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Vancomycin-Resistant *Enterococcus spp*. Isolated From Mastitic cow's milk

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ABSTRACT

This study assessed the recrudescence of *Enterococci* in mastitis cow's milk and investigated their resistance to vancomycin. 300 samples were compiled from separate places and fields in Kirkuk, Iraq during the period from February to May, 2022. The samples were ioculated on the surface of bile esculin agar plates with sodium azide and then incubated at 37°C for 24–48 h. The characteristic pin-point colonies with a zone of black precipitate and morphologically resembling *Enterococci spp.* were further subjected to presumptive identification by Gram' staining, catalase, and oxidase tests. All isolates were kept in BHIB with 30% glycerol at −70°C for further molecular detection. *Enterococci* isolates were tested for their susceptibility to different antibiotics by a disc diffusion technique. Based on the results of the sensitivity test, the ten isolates with the highest level of multiple resistances were selected from each of ***E. faecalis*** and ***E. faecium*** to examine the *van*A, *van*B genes by cPCR. The results of the bacteriological examination revealed that, 61 isolates (20.3%) of *Enterococci* According to phenotypic criteria; 42 isolates were *E. faecalis* and 19 were *E. faecium*. Add this to the confirmatory tests that revealed 25 isolates (8.3%) were *E. faecalis* and 10 isolates (3.3%) were *E. faecium* detected by PCR. Antimicrobial susceptibility tests indicated high levels of multi-resistant E. faecalis *and E. faecium* strains. Vancomycin-resistant strains were 40% and 30% for *E. faecalis and E. faecium,* respectively. The genetic sequences of *E. faecalis* and *E. faecium* isolates and phylogenetic trees were established and registered in GenBank-NCBI. They obtained accession numbers (OP566382) for *E. faecium* and (OP566380) for *E. faecalis,* which became references in Iraq and around the world.

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INTRODUCTION

Enterococci are common, opportunistic pathogens that are typically present in the normal flora of both human and animal intestines. These bacteria are able to survive a wide array of hostile conditions and can persist in the environment for long periods of time **(Nam *et al.*, 2010; Kim, 2022).** Enterococci are one of the mastitis-causing environmental factors because they are able to adapt to various environmental factors and endure in the environment for an extended period of time. As a result, they can infect the mammary glands. **(Bibalan *et al.*, 2015; Al-Dabbagh *et al.*, 2020).** In addition to being linked to warm-blooded animals, enterococci can also be found in soil, surface water, and on plants and vegetables. Additionally, it can contaminate finished goods while food is being processed. **(Pesavento, 2014; Abdeen *et al.*, 2016).** Life-threatening infections have resulted from enterococci resistance to a number of regularly used antibiotics **(http://www.en.wikipedia.org/2019).**

The ability to withstand antibiotics that the members of the Enterococcus genus have acquired through the transmission of transposons, plasmids, mutations, or chromosomal exchange **(Mundy 2000).** Enterococcus pathogens are of global importance due to their propensity to spread antibiotic resistance genes (**Cui *et al.*, 2020**). Enterococci have developed resistance to practically all antimicrobial medicines used to treat them, including vancomycin, which is one of the most effective antimicrobials for treating enterococcal infections (**Emaneini *et al.*, 2016).** Vancomycin-resistant enterococci (VRE) were initially identified in 1998, and their prevalence has

risen dramatically since then. Vancomycin resistance genes are van genes that can be passed on to other Gram-positive bacteria **(Cetinkaya 2000).**

The aims of this study were to isolate Enterococcus from the milk of mastitic cows in Iraq and to describe the genotypic characteristics and vancomycin resistance.

MATERIALS AND METHODS

Sampling

A total of 300 samples of mastitic cow's milk were obtained from various fields and areas in Kirkuk, Iraq. All of the animals were examined clinically. Clinical mastitis was diagnosed when one or more of the following signs were present in an animal: These symptoms included the cardinal indicators of inflammation in one or more udder quarters, as well as aberrant milk features such as clot formation, discoloration, viscosity alterations, an odd odour, and the presence of blood. To isolate and identify *E. faecalis* and *E. faecium*, all samples were labelled, aseptically put in clean, dry, and sterile containers, kept cold, and then transported to a microbiology lab.

