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Antimicrobial Resistance and Virulence Genes of Enterococci Isolated from Water Buffalo's Subclinical Mastitis

Ece KOLDAŞ-ÜRER[1,a], Erhan TEK[2,b], Özkan ASLANTAŞ[2,c,*],Mehmet Ali YILMAZ[3,d] ,Yaşar ERGÜN[1,e]

[1]Hatay Mustafa Kemal University, Faculty of Veterinary Medicine, Department of Obstetrics and Gynecology, Hatay, Türkiye.
[2]Hatay Mustafa Kemal University, Faculty of Veterinary Medicine, Department of Microbiology, Hatay, Türkiye.
[3]International Center for Livestock Research and Training, Lalahan, Ankara, Türkiye.
[a]ORCID: 0000-0003-4262-691; [b]ORCID: 0000-0002-4595-6992; [c]ORCID: 0000-0003-0407-8633; [d]ORCID: 0000-0002-4497-2894; [e]ORCID: 0000-0002-1414-9100

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Abstract: This study aimed to investigate the antimicrobial resistance and virulence genes of enterococci isolated from water buffalo's subclinical mastitis cases. The antimicrobial susceptibilities of the isolates were determined by the disc diffusion method. Identification at the species level of enterococci, virulence [aggregation substance (*asa*1), gelatinase (*gel*E), cytolysin (*cyl*A), enterococcal surface protein (*esp*), and hyaluronidase (*hyl*)] and resistance genes [macrolide (*erm*A, *erm*B, *mef*A/E) and tetracycline (*tet*K, *tet*L, *tet*M, *tet*O, and *tet*S)] were investigated by polymerase chain reaction (PCR). Overall, *Enterococcus* spp. was recovered from 65 of 200 (32.5%) mastitic milk samples, comprising *E. faecium* (n=26), *E. durans* (n=22), *E. faecalis* (n=12), and *E. hirae* (n=5). Most isolates (56.9%) were susceptible to all tested antibiotics. The rest of the isolates showed various rate of resistance against rifampicin (23.1%), tetracycline (21.5%), quinupristin-dalfopristin (10.8%), ciprofloxacin (7.7%), erythromycin (6.2%), and chloramphenicol (3.1%). Out of 65 enterococci, only 16 (24.6%) were detected to have virulence genes, of which 12 were positive for *gel*E, seven were positive for *esp*, two were positive for *asa*1, and one was positive for *hly*A. The gene *cyl*A was not detected in any isolate tested. Resistance to tetracycline was mainly associated with *tet*M. Two erythromycin-resistant isolates were positive for *erm*B, and one was positive for *mef*A/E. This study was the first to report species distribution, antimicrobial susceptibility, and virulence traits of enterococci isolated from subclinical mastitis of water buffaloes in Çorum Province, Türkiye.
Keywords: *Antimicrobial resistance*, *Enterococci*, *Subclinical mastitis*, *Virulence*, *Water buffalo.*

Subklinik Manda Mastitislerinden İzole Edilen Enterokok Türlerinde Antimikrobiyal Direnç ve Virulans Genleri

