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Evaluation of antibiotic resistance and virulence factors of Enterococcus species isolated from clinical samples in Kirkuk city

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Abstract

The genus Enterococcus comprises a broad group of Gram-positive bacteria that are of great importance to human health due to their role as major causative agents of hospital-associated and community acquired infections. The presence of Enterococcus bacteria was investigated in (250) isolated samples from different clinical sources. The isolates were identified by cultural and biochemical tests, as well as using the DL-120 card system. The antibiotic resistance study was performed using the DL-120 card system. Virulence factor such as Gelatinase, proteinase, and hyaluronidase and biofilm formation were detected. Only 16 isolates belonging to the *E. faecalis* species and two isolates belonging to *E. faecium* were obtained by biochemical tests using the DL-120 system. Gelatinase were found in all 18 isolates and among the 18 Enterococci isolates assessed for biofilm production via Congo red agar (CRA), 14 isolates (77.8%) exhibited a positive result demonstrating their capacity for biofilm formation, whereas 4 isolates (22.2%) were negative, while proteinase, and hyaluronidase virulence factors were not detected. The results of the current study showed that Enterococci bacteria showed a high resistance to the Rifampicin, Trimethoprim / Sulfamethoxazole, Cefepime, Ceftriaxone and cefuroxime which MIC were (4,0.5/9.5,4,8,8) respectively, while all of them showed high sensitivity to other antibiotic in all clinical sources. The Enterococci were mostly isolated from urine then from wounds samples. The isolates were high resistance to the third-generation beta-lactam cephalosporin antibiotics. Gelatinase and biofilm production were most prevalent between Enterococcus spp.

Keywords: Enterococcus, Virulence factors, antibiotic resistance.

تقييم مقاومة المضادات الحيوية وعوامل الضراوة لأنواع المكورات المعوية المعزولة من عينات سريرية في مدينة كركوك

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الخلاصة:

يضم جنس المكورات المعوية مجموعة واسعة من البكتيريا موجبة الجرام، وهي ذات أهمية كبيرة لصحة الإنسان نظرًا لدورها كمسبب رئيسي للعدوى المكتسبة من المستشفيات والمجتمع .تم التحقق من وجود بكتيريا المكورات المعوية في (٢٥٠) عينة معزولة من مصادر سريرية مختلفة. تم تحديد العزلات من خلال الاختبارات الزرعيّة والكيميائية الحيوية، بالإضافة إلى استخدام نظام بطاقاتDL-120 . أجريت دراسة مقاومة المضادات الحيوية باستخدام نظام بطاقاتDL-120 . تم الكشف عن وجود عوامل الضراوة مثل الجيلاتيناز، البروتيناز، الهيالورونيداز، وتكوين الأغشية الحيوية. تم الحصول على ١٦ عزلة تنتمي إلى نوع *E. faecalis* وعزلتين تنتميان إلى *E. faecium* من خلال الاختبارات الكيميائية الحيوية باستخدام نظامDL-120 . وُجد الجيلاتيناز في جميع العزلات الثماني عشرة، ومن بين عزلات المكورات المعوية الثماني عشرة التي تم تقييمها لإنتاج الأغشية الحيوية عبر أجار الكونغو الأحمر(CRA) ، أظهرت ١٤ عزلة (٧٧.٨%) نتيجة إيجابية تُثبت قدرتها على تكوين الأغشية الحيوية، بينما كانت ٤ عزلات (٢٢.٢%) سلبية، بينما لم يتم الكشف عن عوامل ضراوة البروتيناز والهيالورونيداز. أظهرت نتائج الدراسة الحالية أن بكتيريا المكورات المعوية أظهرت مقاومة عالية للريفامبيسين، والتريميثوبريم/سلفاميثوكسازول، والسيفيبيم، والسيفترياكسون، والسيفوروكسيم، حيث بلغ الحد الأدنى لتركيز المثبط4) (MIC) ،0.5/9.5،4،8، (8على التوالي، بينما أظهرت جميعها حساسية عالية للمضادات الحيوية الأخرى في جميع المصادر السريرية. عُزلت المكورات المعوية بشكل رئيسي من عينات البول، ثم من عينات الجروح. أظهرت هذه العزلات مقاومة عالية لمضادات السيفالوسبورين بيتا لاكتام من الجيل الثالث. وكان إنتاج الجيلاتيناز والأغشية الحيوية أكثر شيوعًا بين أنواع المكورات المعوية.

الكلمات المفتاحية: المكورات المعوية، عوامل الضراوة، مقاومة المضادات الحيوية.