Isolation and identification

Standard microbiological techniques were used to cultivate the samples that were collected. **(Facklam 1989).** The samples were inoculated with sodium azide on the surface of bile esculin agar plates (Oxoid, UK), then incubated for 24-48 hours at 37°C. Based on Gram staining, catalase, and oxidase tests, as well as growth in brain-heart infusion broth (BHIB) at pH 9.6 to 10.5, 45 °C, and 6.5% NaCl, the suspicious colony was presumed to be identified. For subsequent examination, all isolates were stored in BHIB with 30% glycerol at 70°C.

Antibiotic susceptibility test

Enterococci isolates were tested for their susceptibility to different antibiotics by a disc diffusion technique [CLSI-M100 2018]. The applied antimicrobials included; fluoroquinolones (Ciprofloxacin 10 and Levofloxacin 5), Glycopeptides: (Vancomycin 30), Macrolides: (Azithromycine 15), B-lactamases (Cephalosporins: Cefoxitin 30 and Amoxicillin-Clavulinic Acid acid 20\10) , tetracyclines (Tetracycline 30), phenicols (Florfenicol 25 and chloramphenicol 30), and aminoglycosides (Streptomycin 10 and Gentamycin 10), (Oxoid and high media).

Molecular detection

Material used for extraction of DNA, according to Samboork et al.,1989 QIAamp DNA Mini Kit, no. 51304**,** The QIAamp DNA Mini Kit offers silica-membrane-based nucleic acid purification from many types of samples. The overall hands-on time is 20 minutes because the spin-column method does not require mechanical homogenization.

Oligonucleotide primers

Metabion provided four pairs of primers. (Germany). They follow a distinct sequence and produce distinct products. Specific gene primers were used to confirm the presence of Enterococci at the genus level as shown in table 1.

Table 1: Oligonucleotide primers sequences used in the study

Gene	Primer (5'-3')	Molecular weight	
VanA	CAT- GAC- GTA- TCG- GTA- AAA- TC	885 bp	[Patel 1997]
	ACC- GGG- CAG- RGT- ATT- GAC		
VanB	GTG- ACA- AAC- CGG- AGG- CGA-GGA	433 bp	[Kariyama 2000]
	CCG- CCA- TCC- TCC- TGC- AAA- AAA		
E. faecalis 16S Rrna	GTT- TAT- GCC- GCA- TGG- CAT- AAG-AG	310 bp	[Zoletti 2006]
	CCG- TCA- GGG- GAC- GTT- CAG		
E. faecium adk	TAT- GAA- CCT- CAT- TTT- AAT- GGG	437 bp	[Homan, *et al*., 2002]
	GTT- GAC- TGC- CAA- ACG- ATT- TT		

RESULTS

Preliminary culture results on the selective medium (bile esculin agar) 81 isolates of *Enterococcus spp*. were obtained. They all grew on the selective media and produced the pin-point enterococci colonies that are typical of the species. A zone of black precipitate around each colony showed that they were all resistant to 40% bile and hydrolyzed esculin.

Table 2: Biochemical tests for determination *E.faecalis* isolates from clinical mastitic cow's milk

Test	Result	
	E. faecalis	*E.faecium*
Catalase	-	-
Grow in Temp. (45 C)	+	+
Grow in concentration (6.5% NaCl.)	+	+
Grow in (PH.=9.6-10)	+	+
Grow in the presence of tellurite salts (0.04%)	+	-
Acid production from Sorbitol	+	-
H2S production and motility	-	-
Oxidase	-	-
Lactose	+	+
Glucose	+	+
Fructose	+	+
Arabinose	-	+

The results of biochemical identification test revealed that 42 (14%) isolates were positive for *E. faecalis* and 19 (6%) for *E. faecium.* The results of confirmatory tests showed that 25 (8.3%) isolates were positive for *E. faecalis* and only 10 isolates (3.3%) were positive for *E. faecium* as shown in figures 1 and 2.

