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Isolation and characterization of antibiotics resistance *Enterococcus faecium* from mastitic cow's milk

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Abstract

Antibiotic resistance pathogens are becoming a problem for both humans and animals, as well as environmental health. This study aimed to investigate the occurrence of *Enterococcus faecium* in the milk of cows infected with clinical mastitis and their antibiotic resistance in Kirkuk province from January to May 2022. Of 81 isolates, *Enterococcus species* were obtained through conventional culture methods and biochemical tests. Ten isolates 3.3% were identified to the level of *E. faecium* by standard microbiological methods. The result of the sensitivity test showed that all Ten *E. faecium* isolates gave multi-antibiotic resistance; the highest percentage of resistance by *E. faecium* was to Cephalosporin's group 70%, followed by Azithromycin and Streptomycin 60%. The four isolates that gave high levels of antibiotic resistance were selected to investigate resistance genes in them by PCR. *nor*A, *tet*K, and *aac(6') aph(2'')* genes are found in all four *E. faecium* examined isolates, whereas no isolate contains the *fex*A gene. *E. faecium* target gene was sequenced, analyzed, and registered in Gen-bank-NCBI and obtained accession number OP566382, which became a reference in Iraq and the world.

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Introduction

Bacterial mastitis is one of the most significant diseases affecting high-production dairy cows, causing high financial losses because it affects the dairy industry; it deteriorates the dairy industry and its quality, in addition to the cost of treatment (1). *Enterococcus spp.* is one of the formations of normal physiological gut flora in humans and animals; this opportunistic bacterium is considered an environmental factor that causes mastitis, which is found in the enclosing area of the cow, such as the bedding of housing cows, soil, and the waste product of the animals (2,3). The infections are caused mainly by *E. faecalis, E. faecium, Enterococcus spp.* characterized by their high ability to withstand harsh and different environmental conditions and persist in the environment for long periods, for example, in slaughterhouses, so potential damage of the udder is easy and straightforward (4). Several epidemiological factors can predispose to mastitis, such as season, where winter is a predisposing factor for the infection with mastitis (5). *Enterococcus spp*. are characterized by a high level of resistance to many antibiotics, both by intrinsic and acquired mechanisms, due to their ability to develop and transfer resistance-determining genes to other bacteria, *Enterococcus spp*. Serve as a reservoir of antimicrobial resistance genes; another source of concern is the likelihood of enterococci transmission from irritated udder to humans; the increasing use of raw, unpasteurized milk and products derived from this milk appears to imply the risk of potentially pathogenic and antimicrobial-resistant enterococci being transferred to humans via the food chain (6,7). In addition, *E. faecium* and other lactic acid bacteria have antibacterial ability that can inhibit the growth of other micro-organisms (8). Some Enterococci are inherently resistant to antibiotics as beta-lactam and aminoglycoside groups are considered a medical crisis (9). Polymerase chain reaction assay is a powerful method for detecting the different genes based on the target sequence of the specific gene (10,11).

This investigation was undertaken to evaluate the occurrence of enterococci in the milk of cows with clinical mastitis and to assess their antimicrobial resistance as well as to perform a genetic variation study to identify the polymorphism causing resistance as this study will help to improve understanding of the use of antibiotics in the treatment of mastitis and public health.

Materials and methods

Sampling

Three hundred samples were collected from mastitis cow's milk from different places and fields in Kirkuk / Iraq. All animals were subjected to clinical examination; when one or more symptoms were present, the animal was considered to have clinical mastitis. These symptoms included the typical signs of inflammation in udder quarters, signs of a systemic reaction such as fever, depression, and disturbed appetite, and abnormal milk characteristics such as clot formation, discoloration, viscosity, odor, and presence of blood. All of the samples were labeled, aseptically placed in clean, dry, and sterile containers, preserved in an icebox, and then transported to a microbiology laboratory in a veterinary hospital in Kirkuk province to be examined for the presence and isolation of *E. faecium*.