Özet: Bu çalışmada subklinik manda mastitis vakalarından izole edilen enterokokların antimikrobiyal direnç ve virülans genlerinin araştırılması amaçlanmıştır. İzolatların antimikrobiyal duyarlılıkları disk difüzyon yöntemi ile belirlendi. Enterokokların, tür düzeyinde identifikasyonu, virülans (*asa1*, *gelE*, *cylA*, *esp* ve *hyl*) ve direnç genleri [makrolid (*ermA*, *ermB*, *mefA/E*) ve tetrasiklin (*tetK*, *tetL*, *tetM*, *tetO* ve *tetS*)] polimeraz zincir reaksiyonu (PZR) ile araştırıldı. İncelenen 200 mastitisli süt örneğinin 65'inden (%32,5) *Enterococcus* spp. izole edildi ve izole edilen türler *E. faecium* (n=26), *E. durans* (n=22), *E. faecalis* (n=12) ve *E. hirae* (n=5) olarak identifiye edildi. İzolatların %56,9'u test edilen tüm antibiyotiklere duyarlı bulundu. İzolatların geri kalanı rifampisin (%23,1), tetrasiklin (%21.5), kuinupristin-dalfopristin (%10,8), siprofloksasin (%7,7), eritromisin (%6,2) ve kloramfenikol (%3,1)'e karşı çeşitli direnç oranları gösterdi. İzole edilen 65 *Enterococcus* spp.'nin sadece 16'sının (%24,6) virülans genlerine sahip olduğu tespit edildi. Virulens genlerine sahip izolatların 12'si *gelE*, yedisi *esp*, ikisi *asa1* ve biri de *hlyA* yönünden pozitif bulundu. *cylA* geni incelenen hiçbir izolatta saptanmadı. Tetrasikline direncin esas olarak *tetM* ile ilişkili olduğu saptanırken; eritromisine dirençli iki izolat *ermB* ve bir izolat ise *mefA/E* geni yönünden pozitif bulundu. Bu çalışma, Türkiye'nin Çorum ilinde yetiştirilen mandalarda saptanan subklinik mastitisli süt örneklerinden izole edilen enterokokların tür dağılımı, antimikrobiyal duyarlılık ve virülans özelliklerini bildiren ilk çalışmadır.
Anahtar kelimeler: *Antimikrobiyal direnç*, *Enterococcus* spp., *Manda, Subklinik mastitis*, *Virulens.*

Introduction

Based on the data received from Turkish Statistical Institute (TSI), 63 643 tons of milk were obtained from the registered 185 574 water buffalos in Türkiye in 2021 (TSI, 2021). Water buffalo milk is about 5% of the total world milk production (Atasever and Erdem, 2008), and 0.3% of the milk production in Türkiye (TSI, 2021). Even though milk yield per animal is low in comparison with cows, the quantity and quality of the water buffalo milk are of great importance both for producers and consumers (Şahin and Yıldırım, 2015). Therefore, water buffalo breeding started to be supported by the Ministry of Agriculture and Forestry in recent years (Sarıözkan, 2011).

As in other animal species, there are many factors affecting milk yield in water buffalos, such as the number of lactations, calving season, and diseases. Of these diseases, mastitis is considered a global problem that affects milk yield and quality, thus causing serious economic losses (Singha et al., 2021). Mastitis pathogens are divided into two groups depending on the infection source: contagious or environmental pathogens. Although mastitis control programs are effective against contagious mastitis pathogens, these mastitis control programs are less effective against environmental pathogens, such as enterococci (Yang et al., 2019).

Antimicrobials are widely used for the control and prevention of mastitis cases. However, treatment success is mainly limited due to the antimicrobial resistance against these pathogens (Saini et al., 2019). Another factor that affects the frequency of mastitis cases is the virulence traits of pathogens (Yang et al., 2019). Several virulence factors have been described in enterococci including cytolysin (*cyl*A), hyaluronidase (*hyl*), aggregation substance (*asa*), enterococcal surface protein (*esp*), and gelatinase (*gel*E) (Vankerckhoven et al., 2004). Of these virulence genes, cytolysin is a bacteriocin-type exotoxin, exerts its effects on erythrocytes, leukocytes, and macrophages. Geletinase is zinc-dependent metalloendopeptidase, is capable of hydrolysing gelatine, elastin, collagen, haemoglobin. Aggregation substance is enterococcal surface protein that contributes the formation of mating aggregates facilitating bacterial conjugation. Enterococcal surface protein is known to be involved in biofilm formation. Biofilm production has been shown to play an important role in the exchange of antibiotic resistance genes between cells and to increase their resistance to antibiotics. Hyalurinidase plays a role in destroying mucopolysaccharides of the connective tissue and cartilage and, consequently, in spreading bacteria (Chajęcka-Wierzchowska et al. 2017).