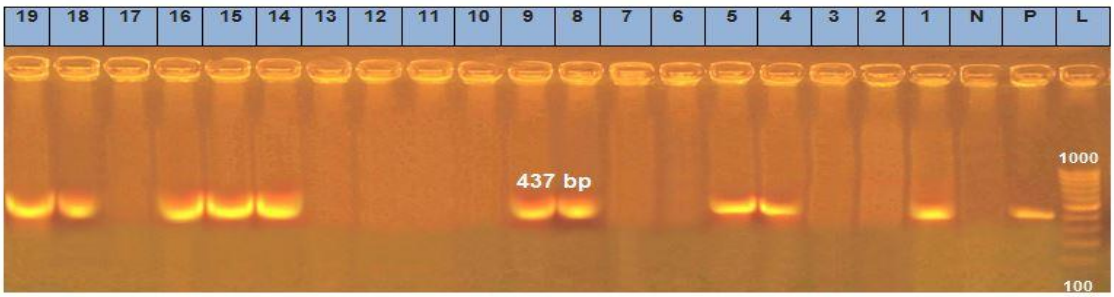

Fig. 1: Agarose gel photo documentation for molecular identification of *E. faecium* lane L molecular weight marker (100 -1000bp) (100bp DNA ladder H3 RTU, BIO- HELIX) Lane p : positive control (at 437 bp.), Lane N : negative control.

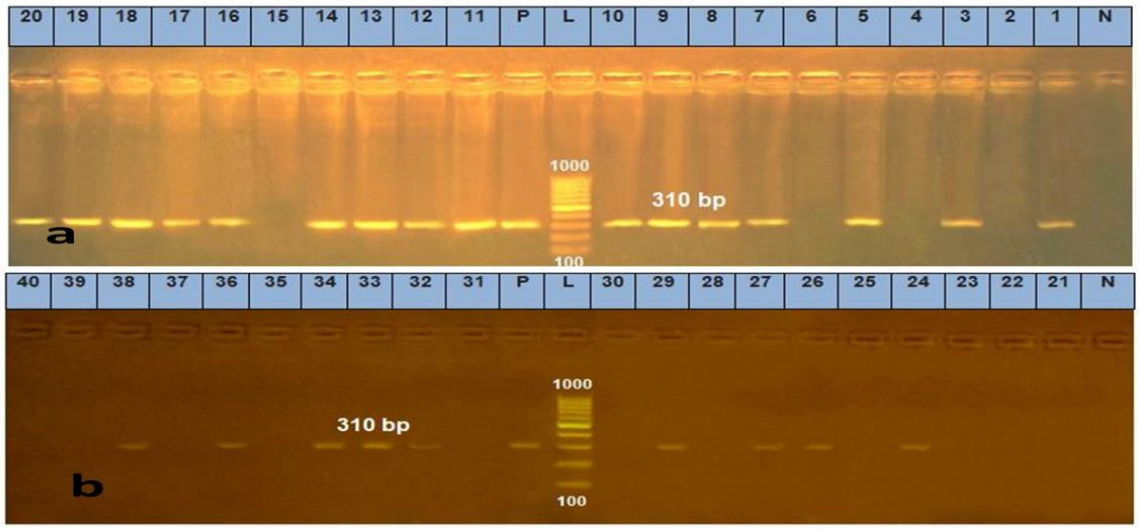

Fig. 2 a & b: Agarose gel photo documentation for molecular identification of *E. feacalis* lane L molecular weight marker (100-1000bp), lane P : positive control (at 310 bp), lane N: negative control.

The results of antimicrobial susceptibility for all positive *E. faecium* (n=10) isolates showed high levels showed high levels to vancomycin resistance (30%) Regarding *E. faecalis* (n=25), 8 isolates (32%) were resistant to Vancomycin as shown in Table (3) and Fig.(3).