Isolation and identification

Enterococci isolates were cultured according to standard microbiological methods. The samples were inoculated on the surface of bile esculin agar plates with sodium azide (Oxoid, Basingstoke, Hampshire, England, UK) and then incubated at 37°C for 24-48 h. The characteristic pin-pointed colonies growing on the agar with a zone of black residue and morphologically resembling enterococci were further subjected to presumptive identification based on Gram staining, catalase, oxidase tests and growth in brain-heart infusion broth (BHI) at pH 9.6 - 10 and 45˚C and with 6.5% NaCl, all the isolates were kept in BHI broth with 30% glycerol at -70°C for further analysis (12).

Antibiotic susceptibility

E. faecium isolates were tested for their susceptibility to Eleven (11) different antimicrobials by a disk diffusion technique (13), comprised of Fluoroquinolones: (Ciprofloxacin and Levofloxacin), Glycopeptides: (Vancomycin), Macrolides: (Azithromycin), B- Lactamase: Cephalosporin's: (Cefoxitin and Amoxicillin- Clavulinic acid), Tetracycline's: (Tetracycline), Phenicols: (Florfenicol and Chloramphenicol), Aminoglycoside: (Streptomycin and Gentamycin).

Molecular detection

The material used to extract DNA_QIAamp DNA Mini Kit, Catalogue no.51304. The QIAamp DNA Mini Kit provides silica-membrane-based nucleic acid purification from different types of samples. The spin-column procedure does not require mechanical homogenization, so the total hands-on preparation time is only 20 minutes (14).

Oligonucleotide primers used in cPCR

Nine pairs of primers were supplied from Metabion (Germany). They have specific sequences and amplify particular products (Table 1).

Table 1: Oligonucleotide primer sequences used in the study

Gene	Primers (5'-3')	Molecular weight	Reference
TetK	GTAGCGACAATAGGTAATAGT GTAGTGACAATAAACCTCCTA	360 bp	(15)
aac(6')aph (2'')	GAAGTACGCAGAAGAGA ACATGGCAAGCTCTAGGA	491 bp	(15)
FexA	GTACTTGTAGGTGCAATTACGGCTGA CGCATCTGAGTAGGACATAGCGTC	1272 bp	(16)
NorA	TTCACCAAGCCATCAAAAAG CTTGCCTTTCTCCAGCAATA	620 bp	(17)
VanA	CATGACGTATCGGTAAAATC ACCGGGCAGRGTATTGAC	885 bp	(18)
VanB	GTGACAAACCGGAGGCGAGGA CCGCCATCCTCCTGCAAAAAA	433 bp	(19)
MphC	GAGACTACCAAGAAGACCTGACG CATACGCCGATTCTCCTGAT	722 bp	(20)
BlaZ	TACAACTGTAATATCGGAGGG CATTACACTCTTGGCGGTTTC	833 bp	(21)
E. faecium adk	TATGAACCTCATTTTAATGGG GTTGACTGCCAAACGATTTT	437 bp	(22)

Results

According to phenotypic criteria, 81 isolates were obtained; they all grew on bile aesculin agar and yielded the typical pin-pointed colonies of enterococci with a zone of black residue, proving that they were tolerant to 40% bile and hydrolyzed esculin. All the isolates were presented microscopically as Gram-positive ovoid cocci arranged chiefly in pairs or short chains. Further confirmation of the recovered isolates revealed that they were catalase test negative, Fermentation of sugar (lactose, fructose, glucose, and arabinose) cheerful, tolerant to high salinity and extreme pH (grew on BHI broth with 6.5% NaCl and at pH 9.6) and exhibited visible growth at ten and 45°C. Differential biochemical tests were carried out for all suspected isolates to investigate the enterococci to the species level and to exclude other bacterial species that are similar to them in some characteristics. Nineteen isolates were supposed to be positive for *E. faecium*. The cconfirmatory test was conducted by PCR to identify *E. faecium* by specific primers (*E. faecium* adk), which produced bands on agarose gel at the position of 437 bp (Table 2 and Figure 1). The results showed that only ten isolates were positive for *E. faecium* 3.3%.

