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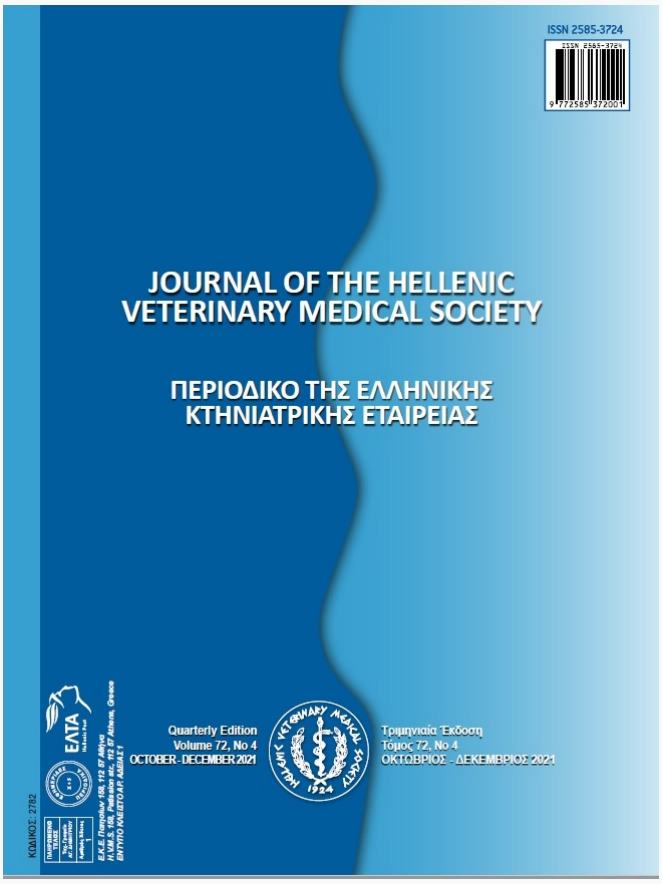

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Research article
Ερευνητικό άρθρο

Methicillin and vancomycin resistant isolates of *Staphylococcus aureus* and *Enterococcus faecalis* recovered from bovine mastitis

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ABSTRACT: Mastitis is the most costly disease in the dairy industry. Selecting the proper antibiotic treatment is beneficial for economic and avoids the emergence of antimicrobial resistance. The objective of the present study was to investigate the prevalence of methicillin and vancomycin resistant isolates of mastitis-causing *Staphylococcus aureus* and *Enterococcus faecalis* as a probable source of transferable vancomycin resistance to staphylococci. A total of sixty-one *Staphylococcus aureus* and eight *Enterococcusfaecalis* isolates were investigated for genotypic and phenotypic antimicrobial resistance.Presence of the *mecA*, *vanA* and *vanB*genes were surveyed by PCR. The MIC (Minimum Inhibitory Concentration) of vancomycin was determined bybroth microdilution test for all the isolates.Moreover, the antibiotic resistance patterns of the isolates to the most common classes of antibiotics used in dairy cattle such as β-lactam, macrolides and tetracyclines were determinedusing the disk diffusion method. Among *Staphylococcus aureus* isolates, one MRSA (methicillin-resistant *Staphylococcus aureus*) isolate was detected while 47.5% of isolates were detected as multidrug-resistant. Furthermore, no phenotypic and genotypicvancomycin-resistance *Staphylococcus aureus* was found. Most of the *Enterococcus faecalis* isolates (6/8) showed high MIC for vancomycin (in the range of 128- 1024 µg/ml) and one *vanA*-type *Enterococcus faecalis* was observed. This study indicates thatsince the source of transferable resistance to vancomycin exists in dairy farms, there is a potential for emerging and spreading VRSA (vancomycin-resistant *Staphylococcus aureus*) in dairy cattle which is a risk to animal and human health.