Table 3: The antimicrobial of *E. faecium and E. faecalis* resistant isolates from mastitic cow's milk samples

Class	Antibiotic	Antibiotic disc concentration	Number of resistant *E. faecium* isolates Total No. (10)	Number of resistant *E. faecalis* isolates Total No. (25)
Glycopeptide	Vancomycin	30	3	8
Tetracycline's	Tetracycline	30	5	17
Aminoglycoside	Gentamycin	10	3	13
	Streptomycin	10	6	11
Macrolides	Azithromycin	15	6	20
Quinolones	Levofloxacin	5	2	10
	Ciprofloxacin	10	4	12
Phenicol	Chlorfenicol	30	3	11
	Florfenicol	25	5	12
B-lactamase	Amoxicilline-Clavulinc acid	20 10	2	9
	Cefoxitin	30	7	18

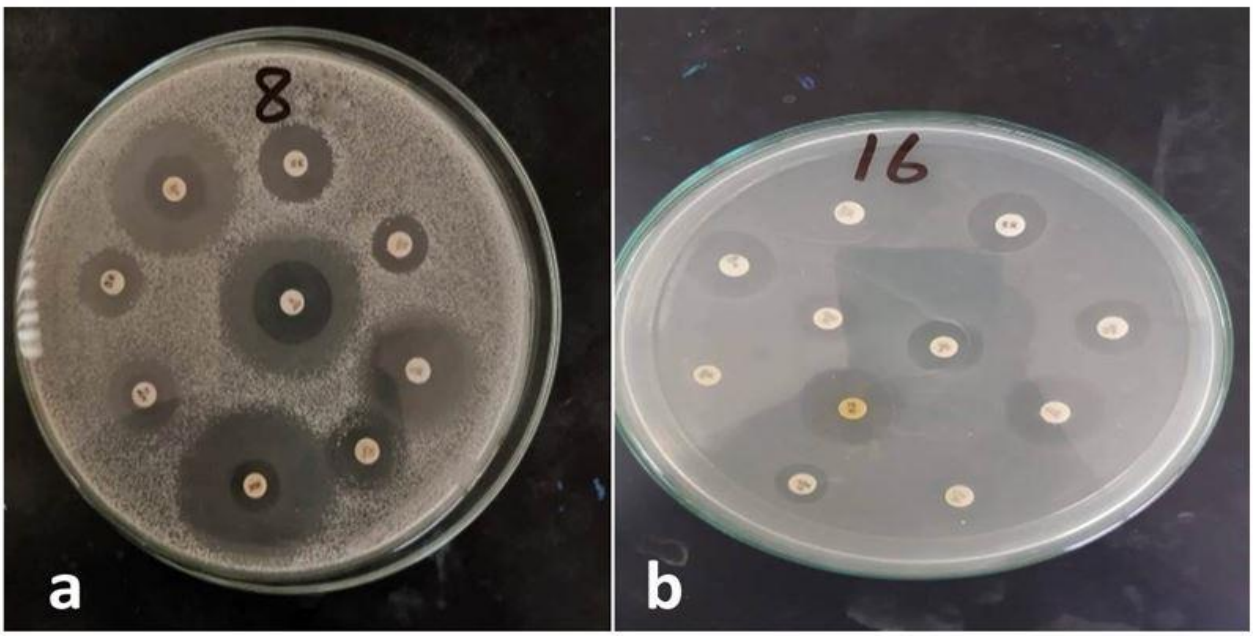

Fig. 3: The antibiogram assay for determination Vancomycin-resistant *E. faecium (a) and E. faecalis* (b) isolates from mastitic cow's milk

Four out of ten *E. faecium* isolates and six out of twenty-five *E. faecalis* isolates, that gave the highest level of resistance were selected, and a confirmatory test by PCR was done to investigate the resistance genes responsible for Enterococci resistance to vancomycin. The results showed that *E. faecium* isolates have the *Van*A gene in two isolates among the four *E. faecium* isolates (50%), and vanB is found in one isolate (25%), As for *E. faecalis*, the results showed that *E.* faecalis *has the VanA* resistance gene in two isolates (33%) , and *van*B only in one isolate (16%), which are responsible for *Enteroccocci* resistance to Vancomycin, as shown in Table (4) and Fig. (4).