Table 2: The percentage of *E. faecium* strains in the collected samples

Samples	Total samples (n)	+ve sample (%)
Milk	300	10 (3.3)

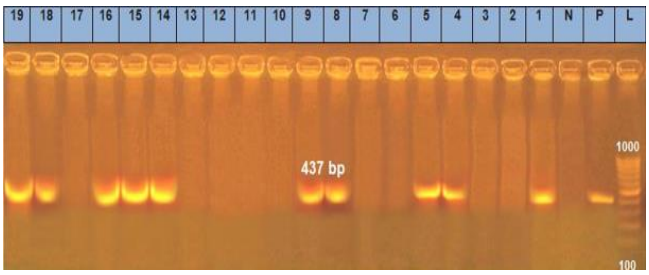

Figure 1: Agarose gel photo documentation for molecular identification of *E. faecium* lane L molecular weight marker (100 -1000bp) lane pos: positive control (at 437 bp.), lane neg.: negative control, lanes 1, 4, 5, 8, 9, 14, 15, 16, 18, and 19 are positive isolates.

The results of the antimicrobial susceptibility test by disc diffusion technique for all positive *E. faecium* isolates showed that high levels of multi-resistant *E. faecium* isolates are of grave concern, with the isolation of three strains resistant to vancomycin at 30%. The highest level of resistance was against the cephalosporins by 70%, while the level of resistance was the lowest against amoxicillin clavulanic by 20%. For the quinolones group levofloxacin, the result showed a low resistance level of 20%, while the resistance level of *E. faecium* strain to ciprofloxacin was 40%. As for the phenol group, the result showed a resistance ratio: florfenicol by 40% and chloramphenicol by 30%. For the aminoglycoside group, the result showed a high level of streptomycin resistance by 60%, while only three isolates showed gentamycin resistance by 30%. Tetracycline *E. faecium* strain as 50%. Finally, the result showed a high resistance to azithromycin by 60% (Table 3). Through the sensitivity test results, the four isolates that gave high multi-antibiotic resistance were selected to investigate resistance genes in them by PCR to identify the polymorphism causing multi-antibiotic resistance (Figures 2 and 3).

Table 3: The antibiogram of *E. faecium Resistance* isolates from mastitis cow's milk samples

Class	Antibiotic	Resistance n (%)
Glycopeptide	Vancomycin	3 (30)
Tetracycline's	Tetracycline	5 (50)
Aminoglycoside	Gentamycin	3 (30)
	Streptomycin	6 (60)
Macrolides	Azithromycin	6 (60)
Quinolones	Levofloxacin	2 (20)
	Ciprofloxacin	4 (40)
Phenicol	Chloramphenicol	3 (30)
	Florfenicol	5 (50)
B-lactamase	Amoxicillin	2 (20)
	Cefoxitin	7 (70)

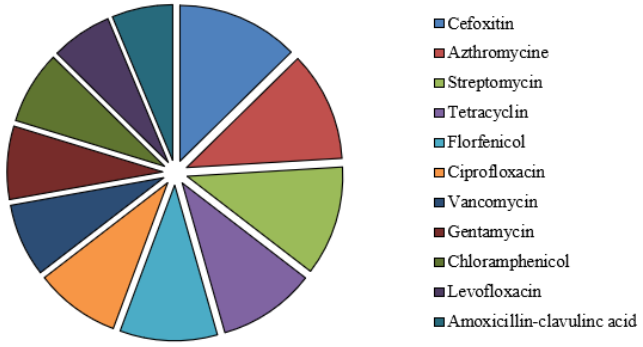

Figure 2: Distribution of Antibiotic resistance of *E. faecium* isolates from mastitic cows' milk.

PCR examination results showed that the resistance genes *norA*, *tetK*, aac(6')*aph* (2") found in all *E. faecium* isolates as 100%, *mph*C, and *Van*A gene found as 50%, *bla*Z, and *van*B found as 25%, whoever *fex*A genes which is responsible for *E. faecium* resistance to phenetoles did not found in any one of *E. faecium* isolates. Still, the sensitivity test results showed that *E. faecium* resistance to florfenicol and chloramphenicol was 40 and 30%, respectively (Table 4 and Figure 4).