Little information exists about enterococci from milk samples of water buffaloes both in Türkiye and in the world. Therefore, in this study, it was aimed to investigate the antimicrobial resistance and virulence genes of enterococci isolated from subclinical mastitic milk samples of water buffaloes in Türkiye.

Materials and Methods

Ethical statements: This study is not subject to HADYEK permission in accordance with Article 8 (k) of the "Regulation on Working Procedures and Principles of Animal Experiments Ethics Committees".

Milk Samples: A total of 200 mastitic milk samples of water buffaloes were collected from family-sized farms located in Çorum, Türkiye, between June 2018 and July 2018. CMT test was applied to buffaloes that did not show clinical signs in mammary tissue and milk, and subclinical mastitis was evaluated by CMT test results.

Bacterial isolation and identification: The milk samples (100 µl) were inoculated into Enterococcosel broth (BD, UK) and incubated at 37 ^{o}C for 24-48 h. When the color change occurred in the Enterococcosel broth tubes, a loopful of culture was inoculated on Vancomycin Resistant Enterococci (VRE) agar plates and incubated for 48 h at 37 ^{o}C. Subsequently, one presumptive colony from each plate was randomly selected and streaked onto Blood agar (Merck, Germany) supplemented with 5% defibrinated sheep blood to obtain pure culture. Following Gram staining and biochemical tests, the isolates were confirmed and identified by the PCR method (Layton et al., 2010).

Antimicrobial susceptibility testing: Antibiotic susceptibilities of the isolates were determined by disc diffusion methods using Mueller Hinton Agar (Merck, Germany), following Clinical and Laboratory Standards Institute (CLSI) criteria (CLSI, 2021). The antimicrobial agents (Bioanalyse, Türkiye) were as follow: ampicillin (10 µg), quinupristin/dalfopristin (15 µg), chloramphenicol (30 µg), tetracycline (30 µg), ciprofloxacin (5 µg), erythromycin (15 µg), vancomycin (30 µg), rifampicin (5 µg), and gentamicin (120 µg). *Staphylococcus aureus* ATCC 25923 was used as a quality control strain. Multidrug resistance (MDR) was defined as resistance against at least 3 antimicrobial agents belonging to different antimicrobial classes (Magiorakos et al., 2012).

Detection of macrolide and tetracycline resistance genes: The presence of macrolide (*erm*A, *erm*B, *mef*A/E) and tetracycline resistance genes (*tet*K, *tet*L, *tet*M, *tet*O, and *tet*S) were investigated using PCR as previously reported by Malhotra-Kumar et al. (2005).

Detection of virulence genes: Virulence genes (*asa1*, *cylA*, *esp*, *gelE*, and *hyl*) were investigated as previously reported by Vankerckhoven et al. (2004).

Results

Isolation and identification: Of the 200 mastitic milk samples, 65 (32.5%) *Enterococcus* spp. were isolated and the distribution of enterococci was detected as follows: 26 *E. faecium* (40%), 22 *E. durans* (33.8%), 12 *E. faecalis* (18.5%), and 5 *E. hirae* (7.7%) (Figure 1-2).