Table 4: The presence of *van*A and *van*B resistant genes for *E. faecium and E. faecalis* from mastitic cow's milk using PCR technique

Samples	*E. faecium*	*E. faecalis*	*van*A	*Van*B
1	-	+	-	-
4	+	-	+	-
5	-	+	-	-
7	-	+	+	-
8	+	-	+	+
9	-	+	-	-
12	-	+	+	+
14	+	-	-	-
16	-	+	-	-
19	+	-	-	-

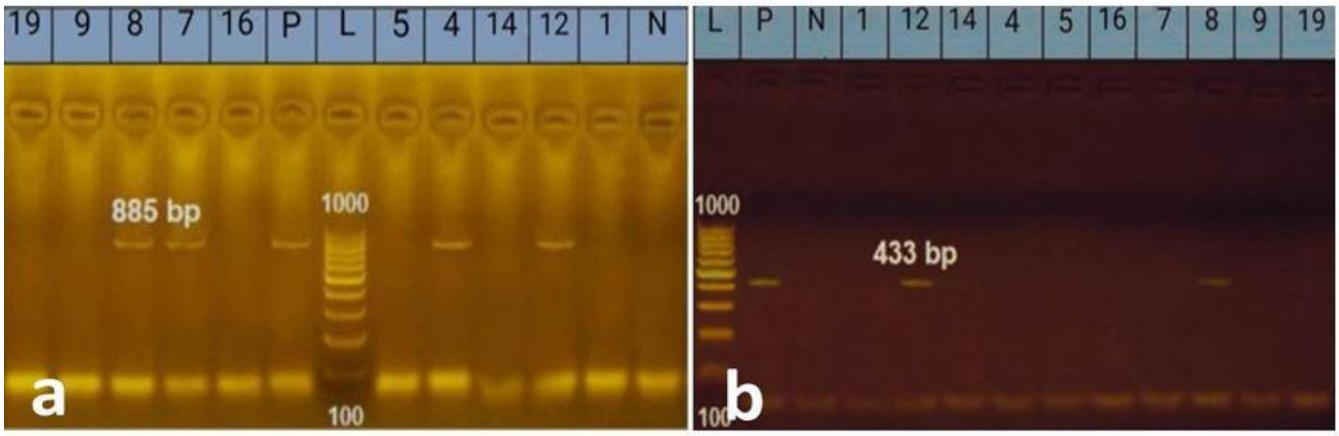

Fig.4: Agarose gel electrophoresis of PCR amplified products of (*van* A) resistant gene at 885 bp.(4, 8) for *E. faecium*, and (12, 7) for *E. faecalis* (a). *van*B resistant gene at 433 bp(8) for *E. faecium* and (12) for *E. faecalis* (b). lane L molecular weight marker, lane p : positive control, lane n: negative control, The size in base pairs (bp.) of each PCR product was indicated for the bands.

Phylogenetic tree and multiple sequences of the local genotype were established for *E. faecium* and *E. faecalis* isolated from mastitic cow's milk in Iraq (Figs. 5&6). The genetic sequences and phylogenetic trees of *E. faecalis* and *E. faecium* isolates were registered in Gen-bank-NCBI. They obtained accession number (OP566382) for *E. faecium* and (OP566380) for *E. faecalis*.