Table 4: The incidence of some resistance genes for *E. faecium* strains from mastitic cow's milk by using of PCR technique

Sample NO.	blaZ	tetK	phC	fexA	norA	aac(6')aph (2")	VanA	VanB
14	-	+	+	-	+	+	-	-
4	+	+	-	-	+	+	+	-
8	-	+	+	-	+	+	+	+
19	-	+	-	-	+	+	-	-
%	25%	100%	50%	0	100%	100%	50%	25%

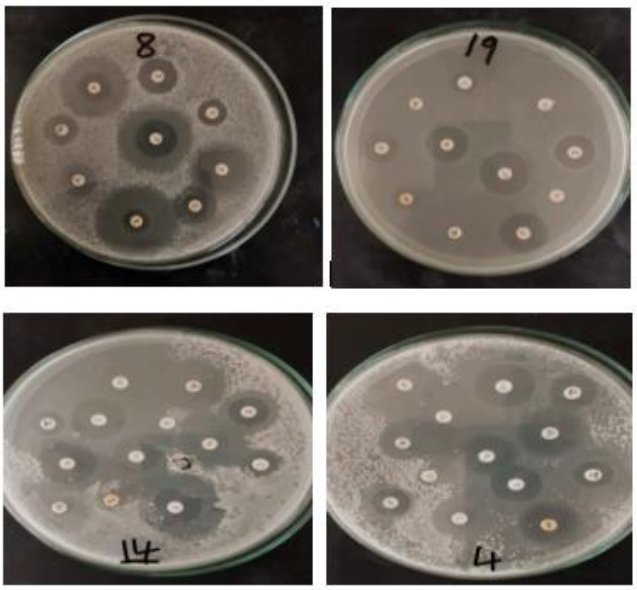

Figure 3: The antibiogram assay for determination of multi-antibiotics resistance genes of *E. faecium* isolates from mastitis cow milk.

When conducting a sequencing procedure on isolate No. 8 of *E. faecium*, which gave a high degree of multiple resistance against antibiotics and showed through the results of the PCR examination that it carries various resistance factors, it was found that the local gene of the isolate that bearing the accession number OP566382 (*E. faecium* Mah-2), is close to the global standard gene of *E. faecium* by 97-100% in comparison with the previous isolates registered in the Gen Bank that gave similarity (Figures 5 and 6).

Discussion

Enterococci are highly resistant to many antibacterial substances by intrinsic and acquired mechanisms (23). Recently, antibiotic resistance has attracted the attention of researchers and has been investigated in several studies (24,25). Several authors have demonstrated the prevalence of Enterococcus spp. in dairy cattle with mastitis and in raw milk (26-28). In this study, *E. faecium* represented 3.3%; this agreement with the study by Rozanska (29), who isolated *E. faecium* from mastitis cow's milk as 2%, and considered lower than the study by LI (30), who isolated *E. faecium* from mastitis cow's milk as 20%. Kim (31) recorded the prevalence of *E. faecium* from mastitis cow milk at 13%; this disparity could be attributed to the incidence of *E. faecium* in different countries, farm management, and climate conditions. According to data recorded in Table 3, antimicrobial susceptibility results showed high levels of contamination with multi-resistant *E. faecium* of grave concern, with the isolation of strains resistant to vancomycin at 30%.