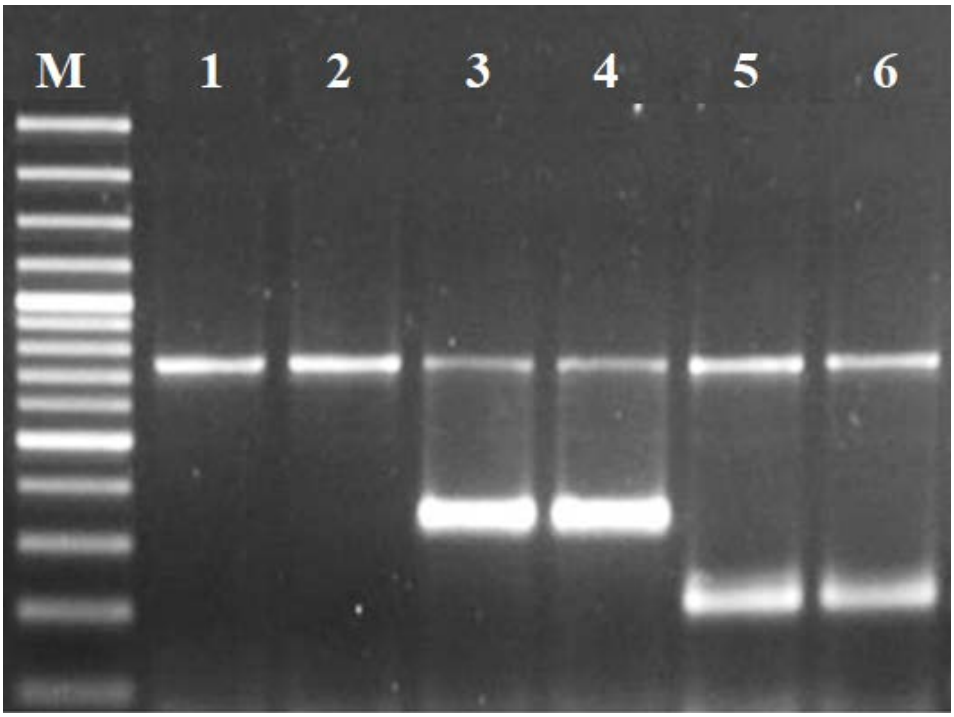

Figure 1. Agarose gel electrophoresis of *Enterococcus* species. Lane M: 100 bp plus molecular marker, Lane 1-2: *Enterococcus* spp. (733 bp), Lane 3-4: *E. faecalis* (360 bp), Lane 5-6: *E. faecium* (214 bp).

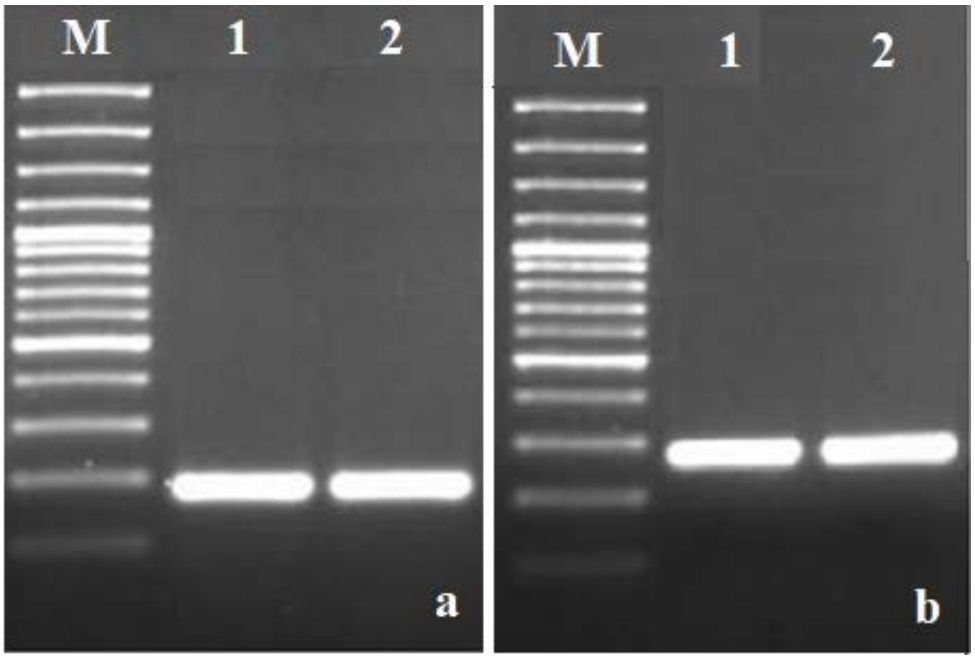

Figure 2. Agarose gel electrophoresis of *E. durans* and *E. hirae* isolates. Lane M: 100 bp plus molecular marker. **Figure 2a.** *E. hirae* (186 bp), F**igure 2b:** *E. durans* (286 bp)