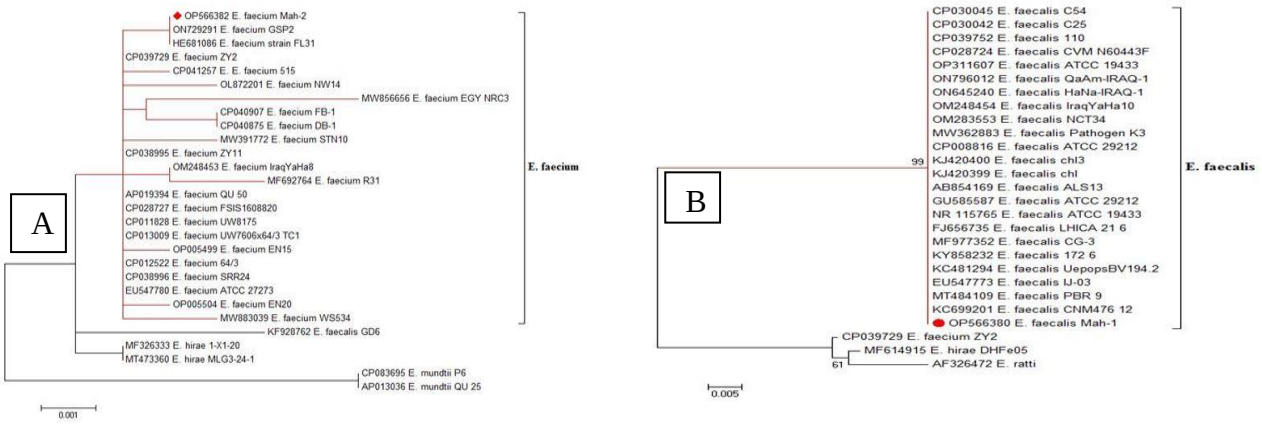

Fig.5: Phylogenetic tree of local genome of *E. faecium* (A) and *E. faecalis* (B) sequencing isolated from mastitic cow's milk

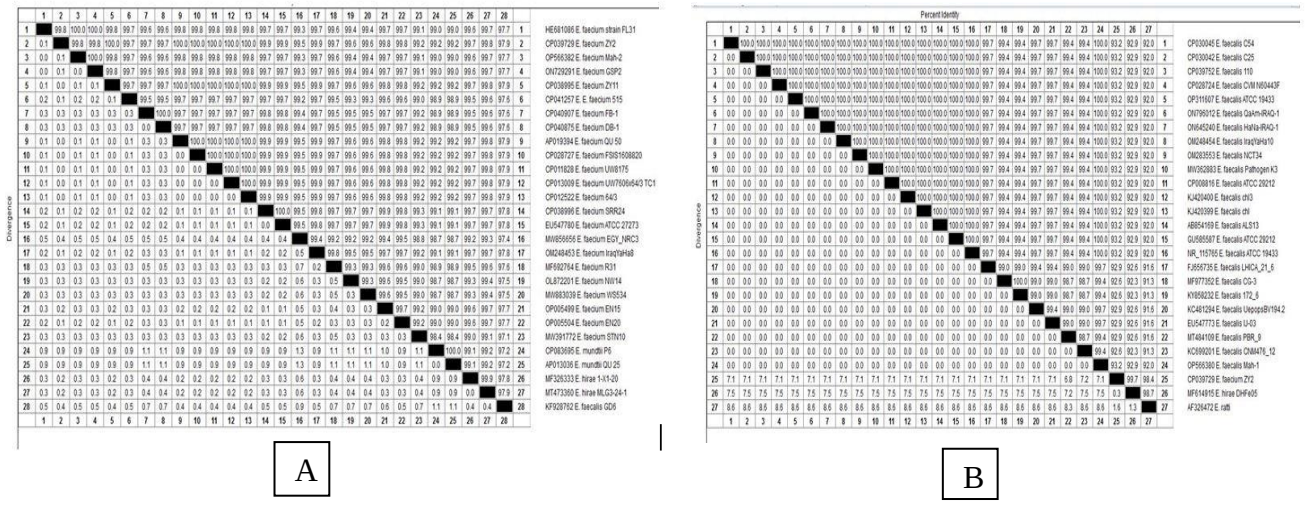

Fig. 6: Alignment of multiple sequences of the local genotype of *E. faecium* (A) and *E. faecalis* (B) isolated from mastitic cow's milk.