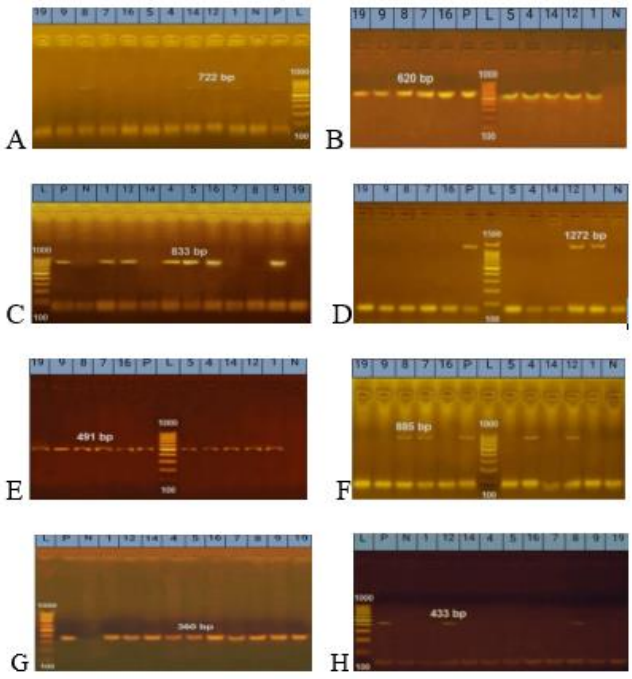

Figure 4: Agarose gel electrophoresis of PCR amplified products of *Enterococcus faecium mph*C resistance gene at 722 bp (A), *E. faecium norA* resistance gene at 620 bp (B), *E. faecium bla*Z resistance gene at 833 bp (C), *E. faecium fex*A resistance gene at 1272 bp (D), *E. faecium aac6* resistance gene at 491 bp (E), *E. faecium van*A resistance gene at 885 bp (F), *E. faecium* (*tet K*) resistance gene at 360 bp (G), *and E. faecium van*B resistance gene at 433 bp (H). lane L molecular weight marker, lane pos.: positive control, lane neg.: negative control, the size in base pairs (bp.) of each PCR product is indicated for the bands.

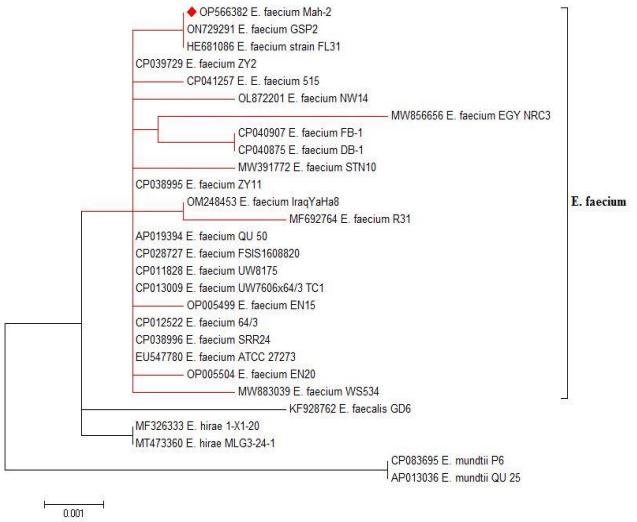

Figure 5: Phylogenetic tree of the local genome of *E. faecium* sequencing isolate from mastitic cows milk.

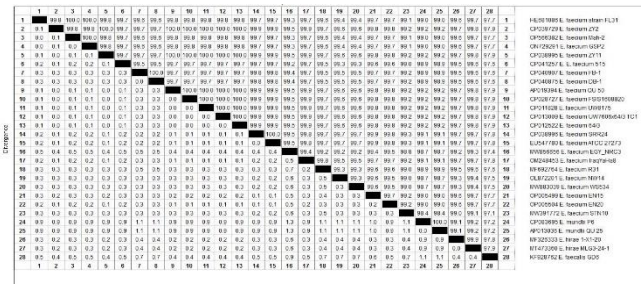

Figure 6: Alignment of multiple sequences of the local genotype of *E. faecium* isolate from mastitic cows milk.