While 37 (56.9%) of the isolates were susceptible to all antibiotics tested, 28 (43.1%) isolates showed various rate of resistance to tetracycline (21.5%, 14/65), rifampicin (23.1%, 15/65), ciprofloxacin (7.7%, 5/65), erythromycin (6.2%, 4/65), quinopristin-dalfopristin (10.8%, 7/65), and chloramphenicol (3.1%, 2/65).

Determinants of erythromycin and tetracycline resistance: In tetracycline-resistant isolates, 12 isolates carried *tet*M and one isolate carried *tet*L. Among erythromycin-resistant isolates,

three isolates were positive for *erm*B and one isolate was positive for *mef*A/E.

Virulence gene profiles of the isolates: In 21.5% (14/65) of the isolates virulence genes were detected. These isolates were found to carry one or more virulence genes. Twelve isolates were positive for *gel*E, seven were positive for *esp*, two were positive for *asa*1 and one was positive for *hly*A. The gene *cyl*A was not detected in any isolate tested (Figure 3). Resistance phenotypes, virulence, and resistance genes determined in *Enterococcus* spp. are given in Table 1.

Table 1. Resistance phenotypes, virulence and resistance genes determined in *Enterococcus* spp.

Species	**Resistance Phenotype***	**Resistance Genotype**	**Virulence Gene(s)**
E. faecium (n=2)	CIP, RA	-	-
E. faecium	E	*mef*A/E, *tet*K	-
E. faecium	E	-	-
E. faecium	RA	-	*gel*E, *esp*
E. faecium	TE, SYN	-	-
E. faecium (n=2)	RA	-	-
E. faecium (n=3)	CIP	-	-
E. faecalis	TE, RA, SYN	*tet*M	-
E. faecalis	TE, RA, SYN	*tet*M	*gel*E, *esp*
E. faecalis	TE	*tet*M	*gel*E, *esp*
E. faecalis	SYN	-	*asa*1, *gel*E
E. faecalis	SYN	-	*gel*E
E. faecalis (n=2)	TE, RA	*tet*M	*gel*E, *esp*
E. faecalis	TE, RA	*tet*M	*gel*E
E. faecalis	TE, RA	*tet*M	*gel*E
E. faecalis	E, TE, RA, SYN, C	*erm*B, *tet*M	*esp*
E. faecalis	E, TE, RA, SYN, C	*erm*B, *tet*M	*esp*
E. durans (n=3)	TE	*tet*M	-
E. durans	TE	*tet*M, *tet*L	*asa*1, *gel*E
E. durans (n=2)	RA	-	-
E. hirae (n=1)	Sensitive	-	*hlyA*
E. durans (n=1)	Sensitive	*erm*B	-
E. durans (n=15)	Sensitive	-	-
E. hirae (n=4)	Sensitive	-	-
E. faecium	Sensitive	-	*gel*E, *esp*
E. faecalis	Sensitive	-	*gel*E
E. faecium	Sensitive	-	*gel*E
E. faecium (n=13)	Sensitive	-	-