Keywords: Bovine mastitis; VRSA; MRSA;VRE; MDR

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INTRODUCTION

Bovine mastitis is one of the most costly concerning in dairy farms. The principles of prevention and control programs of mastitis are the improvement of milking hygiene and antimicrobial application.To date, many bacterial pathogens are identified as causes of intra mammary infections (IMI). *Staphylococcus aureus* (*S. aureus*) is the cause of the most common types of chronic and contagious mastitis. It is also responsible for various types of infections in human and other animals (Ruegg, 2017). Antimicrobial resistance of *S. aureus* has attracted a lot of attention, thus numerous studies has been conducted all around the world to surveil it (Jamali et al., 2014; Wang et al., 2016; Li et al., 2017; Zaatout et al., 2019). As emerging of methicillin (oxacillin) resistant *S. aureus* (MRSA) and vancomycin-resistant *S. aureus* (VRSA) strains has led the therapeutic programs face a big challenge (Tarai et al., 2013), it is essential to monitor the development and expansion of MRSA and VRSA. Resistance to vancomycin in *S. aureus* is an acquired antimicrobial resistance from enterococci through the acquisition of the genes *vanA* and/or *vanB* (Courvalin, 2006). Different species of enterococci are considered as environmental mastitis-causing pathogens. To date, no report of genotypic resistance to vancomycin in *S. aureus* has been recorded in dairy cattle. Monitoring the development of antimicrobial resistance especially the acquired type is necessary in food-producing animals and dairy cattle is not an exceptional. Our purpose of the present study was to investigate the prevalence of MRSA and VRSA among *S. aureus* recovered from bovine mastitis milk. We also aimed to detect vancomycin resistance enterococci from mastitis milk as a probable and possible source of transmission of vancomycin resistance to *S. aureus*. We also covered the antibiotic resistance patterns of *S. aureus* and *Enterococcus faecalis* (*E. faecalis*) isolates to the most common classes of antibiotics used in dairy cattle such as β-lactam, macrolides and tetracyclines.

MATERIALS AND METHODS

Bacterial isolates

Sixty-one isolates of *S. aureus* and eight isolates of *E. faecalis*were investigated in the current study. The isolates belonged to subclinical bovine mastitis which were submitted to Veterinary Hospital of Ferdowsi University of Mashhad. Sampling and microbial culture were conducted according to National Mastitis Council guidelines. Conventional biochemical tests were carried out in order to confirm bacterial species (National Mastitis Council (U.S.), 2004).

DNA extraction

Bacterial DNA was extracted by GeneAll Exgene™ Cell SV kit (GeneAll, South Korea) following the manufacture's instructions.

Molecular confirmation of S. aureus

Molecular confirmation was performed by amplification of the*S. aureus*-specifi c*nuc*g ene as described by Graber et al. (2007). The primer (Macrogen, South Korea) sequenceand PCR condition are mentioned in table 1.

Molecular detection of methicillin (oxacillin) and vancomycin resistance genes

All the isolates were tested for the presence of genes *vanA* and *vanB*. To detect MRSA, all the *S. aureus* isolates were investigated for the presence of *mecA*. The primers' characteristics (Macrogen, South Korea) and PCR conditions are listed in table 1. All PCR products were analyzed by 1.2% agarose gel (w/v) (DENAzist Asia, I. R. Iran) and Green Viewer safe stain (0.01 v/v) (SinaClon, I. R. Iran).

Antimicrobial susceptibility testing

A standard agar-disk diffusion (Kirby-Bauer) was performed for all *S. aureus* and *E. faecalis* isolates according to CLSI interpretive criteria using

Table 1. PCR conditions and primers used in this study

Gene	Sequence (5' to 3')	Product size (bp)	No. of cycles T_a, Time	Ref.
nuc	CTGGCATATGTATGGCAATTGTT TATTGACCTGAATCAGCGTTGTCT	664	35 cycles 60°C, 1 min	(Graber et al., 2007)
mecA	AAAATCGATGGTAAAGGTTGGC AGTTCTGCAGTACCGGATTTGC	533	40 cycles 55°C, 30 sec	(Murakami et al., 1991)
vanA	CATGAATAGAATAAAAGTTGCAATA CCCCTTTAACGCTAATACGATCAA	1030	30 cycles 58°C, 30 sec	(Clark et al., 1993)
vanB	GTGACAAACCGGAGGCGAGGA CCGCCATCCTCCTGCAAAAAA	433		

Mueller-Hinton agar plates (Merck, Germany) and antibiotic disks (Padtan Teb, I. R. Iran) for penicillin (10 units), ampicillin (10 μg), erythromycin (15μg) and tetracycline (30μg) (Bauer et al., 1968). Based on CLSI guideline, phenotypic resistance to vancomycin (Sigma-Aldrich, Germany) determined by broth microdilution method for both genus and disc diffusion test for vancomycin was carried out for *E. faecalis* isolates (CLSI, 2017). *S. aureus* ATCC 25923 and *S. aureus* ATCC 29213 were used as quality control strains for disk diffusion method and broth microdilution method, respectively.

