# MOLECULAR CHARACTERIZATION OF MULTI-DRUG RESISTANCE ENTEROCOCCUS FAECALIS ISOLATED FROM MASTITIC COWS MILK

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**ABSTRACT**

**The study objects were to assess *Enterococcus faecalis* prevalence in milk from mastitis infected cows, and access their antibiotic resistance as well as investigate their resistance genes. A total of 300 samples were tested during the period from February to May in 2022. The results of initial examination showed that only 81 isolates as *E. species* according to phenotypic criteria. Confirmatory test was conducted, the results showed that only 25 isolates by 8.3% *E.faecalis* isolates. Antimicrobial susceptibility test indicated high levels of contamination with multi-drug resistant *E.faecalis* of with isolation of strains resistant to Vancomycin as 32%. The most high level of *E.faecalis* resistance was observed to Azithromycin, Cephalosporines, by 80 and 72% respectively. Adopt on results of susceptibility test, the six most isolates that gave high (MDR) were selected to investigate resistance genes in them by PCR, *nor*A, *tet*K and *aac6* genes are found in all of the isolate. *E. faecalis* gene was sequenced, analyzed, and registered in Gen-bank-NCBI and obtained the accession number (OP566380) that became a reference in Iraq and the world.**

**Keywords: PCR, bacteria, gene, isolates, strains, contamination**

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## التوصيف الجزيئي لبكتريا المكورات المعوية متعددة المقاومة للمضادات الحيوية المعزولة من حليب الابقار المصابة بألتهاب الضرع

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**المستخلص**

هدفت الدراسة إلى تقييم انتشار بكتريا ***Enterococcus faecalis*** في حليب الأبقار المصابة بالتهاب الضرع، والتحري عن مقاومتها للمضادات الحيوية والكشف عن جينات المقاومة فيها. تم اختبار 300 عينة جمعت خلال الفترة من شباط إلى ايار في عام 2022. وأظهرت نتائج الفحص الأولي أن 81 عزلة فقط تعود لـ Enterococcus Species وفقا لمعايير النمط الظاهري. تم إجراء اختبار تأكيدي، وأظهرت النتائج أن 25 عزلة بنسبة 8.3٪ تعود لجنس ***E.faecalis***. أشار اختبار الحساسية لمضادات الجراثيم إلى وجود مستويات عالية من التلوث بسلالات ***E. faecalis*** متعددة المقاومة التي تثير قلقًا خطيرًا مع عزل السلالات المقاومة للفانكومايسين بنسبة 32٪. لوحظ أعلى مستوى مقاومة كان ضد الأزيثروميسين ، سيفالوسبورين ، بنسبة 80٪ ، 72٪ على التوالي. بالاعتماد على نتائج اختبار الحساسية ، تم اختيار أكثر ست عزلات أعطت نسبة عالية من (MDR) لفحص جينات المقاومة فيها عن طريق تفاعل البلمرة المتسلسل (PCR). تم العثور على جينات norA و tetK و aac6 في جميع العزلات. تم دراسة تسلسل الجيني لـ ***E. faecalis*** وتحليله وتسجيله في Gen–bank– NCBI والحصول على رقم الانضمام (OP566380) الذي أصبح مرجعا في العراق والعالم.

**الكلمات المفتاحية: تفاعل البلمرة المتسلسل، بكتريا، جين ,معزولات ,سلالات ,تلوث**



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## INTRODUCTION

Mastitis is a major economic illness defined as a mammary gland infection characterized by physical and chemical changes in milk and glandular tissue abnormalities (6, 7). *Enterococcus spp.* is among the environmental factors that induce mastitis.(15, 20) these opportunistic microorganisms are frequently present in both humans' and animals' gut micro-biota, which are discharged into the environment with feces, there for its present in the surrounding area, such as bedding of housing cows, dirt, and animal waste products, resulting contamination of the udder easily(23, 24). Recently, enterococci have gained resistance to a number of antimicrobial drugs, antibiotic resistance genes in enterococci are frequently carried on transposons, which are easily transmitted to other bacteria, leading to antibiotic resistance spreading and multi antibiotic resistance(16). In both human and veterinary medicine, antimicrobial resistance appears to be the most pressing problem (14). Additionally, lactic acid bacteria such as enterococci and others have antimicrobial properties that prevent the development of other microorganisms (2). PCR, or polymerase chain reaction, is a effective techniques for identifying various genes depending on their target sequences. There is insufficient information from Iraq's Kirkuk province about the occurrence of E. faecalis in mastitic cow's milk or their susceptibility to antimicrobials, so This study was conducted to assess the presence of *E. faecalis* in milk of clinical mastitis cows and assess their antimicrobial resistance, well this studty will contribute to a better understanding of how antibiotics are used to treat mastitis and other public health issues.