The highest resistance level was observed to Cefoxitin at 70% and about 60% for each of Azithromycin and Streptomycin. The result showed a middle level of resistance at 50% for each Florfenicol and Tetracycline, while the lowest level was for Amoxicillin-clavulanic and Levofloxicillin by 20%. For Gentamycin 30%, Chloramphenicol 30% and Ciprofloxacin 40%. Enterococci exploit several intrinsic and acquired antibiotic resistance mechanisms (32,6). Rozanska (29) found *E. faecium* resistant to Vancomycin by 3%, contrasting with our result. Our result, considered lower than EL-zaman (33), found that Vancomycin-resistant *E. faecium* by 66%. Tekin (34) recorded that 7 (88%) *E. faecium* isolates were positive for van genes, but there was no Vancomycin resistance in all of the isolates. Also, Kim (31) recorded no *E. faecium* Vancomycin resistance by 0%. Many studies found *E. faecium* resistance to B-lactam in high levels (9,35), which agrees with our Cephalosporin result. Our results showed *E. faecium* resistance to phenicol, although the results of the PCR examination showed that none of the *E. faecium* isolates had the fexA gene, which is responsible for *E. faecium* resistance to phenicols. This resistance may be due to the common and prolonged usage of these antibiotics in the dairy industry for prophylaxis and treatment of mastitis-diseased cattle. Our result agrees with Montiron (36), who found *E. faecium* resistance to Chloramphenicol as (40%); our impact is considered higher than the result in studies conducted by Kim and Olivera (31,37) by 9 and 0%. The report given by Kuyucuoğlu (38) mentioned that *E. faecium* resistance to Tetracycline was 62%, which is almost similar to our result, lower than the result in Chain by Yang (39), who recorded Tetracycline resistance by 88%. A study in Korea by Know (40) found resistance to ciprofloxacin at 69%, which is higher than our result. Finally, streptomycin resistance is mentioned in the study by Jackson (41) by 1%, which contrasts with our development. Generally, enterococci antibiotic resistance levels vary by species, drug, and country (29). NCBI analyzes sequencing results to know the genetic variation, which shows that the local genome of *E. faecium* isolate is close to HE681086 in Saudi Arabia, ON729291 in India by 100%, while CP083695 in China, AP013036 in Japan is the furthest in the phylogenic tree.

Conclusion

Multi-antibiotic resistance enterococci are a severe hazard to both cattle and owners because they increase the cost of disease treatment and sometimes result in fatalities. In this study, multi-antibiotic resistance *E. faecium* is prevalent in Kirkuk- Iraq, during winter and might spread further if preventative steps are not taken. Moreover, *E. faecium* isolated from mastitis cow's milk is becoming resistant to an increasing number of antibiotics due to inappropriate use of antibiotics and an insufficient treatment period.

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Conflict of interest

No conflict of interest.

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عزل وتوصيف المكورات المعوية متعددة المقاومة للمضادات الحيوية من حليب الأبقار المصابة بالتهاب الضرع

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الخلاصة

أصبحت مسببات الأمراض المقاومة للمضادات الحيوية مشكلة لكل من البشر والحيوانات وكذلك الصحة البيئية. هدفت هذه الدراسة إلى التحقق من وجود بكتيريا المكورة المعوية البرازية في لبن الأبقار المصابة بالتهاب الضرع السريري ومقاومتها للمضادات الحيوية في محافظة كركوك خلال الفترة من كانون الثاني إلى أيار ٢٠٢٢. تم الحصول على ٨١ عزلة من المكورات المعوية من خلال طرق الاستزراع التقليدية والاختبارات البيوكيميائية. تم تحديد عشر عزلات (٣,٣٪) لمستوى المكورة المعوية البرازية بالطرق الميكروبيولوجية القياسية. أظهرت نتيجة اختبار الحساسية أن جميع عزلات المكورة المعوية البرازية أعطت مقاومة متعددة للمضادات الحيوية، وكانت أعلى نسبة مقاومة لمجموعة السيفالوسبورين ٧٠٪، يليها الايزويرثرومايسين والستبتومايسين ٦٠%. تم اختيار أكثر أربع عزلات ذات مقاومة عالية للمضادات الحيوية للتحري عن جينات المقاومة فيها بواسطة تقنية تفاعل البلمرة المتسلسل. تم العثور على جينات norA و tetk و aph(2) aac(6) في جميع عزلات المكورة المعوية البرازية الأربعة التي تم فحصها ، بينما لا تحتوي أي عزلة على جين fexA. تم تحليل تسلسل جين بكتريا المكورة المعوية البرازية، وتسجيله في بنك الجينات والحصول على رقم الانضمام الذي أصبح مرجعا في العراق والعالم.