RESULTS

S. aureus

Sixty-one isolates were confirmed as *S. aureus* based on biochemical reactions, coagulase test and possessing the *nuc* gene. Ten different antibiotic resistance patterns were obtained according to the combination of the results of agar-disk diffusion (penicillin, ampicillin, erythromycin and tetracycline), broth microdilution method (determination of MIC for vancomycin) and molecular detection of *vanA* and *vanB* (involved in vancomycin resistance), and *mecA* (responsible for oxacillin resistance).

Only one isolate (1.6%) was detected positive for *mecA* and considered as MRSA, while no VRSA isolate was found. The MIC of all the tested isolates for vancomycin was ≤2 μg/ml and none of them carried *vanA* and/or *vanB* genes.

According to the definition of multidrug-resistance (MDR) in veterinary medicine"an isolate which is not susceptible to at least one agent in at least three antimicrobial classes" (Sweeney et al., 2018), 29 isolates (47.5%) showed multidrug-resistance while 18% of isolates were detected susceptible or resistant to one antibiotic agent. To sum up, the most frequent antibiotic resistance patterns are simultaneous resistance to penicillin and ampicillin (34.4%) and penicillin, ampicillin and erythromycin (27.8%). Antibiotic resistance patterns for *S. aureus* isolates are described in details in figure 1.

E. faecalis

Most of the *E. faecalis* isolates (6/8 isolates) were resistant to all the tested antibiotics (penicillin, ampicillin, erythromycin, tetracycline and vancomycin). From the rest, one isolate showed complete susceptibility and the other one identified as resistant to penicillin and ampicillin. The MIC of vancomycin for multidrug-resistant isolates (6/8) was high and in the range of 128- 1024 μg/ml. The other two isolates were susceptible to vancomycin according to the MIC. The *vanA* gene was only detected in one isolate (MIC: 1024 μg/ml) and no isolate was identified positive for the presence of *vanB*. The results of the study for *E. faecalis* are presented in details in figure 2.

DISCUSSION

In the current study, 61 isolates of *S. aureus* were investigated for antibiotic resistance against different classes of antibiotics such as β-lactam (penicillin, ampicillin, oxacillin), macrolides (erythromycin), tetracyclines (tetracycline) and polypeptide antibiotics (vancomycin). Phenotypic and genotypic resistance to vancomycin was studied and all the *S. aureus* isolates were found to be susceptible to vancomycin. The transferable genes *vanA* and *vanB* are responsible for inducible resistance to vancomycin and*S. aureus* acquisition of the genes from enterococci has been proved (Courvalin, 2006). To date no report of simultaneous genotypic and phenotypic resistance to vancomycin among bovine mastitis causing strains of *S. aureus* has been recorded. A probable explanation could be that the some of the reports from the presence of VRSA were based on the results of the application of agar-disk diffusion which is not acceptable today (Sharma et al., 2015). Furthermore, the majority of those studies which were applied broth microdilution test or E-test to investigate phenotypic resistance to vancomycin, targeted only *vanA* for molecular investigation which is responsible for high level of vancomycin resistance and ignore *vanB*, while the gene *vanB* involves in variable levels of vancomycin resistance (Courvalin, 2006). Only Bhattacharyya et al. reported VRSA based on application of broth microdilution test and investigating for genes *vanA* and *vanB*, although they did not detect any genotypic positive strain (Bhattacharyya et al., 2016).

Low prevalence of MRSA (1.6%) was observedin the study and it agrees with researches done by Gentilini et al. (2000) and Erskine et al. (2002), while high prevalence of MRSA and outbreaks of subclinical mastitis due to oxacillin resistant strains have been reported (23.3- 83%) (Hata, 2016; Guimarães et al., 2017). MRSA is a healthcare-acquired pathogen and its transmission between human and cows has been shown (Sato et al., 2017), thus monitoring the state of MRSA in both human and dairy cattle is necessary.