## MATERIALS AND METHODS

**Sampling and isolation:** according to Facklam1989 (8). 300 samples of mastitic cow's milk were taken from various locations throughout the Kirkuk Governorate in Iraq. Animals were given a clinical examination, and if any of the following signs emerged, the animal was diagnosed with clinical mastitis: These signs included conventional indicators of udder quarter inflammation, abnormal milk characteristics like clot formation, discoloration, changes in viscosity, an unusual odor, and the presence of blood, systemic reaction symptoms can include fever, depression, and altered appetite. All samples were tagged and aseptically deposited in clean, dry, and sterile containers before being sent to a microbiology facility for testing the presence of *E.faecalis.* Standard microbiological methods were utilized to culture enterococci isolates. The samples were plated on bile esculin agar surfaces and immunized with sodium azide. (Oxoid, Basingstoke, Hampshire, England, UK), then incubated at 37°C for 48 hours, Black color was seen in the medium surrounding typical colonies. Presumptive identification of bacteria that resembled enterococci was done using gram stain, catalase test, oxidase test, growth in brain-heart infusion broth (BHI) at pH 9.6 - 10.5, 45 C, and 6.5% NaCl, as well as sugar fermentation tests. The isolates were kept in BHI broth with 30% glycerol at 70°C for subsequent analysis utilizing a number of different biochemical tests to establish the genus level.

**Antibiotic susceptibility**

A disk diffusion technique was used to assess *E.faecalis* isolates for antibiotic susceptibility (4), comprised Fluoroquinolones: (Ciprofloxacin and Levofloxacin), Glycopeptides: (Vancomycin), Macrolides: (Azithromycine), B- Lactamase: (Cephalosporin's: Cefoxitin) and Augmentin (Amoxicillin-Clavulinic acid), Tetracycline's: (Tetracycline), Phenicols: (Florfenicol and Chloramphenicol), Aminoglycoside: (Streptomycin and Gentamycin)

**Molecular detection**

Material used for extraction of DNA, according to Samboork et al.(21) QIAamp DNA Mini Kit, no. 51304**),** The silica-membrane-based nucleic acid purification from many types of samples is offered by the QIAamp DNA Mini Kit. The overall hands-on time is 20 minutes because the spin-column method does not require mechanical homogenization.

**Oligonucleotide primers**

Metabion provided nine 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 (Table 1).

**Table 1. Sequences of oligonucleotide primers used during the study**

| Gene | Primer (5'-3') | Molecular weight | |
|---|---|---|---|
| *TetK* | GTA- GCG- ACA- ATA- GGT- AAT- AGT<br>GTA- GTG- ACA- ATA- AAC- CTC- CTA | 360 bp | Duran *et al.*, 2012 |
| *aac(6')aph (2'')* | GAA- GTA- CGC- AGA- AGA- GA<br>ACA- TGG- CAA- GCT- CTA- GGA | 491 bp | |
| *FexA* | GTA- CTT- GTA- GGT- GCA- ATT- ACG- GCT- GA<br>CGC- ATC- TGA- GTA- GGA- CAT- AGC- GTC | 1272 bp | Kehrenberg and Schwarz, 2013 |
| *NorA* | TTC- ACC- AAG- CCA- TCA- AAA- AG<br>CTT- GCC- TTT- CTC- CAG- CAA- TA | 620 bp | Pourmand et al., 2014 |
| *VanA* | CAT- GAC- GTA- TCG- GTA- AAA- TC<br>ACC- GGG- CAG- RGT- ATT- GAC | 885 bp | Patel *et al.*, 1997 |
| *VanB* | GTG- ACA- AAC- CGG- AGG- CGA- GGA<br>CCG- CCA- TCC- TCC- TGC- AAA- AAA | 433 bp | Kariyama *et al.*, 2000 |
| *MphC* | GAG- ACT- ACC- AAG- AAG- ACC- TGA- CG<br>CAT- ACG- CCG- ATT- CTC- CTG- AT | 722 bp | Schlegelova *et al.*, 2008 |
| *BlaZ* | TAC- AAC- TGT- AAT- TCG- GAG- GG<br>CAT- TAC- ACT- CTT- GGC- GGT- TTC | 833 bp | Bagcigil *et al.* 2012 |
| *E. faecalis 16S Rrna* | GTT- TAT- GCC- GCA- TGG- CAT- AAG- AG<br>CCG- TCA- GGG- GAC- GTT- CAG | 310 bp | Zoletti *et al.*, 2006 |