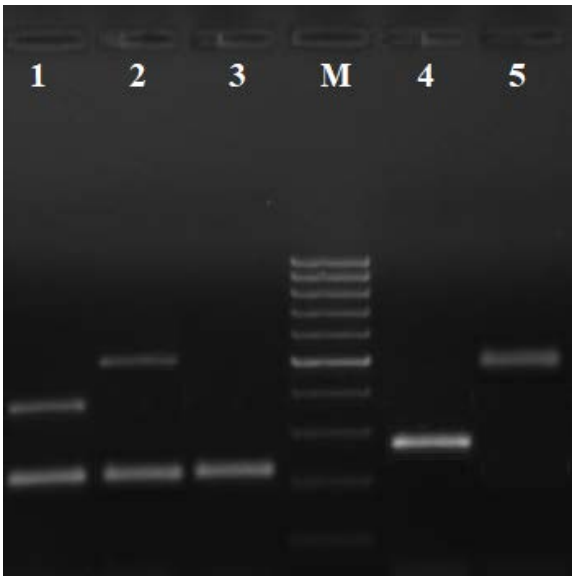

Figure 3. Agarose gel electrophoresis of virulence genes. Lane M: 100 bp molecular marker. Lane 1: *asa1* (375 bp)+*gelE* (213 bp), Lane 2: *esp* (510 bp)+*gelE* (213 bp), Lane 3: *gelE*, Lane 4: *hlyA* (276 bp), Lane 5: *esp*

Discussion

Although enterococci are considered as commensal inhabitants of gastrointestinal microbiota both in humans and animals, these agents have increasingly been reported in mastitic milk samples (Yang et al., 2019). In this study, the prevalence of enterococci was determined as 32.5%. In different studies carried out in Türkiye, the prevalence of enterococci in milk samples of bovine subclinical mastitis cases was reported to be 0.7% (3/421) in Samsun (Gürler et al., 2015), 10.9% (43/392) in Afyon (Kuyucuoğlu, 2011), and 16% (96/600) in Aydın (Herkmen and Türkyılmaz, 2016). On the other hand, in the studies conducted abroad, the prevelance of enterococci were reported as 4.8% (105/2185) in Korea (Nam et al., 2010), 15.23% (27/177) in Canada (Cameron et al., 2016), 16.7% (112/669) in the Czech Republic (Cervinkova et al., 2013), and 21.3% (426/2000) in Poland (Różańska et al., 2019). In the above-mentioned studies, the presence of different Enterococcus species has also been reported. In the majority of these studies, *E. faecalis* was identified as the predominant species (Kuyucuoğlu, 2011; Nam et al., 2010; Cameron et al., 2016; Różańska et al., 2019). In contrast, *E. faecium* and *E. durans* were found to be the predominant species in this study. While similar observation was reported by Kateete et al. (2013) in Uganda, Klimiene et al. (2011) found *E. durans* as the predominant species in Lithuania. Variations in prevalence rates and species distribution could be attributed to the isolation methods, geographical origins of the samples, and differences in rearing conditions.

Despite enterococci having intrinsic resistance to antimicrobials such as beta-lactams, lincosamides, cephalosporins, trimethoprim, and aminoglycosides (low level), emergence and dissemination of acquired resistance is mainly related to over-and misuse of antimicrobial agents e.g. tetracyclines, ciprofloxacin, daptomycin, erythromycin, linezolid, quinupristin-dalfopristin, and vancomycin. While most of the isolates were susceptible to antimicrobials tested, the rest showed moderate levels of resistance to rifampicin (23.1%) and tetracycline (21.5%) in this study. In previous studies, high resistance rates to tetracycline and erythromycin has been reported in enterococci isolated from subclinical bovine mastitis cases (Yang et al., 2019; Kuyucuoğlu, 2011; Nam et al., 2010). The high resistance observed for these agents could be explained by the long-term and widespread use of these antimicrobials in food-producing animals (Yang et al., 2019).