DISCUSSION

In this study, the prevalence rate and vancomycin resistance of enterococci isolated from mastitis-infected cow milk were investigated. This type of clinical research is beneficial to antimicrobial usage practices and public health. Because commensally occurring bacteria, such as enterococci, have natural gene transfer mechanisms that might result in various resistances **(Nam, 2010, Jackson, 2011).**

The current investigation found that enterococci are prevalent in mastitic milk and have a high rate of vancomycin resistance. As a result, a new strategy for treating mastitis in Iraqi cows should be implemented. Opinions on the presence of enterococci in mastitic cow's milk differ. The current investigation discovered, using traditional microbiological techniques, that the incidence of *Enterococci spp.* in the milk of infected cows with clinical mastitis was 27% (3.3% for *E. faecium* and 8.3% for *E. faecalis*). These results are higher than the results obtained by earlier workers who isolated enterococci from 10.9% of mastitic milk samples in Turkey **(Kuyucouğlu, 2011).** On the other hand, the present results are lower than those of previous authors, who found *enterococci* in 60% of mastitic milk samples collected in Iraq **(Hamzah, 2018).** This variation may be linked to the prevalence of *Enterococcus spp.* in various countries, farm management practises, climate conditions, and the high sensitivity of detection technologies.

The result of the sensitivity test showed that high levels of resistant *E. faecium* isolates were of serious concern, with the isolation of three strains resistant to Vancomycin representing 30%. For *E. faecalis*, 8 isolates were resistant to Vancomycin representing 32%. Similar findings were reported by earlier workers who found that the incidence of resistance to Vancomycin is 25% in Iraq **(Mohammed, 2013).** In contrast, Kececi et al. mentioned that there is no Vancomycin-resistant *Enterococcus faecalis* or *Enterococcus faecium* isolated from cattle milk, although they both carry the vanB gene **(Tekin, 2016).** In addition, Kim et al, did not record Enterococci resistance to vancomycin in South Korea **(Kim, 2022).** However, there are many reports recording a high level of Enterococci resistance to Vancomycin such as a study conducted in Egypt that found that *E. faecium* resistance to Vancomycin is 66% **(El-Zamkan, 2021).** In another study conducted in Egypt, a high level (100%) of Vancomycin resistance was reported in *Enterococcus faecalis* isolated from different food sources **(Abdeltawab, 2019).** In a comprehensive study conducted in Nigeria, it was found that VRE is 63.1% for *E. faecium* and 36.9% for *E. faecalis* **(Orababa, 2021).** The resistance of enterococci can be explained by the wide and inappropriate use of antibiotics in the treatment of mastitis infections.

The degree of antibiotic resistance in enterococci typically varies by species, drug, and nation **(Rózanska, 2019).** According to the data mentioned in table (4), the *van*A gene was found in two isolates representing 50%, one of which carried each of the *van*A and *van*B genes. As regards *E. faecalis, the van*A gene was found in two isolates representing 33%, and one of them carried each of the *van*A and *van*B resistance genes. The *van*B gene was found in only one isolate among the six *E. faecalis* isolates (16%). Similar findings were recorded before **(Abdeltawab, 2019, Rózanska *et al.*, 2019).**

Sequencing results were analyzed by NCBI to determine the genetic variation, which showed that the local genome of *E. faecium* isolated from Iraq is close to HE681086 in Saudi Arabia and ON729291 in India by 100%, while CP083695 in China and AP013036 in Japan are the furthest in the phylogenic tree. Regarding *E. faecalis*, the result showed that the local genome of *E. faecalis* isolated from Iraq is close to standard global isolates previously registered in the gene bank by 99%.

CONCLUSION

Iraq has a 27% prevalence of *Enterococci spp.* in mastitic cow's milk, and they have a high rate of vancomycin antibiotic resistance. *E. faecalis was the* predominant species *(8.3%), and E. faecium* was 3.3%. Additionally, due to improper antibiotic use and a short treatment duration, the isolated E. spp. from mastitic cow's milk displayed numerous antibiotic resistances. The genetic sequences and phylogenetic trees of *E. faecalis* and *E. faecium* isolated in this study were established and registered in GenBank-NCBI.

Conflicts of interest

The writers declare that there are no potential conflicts of interest related to the creation or dissemination of this article.

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