## RESULTS AND DISCUSSSION

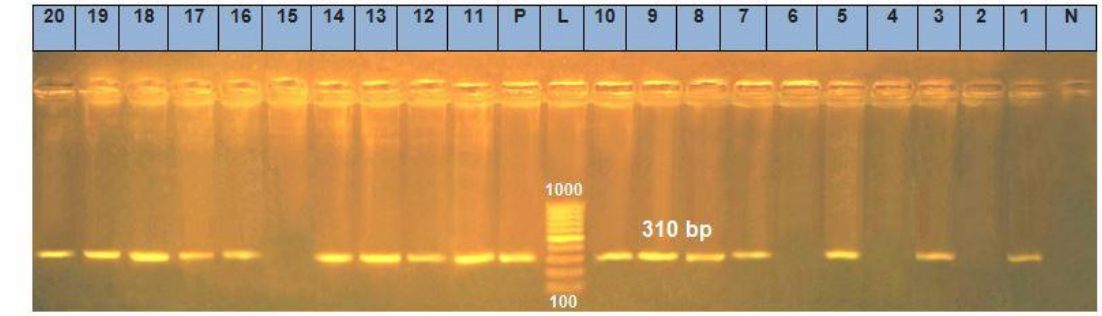
### Isolation and detection of enterococcus

300 samples of mastitic cow's milk were examined for this study, Enterococci were diagnosed depending on primary culture on the selective medium (bile esculine azide agar) and biochemical characteristics of *Enterococci.* Only 81 isolates were positive for *Enterococcus spp*. Concurring result (10) which isolates *E.spp.* as 31% from mastitis milk samples, in contrast with result in Iraq did not found *Enterococcus Spp*. from mastitis cases in cattle. Differential biochemical tests were carried out for all suspected isolates in order to investigate the enterococci to the species level and to exclude other bacterial species that are similar to them in some characteristics, so based on the results of the biochemical assays Table (2), 42 isolates were positive for *E.faecalis*.

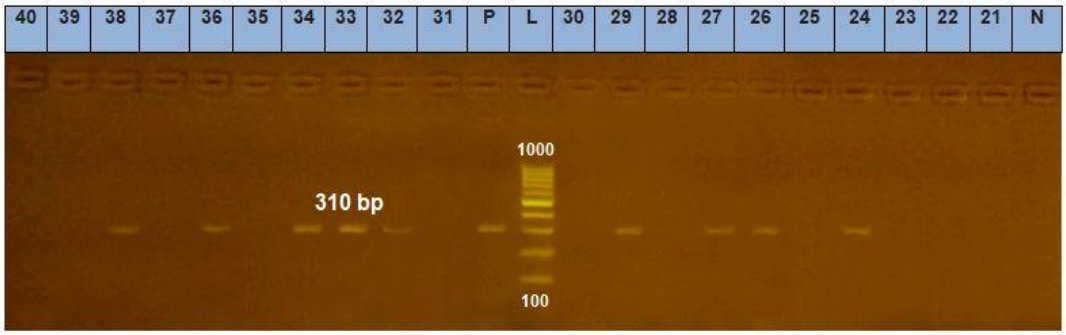
**Table 2. Biochemical tests for determination *E.faecalis* isolates from clinical mastitic cows milk**

| Test | Result | Test | Result |
|---|---|---|---|
| Catalase | - | Manitol | + |
| Grow in Temp. (45 C) | + | Ribose | + |
| Grow in concentration (6.5% NaCl.) | + | Sucrose | + |
| Grow in ( PH.=9.6-10) | + | Glycerol test | + |
| Grow in the presence of tolerite salts (0.04%) | + | Arabinose | - |
| Acid production from Sorbitol | + | Raffinose | - |
| $H_2S$ production and motility | - | Neolin | - |
| Oxidase | - | | |