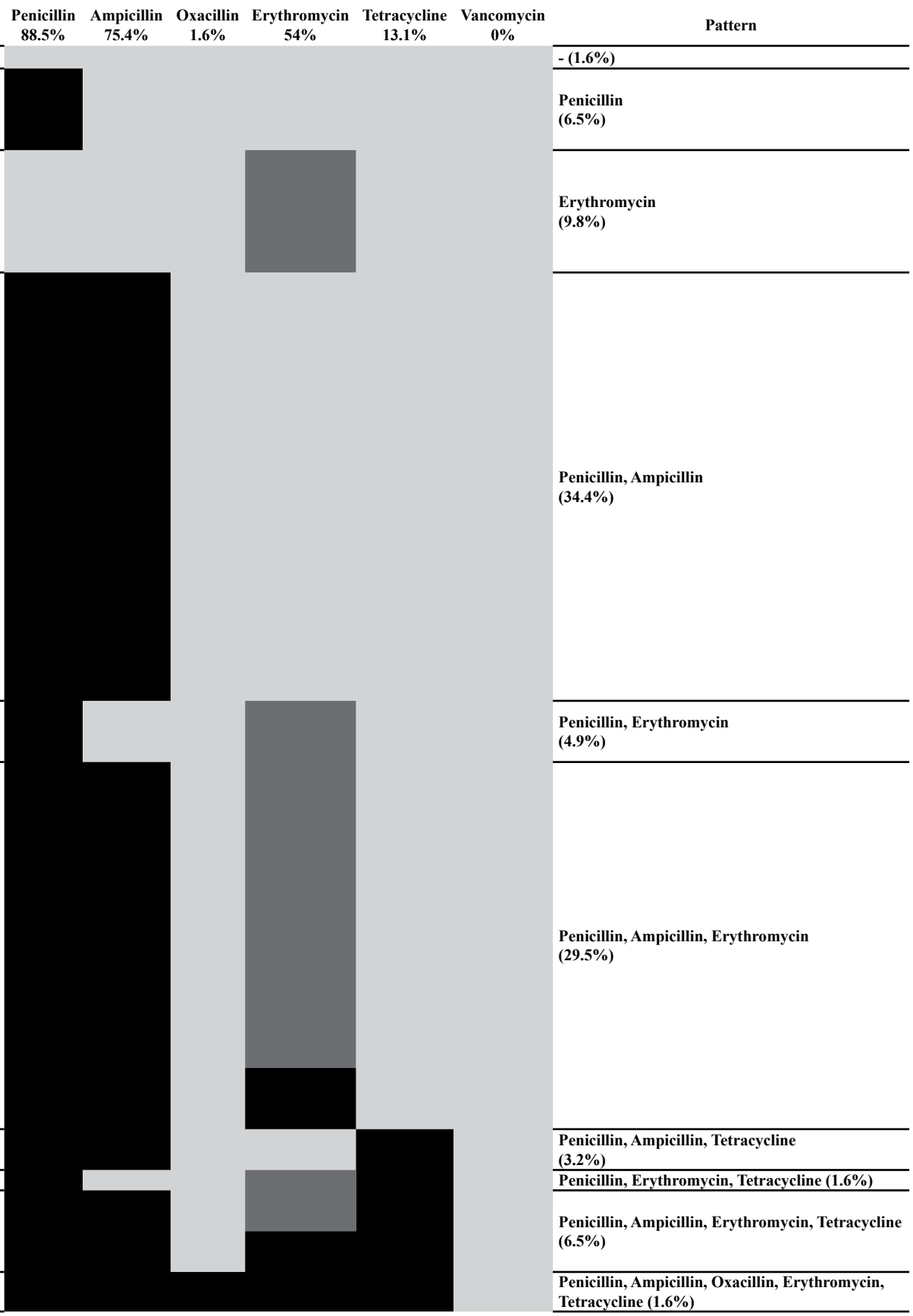

Figure 1: Antibiotic resistance patterns for *S. aureus* isolates. Black indicates resistance, dark gray indicates intermediate resistance, and light gray indicates sensitive.