Horizantal transfer of antimicrobial resistance genes between bacteria is of important concern for both human and veterinary medicine (Aslam et al., 2012). In this study, the *tet*M was the most frequent resistance gene detected among the tetracycline-resistant isolates. Similarly, the dominance of *tet*M in tetracycline-resistant enterococci from different sources such as meat (Yılmaz et al., 2016), cheese (Kürekci et al., 2016), and dogs (Boyar et al., 2017) have been reported in previous studies conducted in Türkiye. Kim et al. (2019) explained the widespread occurrence of *tet*M (providing ribosomal protection) in tetracycline-resistant enterococci by localization of this gene on conjugative transposons such as Tn916, Tn1545, and Tn5385 leading to the easy spread of this gene among enterococci. Moreover, among four erythromycin-resistant enterococci, *erm*B in three isolates, and *mef*A/B in one isolate was detected. But, one isolate did not carry any gene studied. Similarly, previous studies revealed the *erm*B gene was the most dominant gene found in enterococci from animals (Boyar et al., 2017; Aslantaş, 2019) and food of animal origin (Yılmaz et al., 2016).

Enterococci are capable of producing various virulence factors that play an important role in the pathogenesis of the infections they cause (Mundy, 2000). The five virulence genes examined in this study were reported to contribute to the virulence of enterococci (Vankerckhoven et al., 2004). Of these virulence genes, the *gel*E is a metalloproteinase, capable of hydrolyzing casein, hemoglobin, collagen, gelatine, elastin as well as various peptides and proteins (Chajęcka-Wierzchowska et al., 2017). In this study, the *gel*E was found in 18.5% of the

isolates. In contrast, Yang et al. (2019) reported a higher prevalence rate (70.4%) in *E. faecalis* isolates. Herkmen and Türkyılmaz (2016) found *gel*E gene in 30.7% of *E. faecium* isolates. Another virulence factor, the aggregation substance encoded by the *asa*1 gene is an enterococcal surface protein contributing to the formation of mating aggregates that facilitates the conjugation of bacteria (Sava et al., 2010). In the current study, 3.1% of the isolates carried the *asa*1 gene. In contrast, a higher rate of prevalence (24.7%) of this gene was reported by Yang et al. (2019) in China. The *esp* gene encoding enterococcal surface protein has been reported to be related to biofilm formation in enterococci (Sava et al., 2010). This gene was found in seven (10.8%) isolates in this study. The prevalence of this gene was reported as 85.2% by Yang et al. (2019) and 30.7% by Herkmen and Türkyılmaz (2016). Hyaluronidase is an enzyme with a molecular weight of approximately 45 kDa and encoded by the *hyl* gene. The enzyme plays important role in degrading the connective and cartilage tissue glycosaminoglycans (mucopolysaccharides), consequently leading to the spread of the bacteria (Chajęcka-Wierzchowska et al., 2017). This gene was detected in only one (1.5%) *E. hirae* isolate in the study. Similarly, in China, Yang et al. (2019) reported that they found the *hyl* gene only in a few *E. faecalis* isolates (2.5%, 2/81) from subclinical bovine mastitis.

In conclusion, the results of the study showed that enterococci isolated from subclinical buffalo mastitis had low levels of resistance to antimicrobials tested and revealed a low carriage rate of virulence genes. This might be explained by no or low transmission of virulent and antimicrobial-resistant enterococci via human intervention.

Conflict of Interest

The authors stated that they did not have any real, potential or perceived conflict of interest.

Ethical Approval

This study is not subject to HADYEK permission in accordance with Article 8 (k) of the "Regulation on Working Procedures and Principles of Animal Experiments Ethics Committees".

Similarity Rate

We declare that the similarity rate of the article is 14% as stated in the report uploaded to the system.

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Author Contributions

Motivation / Concept: ÖA, EKÜ, MAY
Design: ÖA, EKÜ, YE
Control/Supervision: ÖA
Data Collection and Processing: ET, ÖA, MAY
Analysis and Interpretation: ET, ÖA, EKÜ
Literature Review: ÖA
Writing the Article: ÖA, EKÜ
Critical Review: ÖA

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[*]**Correspondence:** Özkan ASLANTAŞ

Hatay Mustafa Kemal University, Faculty of Veterinary Medicine, Department of Microbiology, Hatay, Türkiye.

e-mail: aslantas@mku.edu.tr