Confirmatory test was conducted by PCR for identification *E.faecalis* by specific primers (*E.faecalis 16rRNA*), generated bands at the location of 310 bp on an agarose gel. as show in table (3), Figure (1), the results showed that only 25 out of 42 isolates were positive for *E.faecalis* by 8.3%, this results agreement with results of study in Turkey (14) which found *E.faecalis* from mastitic cow's milk samples by 11%. This result considered lower than study in Iraq (10) found *E.faecalis* as 67%, this disparity could be attributed to farm management, climate conditions, and the varying number of samples collected. Furthermore, environmental issues, such as the existence of a plumbing system in the vicinity of where milk samples were gathered, are believed to influence the results (19).




a




b

**Figure 1. Agarose gel photo documentation for *E.feacalis* molecular identification lane L molecular weight marker (100-1000bp), lane pos : positive control (at 310 bp), lane neg: negative control, lane 1, 3, 5, 7, 8, 9, 10, 11, 12, 13, 14, 16, 17, 18, 19, 20, 24, 26, 27, 29, 32, 33, 34, 36 and 38 are positive isolates**

**Table 3. The percentage of *E.faecalis* in the collected samples by PCR**

| Samples brands | Total Number of samples | *E.faecalis* isolation | |
|---|---|---|---|
| | | +Ve. | % |
| Milk of cows with mastitis | 300 | 25 | 8.3 |

**Antimicrobial sensitivity test**

The use of the disc diffusion technique was made to analyze antimicrobial susceptibility for all positive *E.faecalis* isolates in order to investigate antibiotic resistance. According to the inhibitory zone readings of various antibiotic discs. The findings revealed high levels of contamination with multi-antibiotics resistant *E. faecalis* strains of serious concern, with 32% of isolates resistant to vancomycin. Azithromycin, cephalosporin groups, and tetracycline had the highest levels of resistance, with 80%, 72%, and 68%, respectively. while less to Amoxicillin-clavulinc by 36%. As for Aminoglycosides: Gentamycin and streptomycin as 52%, 44% respectively and about 44%, 48% to each of chloramphenicol and florfenicol respectively, finally Quinolones: levofloxacin and ciprofloxacin 40% and 48% respectively Table (4). A study in Iraq conducted by Ghaidaa (9) found resistance to vancomycine by 25% which agreement with our result, and disagreement with Kim (13) in Korea who did not found resistance to vancomycin by 0%. Whereas study in Egypt by Ashraf (1) recorded high level of Enterococci resistance to vancomycin as 100%, a high rate of resistance to enterococci, which can be explained by the wide and inappropriate use of antibiotics in the treatment of mastitis infection (13). Ashraf (1) mentioned that no resistance to B-lactam family which is disagreement with our results. Rosa (19) recorded that *Enterococcus spp*. resistance to phenols by 44% which considered agreement with our results. Whereas study by Kim in Korea (13) found phenols resistance *E.faecalis* as 22% , which considered lower than these results, this same study confirmed that the prevalent use of Antibiotic in milking cows apparently led in significantly more antibiotic-resistant Enterococcus spp. being detected in bovine mastitis milk. Quinolones also can be used in enterococci infections. Some studies conducted quinolones resistance as study by Ghaidaa (9) mentioned that about 22% of *E. spp.* are resistance to quinolones which considered lower than our results. Generally, antibiotics resistance levels in enterococci typically vary by species, drug, and country(19).

**Table 4.The antibiogram of *E.faecium resistance* isolates from mastitis cows milk samples**

| Class | Antibiotic | Number of resistance *E.faecalis* isolates (25) | % |
|---|---|---|---|
| Glycopeptide | Vancomycin | 8 | 32 |
| Tetracycline's | Tetracyclin | 17 | 68 |
| Aminoglycoside | Gentamycin | 13 | 52 |
| | Streptomycin | 11 | 44 |
| Macrolides | Azthromycin | 20 | 80 |
| Quinolones | Levofloxacin | 10 | 40 |
| | Ciprofloxacin | 12 | 48 |
| Phenicol | Chlorfenicol | 11 | 44 |
| | Florfenicol | 12 | 48 |
| B-lactamase | Amoxicillin-Clavulinic | 9 | 36 |
| | Cefoxitin | 18 | 72 |