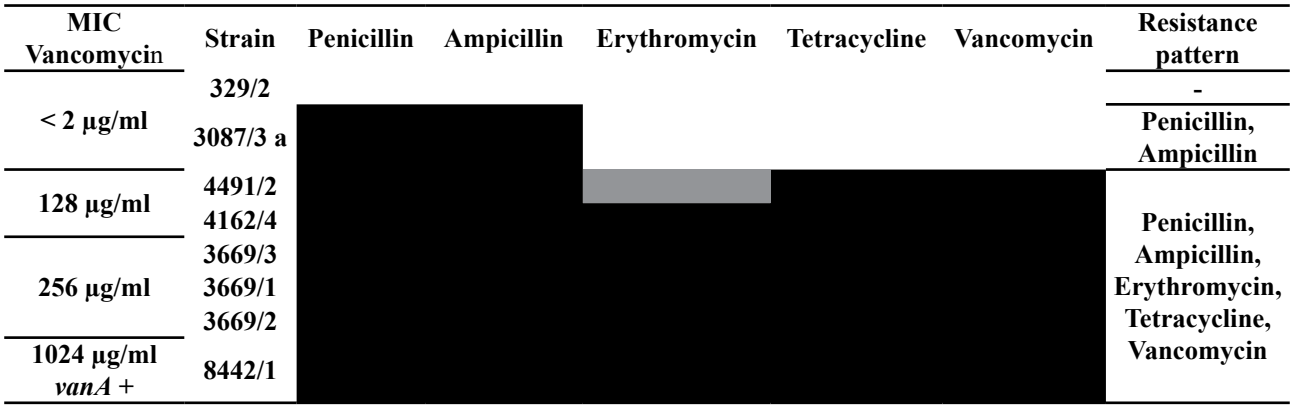

Figure 2: Antibiotic resistance patterns for *E. faecalis* isolates. Black indicates resistance, dark gray indicates intermediate resistance, and light gray indicates sensitive.

High incidence of resistance to penicillin (88.5%) and ampicillin (75.4%) were identified among *S. aureus* isolates which is as high as records (84- 94%) reported by other researchers(Wang et al., 2016; Yang et al., 2016). The high reported incidence can be the result of routine application of antibiotics for dry cow therapy and lactation therapy which cause pressure for selection of resistant strains. Prevalence of resistant isolates of *S. aureus* against erythromycin and tetracycline was determined 54% and 13.1%, respectively. Different studies reported various ranges for prevalence of resistant isolates to erythromycin. While Wang et al. (2016) reported 68.6% of erythromycin resistance, Ruegg et al. (2015) recorded 8.6%. On the contrary, there is a narrow spectrum of tetracycline resistance prevalence from 3- 17% based on different reports (Oliver and Murinda, 2012; Ruegg et al., 2015).

Monitoring the antimicrobial resistance should not be limited to contagious or prevalent causes of mastitis and pathogens which are the origin of antimicrobial resistance should also be included. The main goal of studying antimicrobial resistance of *E. faecalis* isolates in the current study was to find phenotypic and genotypic vancomycin-resistant strain as a probable and possible source of transferring of vancomycin resistance to *S. aureus*. Noticeably, most of the tested isolates (6/8) were resistant to vancomycin and one isolate carried resistance gene *vanA*. The presence of *vanA* type- *E. faecalis* is a caution that there is a potential of emerging VRSA in dairy cattle which is a risk to dairy cattle and human health. Furthermore, this type of *E. faecalis* pose a threat to human health by freely spreading of resistance gene to human enterococci that should not be ignored (Angulo et al., 2006).

High incidence of resistance against penicillin, ampicillin, erythromycin, tetracycline and vancomycin in *E. faecalis* isolates was observed during the present study. High-level resistance to erythromycin and tetracycline in enterococci acquired from bovine mastitis has been recorded from China, Korea and Poland (Nam et al., 2009; Różańska et al., 2019; Yang et al., 2019). Although the number of investigated *E. faecalis* isolatesin the current study was not high, the presence of high proportion of MDR (6/8) isolates warns us about using antimicrobial agents cautiously in dairy cattle.

CONCLUSIONS

The current study showed the existence of the source of vancomycin resistance in dairy cattle. Although no VRSA was isolated, the risk of emerging and spreading of VRSA in dairy cattle should not be underestimated. Moreover, bovine vancomycin resistant enterococci (VRE) isolates can be the source of vancomycin resistance for human enterococci and staphylococci and act as a human health hazard.

CONFLICT OF INTEREST

None declared by the authors.

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