCCATCGATAGTGCCACATGC     441       R: GTCACAAGTACTATAGCATGC     575       agrII     F: ATGCACATGGTGCACATGC     575       R: TATTACTAATTGAAAAGTGGCCATAGC     575       agrIII     F: ATGCACATGGTGCACATGC     323       coa     F: CGAGACCAAGATTCAACAAG     970       R: AAAGAAAACCACTCACATCA     F: GGCTTCAGTGCTTGTAGG     200		
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etBF: ACGGCTATATACATTCAATT R: TCCATCGATAATATACCTAA200agrIF: ATGCACATGGTGCACATGC R: GTCACAAGTACTATAAGCTGCGGAT441agrIIF: ATGCACATGGTGCACATGC R: TATTACTAATTGAAAAGTGGCCATAGC575agrIIIF: ATGCACATGGTGCACATGC R: GTAATGTAATAGCTTGTATAATAATACCCAG323coaF: CGAGACCAAGATTCAACAAG F: GGCTTCAGTGCTTGTAGG970IIIF: GGCTTCAGTGCTTGTAGG200		
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agrIF: ATGCACATGGTGCACATGC R: GTCACAAGTACTATAAGCTGCGAT441agrIIF: ATGCACAATGGTGCACATGC R: TATTACTAATTGAAAAGTGGCCATAGC575agrIIIF: ATGCACATGGTGCACATGC R: GTAATGTAATAGCTTGTATAATAATACCCAG323coaF: CGAGACCAAGATTCAACAAG R: AAAGAAAACCACTCACATCA F: GGCTTCAGTGCTTGTAGG970		
agrI     R: GTCACAAGTACTATAAGCTGCGAT     441       agrII     F: ATGCACATGGTGCACATGC     575       R: TATTACTAATTGAAAAGTGGCCATAGC     575       agrIII     F: ATGCACATGGTGCACATGC       agrIII     F: ATGCACATGGTGCACATGC       323     R: GTAATGTAATAGCTTGTATAATAATACCCAGG       coa     F: CGAGACCAAGATTCAACAAG       F: GGCTTCAGTGCTTGTAGG     970		
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coa R: AAAGAAAACCACTCACATCA 970 F: GGCTTCAGTGCTTGTAGG		
F: GGCTTCAGTGCTTGTAGG		
-164		
cija 980 R: TTTTCAGGGTCAATATAAGC		
F: CAAGCACCAAAAGAGGAA		
X-region 320 B: CACCAGGTTTAACGACAT		
IgG binding F. CACUTOCIOCAATOCIOCO 920		

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

#### 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 trimethoprim-sulfamethoxazole (77.77%),and (72.22%)antibiotic agents. Prevalence of against nitrofurantoin (22.22%),resistance 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	No samples resistance against each antibiotic agent (%)										
positive)	P10*	CZ30	CIP5	CC2	AZM15	E15	<b>MUP200</b>	RA5	<b>TE30</b>	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  $\mu$ g/disk), CZ30: cefazolin (30  $\mu$ g/disk), CIP5: ciprofloxacin (5  $\mu$ g/disk), CC2: clindamycin (2  $\mu$ g/disk); AZM15: azithromycin (15  $\mu$ g/disk), E15: erythromycin (15  $\mu$ g/disk), MUP200: mupirocin (30  $\mu$ g/disk), RA5: rifampin (5  $\mu$ g/disk), TE30: tetracycline (30  $\mu$ g/disk), SXT25: trimethoprim-sulfamethoxazole (25  $\mu$ g/disk), and F300: nitrofurantoin (300  $\mu$ g/disk) antibiotic agents.

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

Samples (No	No samples positive for each gene (%)											
positive)	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 distribution of the 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.

Somulas (No										
positive)	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)

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

190	se1	se2	se3	se4	se5	
99.8	7	0	0	3	49	Strain 26
	7	0	0	0	60	Strain 31
	7	4	0	0	49	Strain 20
99.6	7	4	0	0	49	Strain 30
	7	4	0	0	49	Strain 34
	7	4	0	0	49	Strain 35
100.0	7	4	0	0	49	Strain 36
	7	4	0	0	60	Strain 25
	7	4	4	1	49	Strain 19
99.6	7	4	4	1	49	Strain 21
	7	4	4	1	49	Strain 27
100.0	7	4	4	1	49	Strain 28
100.0	7	4	4	0	49	Strain 23
0.0 99.8	7	4	5	0	49	Strain 29
99.7	7	4	6	3	49	Strain 24
99.9	7	0	4	1	49	Strain 32
	7	0	5	0	49	Strain 33
	7	4	5	1	0	Strain 22

MLVA (<All Characters>) MLVA

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

#### DISCUSSION

Bovine mastitis isan economically significant disease of the dairy industry worldwide. Although numerous pathogens are listed, Staphylococcus spp. a n d 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 etal., 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. predominant More recently, the 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 coagulase-negative commonly isolated subclinical Staphylococcus species in 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, trimethoprim-sulfamethoxazole and 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. erythromycin antibiotics. and 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 trimethoprimsulfamethoxazole (73.91%)antibiotic agents. Reversely, S. epidermidis strains harbored the of lowest prevalence 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,

erythromycin, trimethoprim clindamycin, 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, ofloxacin, penicillin, rifampicin, oxacillin, tetracycline, teicoplanin, trimethoprim, and antibiotic 92.30%, vancomycin agents were 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 present in different are many 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 genes resistance 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

reported Momtaz (2019)that aacA-D (69.56%), tetK (56.52%), mecA (45.65%), msrA (3) 9.13%), 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*, *p* vl, 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 a fundamental basis populations and for subdividing the species (Gomes-Fernandes et al., 2017). It encodes а 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 was accompanied with considerable which 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 sources of resistant and were 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 particularly, their milk samples, 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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