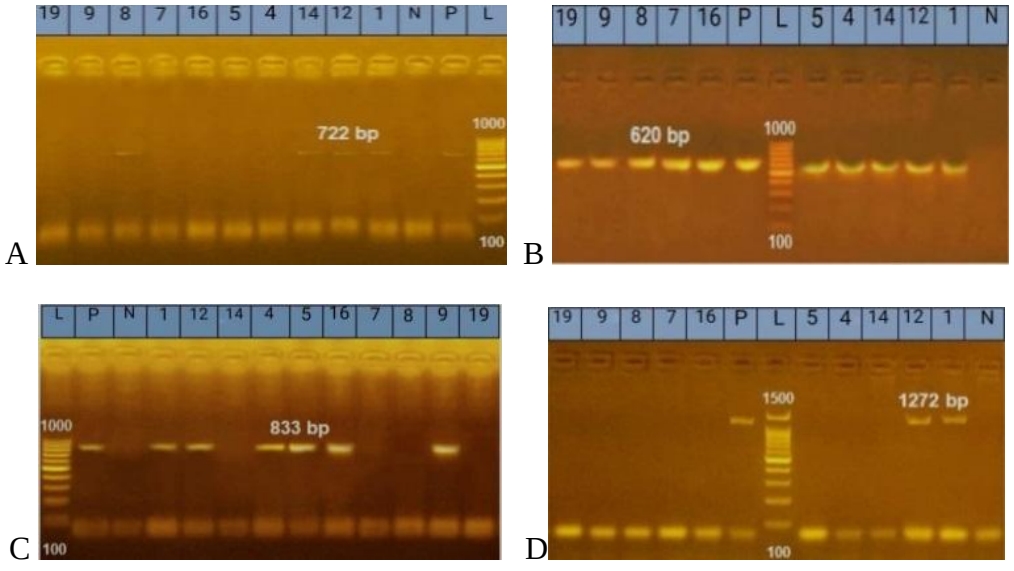
Through the results of the sensitivity test, the six most isolates that gave high level of multi drug resistance{MDR} were selected to investigate resistance genes in them by PCR, to identify the polymorphism causing MDR.

**Detection of resistance genes**

Tetracyclines' tetK gene was targeted for detection by PCR, *fexA* for chloramphenicol, *mphC* for macrolides, *norA* for quinolones, and *vanA* and *vanB* for vancomycin genes, *aac6* for aminoglycoside and *blaz* for B-lactam family in different antibiotic resistant *E.Faecalis*.Antimicrobial Resistance Genotypes by PCR, showed that all the *E.faecalis* isolates harbored at least 4 out of 8 antimicrobial resistance genes all have been checked, *tet*K, *nor*A, and *aac*(6')*aph* (2") genes are detected in all of six positive *E.faecalis* isolates as 100%. *van*A gene was detect in two *E.faecalis* isolates, *van*B gene was detect in only one of *E.faecalis* isolates. *blaz* gene was detect in five of *E.faecalis* isolates. *mph*C gene was detect in two *E.faecalis* isolates, and *Fex*A gene was detect in two *E.faecalis* isolates as show in table (5) and Figure 2 ( A, B, C, D, E, F, G and H).

**Table 5. Prevalence of *E.faecalis* and resistance factors in samples of mastitic cows milk**

| Sample | *E. faecalis* | blaZ | TetK | mphC | FexA | norA | aac(6')aph (2'') | VanA | VanB |
|---|---|---|---|---|---|---|---|---|---|
| 1 | + | + | + | + | + | + | + | - | - |
| 12 | + | + | + | + | + | + | + | + | + |
| 5 | + | + | + | - | - | + | + | - | - |
| 16 | + | + | + | - | - | + | + | - | - |
| 7 | + | - | + | - | - | + | + | + | - |
| 9 | + | + | + | - | - | + | + | - | - |




A B C D

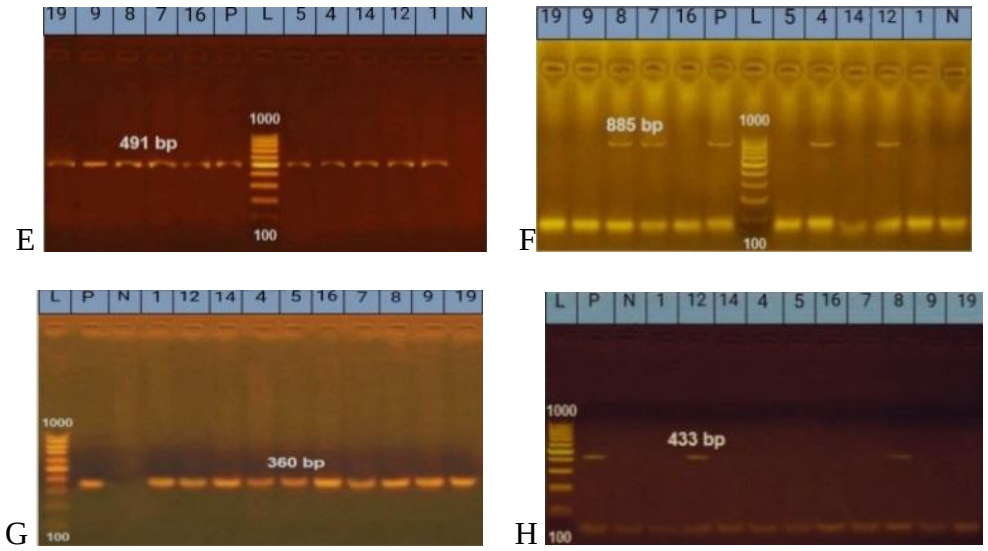



**Figure 2. PCR amplified products of *E. faecalis* electrophoresed on agarose gel *mph*C resistance gene at 722 bp.(A) , *E. faecalis norA* resistance gene at 620 bp. (B), *E. faecalis blaZ* resistance gene at 833 bp.(C), *E. faecalis fex*A resistance gene at 1272 bp.(D), *E. faecalis aac6* resistance gene at 491 bp.(E), *E. faecalis van*A resistance gene at 885 bp.(F), *E. faecalis* (*tet K*) resistance gene at 360 bp.(G), *and E. faecalis 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**

Sequencing results are analyzed by NCBI to know the genetic variation which show that the local genome of *E. faecalis* isolate is close is close to the global standard gene of *E.faeclis* by 99% in comparison with the previous isolates registered in the Gen Bank NCBI that gave similarity, while CP039729 in China , MF614915 in India and AF326472 in USA. are the furthest in the phylogenic tree by 61%, as show in Figures 3 and 4.

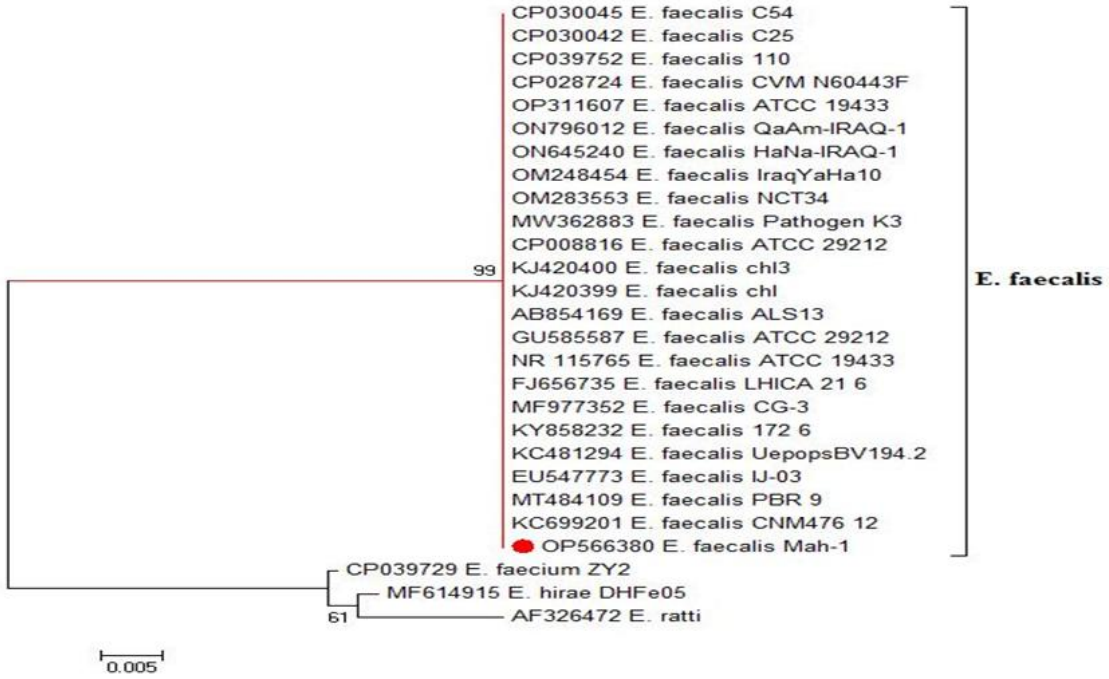



**Figure 3. Phylogenetic tree of local genome of *E.faecium* sequencing isolate from mastitic cows milk**

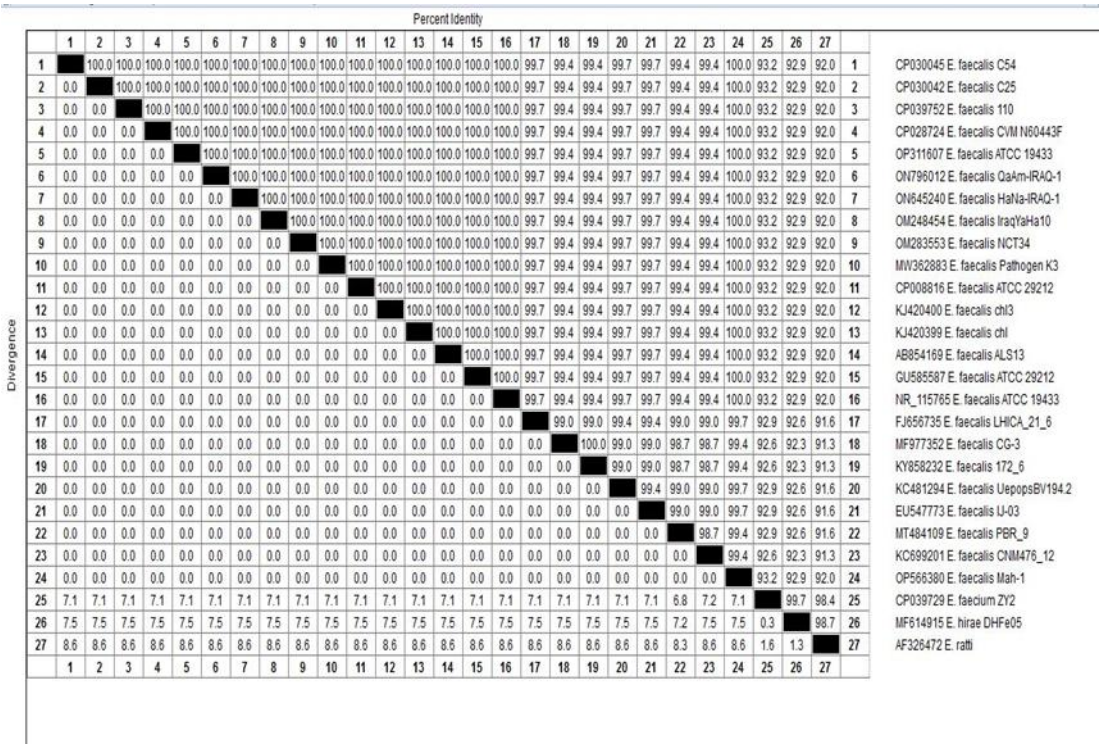



**Figure 4. Alignment of multiple sequences of the local genotype of *E.faecium* isolate from mastitic cows milk**

## CONCLUSIONS

This investigation showed that *E. faecalis* was frequently found in mastitic cow's milk, and it proved that *E. faecalis* is common contaminants in Kirkuk environment and fields, also confirmed the high rate of their antimicrobial resistance with vancomycin resistant which could pose a concern to people. Last but not least, E. faecalis isolated from clinical samples is developing resistance to a growing number of antibiotics as a result of improper antibiotic administration and inadequate treatment duration.

## CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

## DECLARATION OF FUND

The authors declare that they have not received a fund.

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