Introduction

The genus Enterococcus comprises a broad group of Gram-positive bacteria that are of great importance to human health due to their role as major causative agents of hospital-associated and community acquired infections. Enterococcus are resilient and versatile species capable of surviving harsh conditions [1]. The genus Enterococcus is one of the most widespread bacterial genera in nature. It can be found in a variety of habitats, including soil, plants, fresh and salt water, wastewater, food, and the gastrointestinal tract of animals, including mammals, birds, fish, reptiles, insects, and humans [2]. Enterococci, specifically Enterococcus faecalis and Enterococcus faecium, exhibit numerous virulence characteristics that enhance their capacity to induce infections. Factors such as adhesins, including aggregation substance (AS), collagen-binding protein (Ace), cell wall adhesin (Efa A), and enterococcal surface protein (Esp), facilitate colonisation and biofilm development. Furthermore, they produce toxins such as cytolysin (Cyl), gelatinase (GelE), and hyaluronidase (Hyl), which can harm host tissues[3]. *Enterococcus* is now well established as one of the major pathogens in

community and hospitals and is becoming increasingly resistant to antibacterial agents. Currently, most enterococcal infections in hospitals are caused by either *Enterococcus faecalis* or *Enterococcus faecium* [4]. *E. faecalis* is the most pathogenic species, but *E. Faecium* is of increasing importance because it is often more resistant to antibiotics [5]. Although Enterococcus is normally a commensal in the gastrointestinal tract, oral cavity, and vagina of humans, it has become an opportunistic pathogen, primarily causing infections in patients admitted to intensive care units, those with severe underlying disease, or those with immunodeficiency. Therefore, disease severity and immunosuppression can be directly related to prolonged hospitalization and/or indiscriminate antibiotic use, which are major risk factors for hospital-acquired drug-resistant *Enterococcus* [6]. Antibiotic treatment may lead to the creation of various niches, including the elimination of commensal gut microbes and their subsequent replacement by opportunistic pathogens[3]. *Enterococcus* can cause a variety of clinical infections, the most common of which are urinary tract infections (UTIs) or intraperitoneal infections (ITIs). Intraabdominal infections, rarely causing meningitis, osteomyelitis, septic arthritis, endocarditis, bacteremia, wound and burn infections, or pneumonia [7]. The discovery of antibiotics has had a significant impact on reducing the rates of infectious coccal infections, but the indiscriminate use of these antibiotics has led to the emergence of bacterial strains resistant to one or more antibiotics[8]. One of the important mechanisms that bacteria possess to resist beta-lactam antibiotics is the production of beta-lactamase enzymes, which attack the beta-lactam ring found in the nucleus of penicillin and cephalosporins, rendering the antibiotic ineffective. These enzymes are encoded by genes Carried on the chromosome or plasmid [9,10]. Therefore, our study was aimed to isolates enterococcus spp. from various clinical samples and diagnosing these isolates based on phenotype and chemical tests, then investigating some virulence factors and drug sensitivity to certain antibiotics and identifying the resistance patterns of enterococci to antibiotics.

Materials and Methods

Sample Collection

Two hundred and fifty different clinical samples were collected from patients attending and inpatients at Kirkuk Teaching Hospital and Azadi Teaching Hospital, as well as from external private laboratories in Kirkuk Governorate, for the period from 2 December 2024 to 30 May2025. The samples were collected

from all ages and both sexes. The patient's medical information was recorded on a form that included name, gender, age and medical problems.

Diagnostic methods

These isolates were identified based on cultural, microscopic and biochemical tests. Several cultural media including blood agar, MacConkey agar, and mannitol salt media were employed to assess colony morphology, including color, shape, texture, size, odor, and the appearance of colony edges. Additionally, the isolates' ability to produce hemolysin and cause hemolysis on blood agar was evaluated. Microscopic characterization was performed using Gram staining techniques. A set of biochemical tests, such as Catalase, oxidase, PYR, citrate, urease and Indole, was conducted for further identification of bacterial isolates. For confirmatory testing, the DL-120 strip test was ultimately utilized.

Virulence factor

Gelatinase, proteinase, biofilm and hyaluronidase virulence factors were detected according to collee et al.,[11].

Gelatinase test

Isolates were cultured on gelatinized medium and then incubated at 37°C for 18–72 hours. Liquefaction of the gelatin, even after 15 minutes at 4°C, is considered positive.

Detection of Proteinase Production

Isolates were activated on heart and brain infusion agar using the streaking method, then incubated at 37°C for 24 hours. A portion of the sample was then transferred to the medium using the streaking method, and the plates were then incubated at 37°C for 24 hours. The presence of lytic areas around the growing colonies indicates a positive test.

Detection of the bacterial ability for the biofilm production

The congo red agar method was used to detect the bacterial ability to produce the biofilm; this was performed by culturing the bacterial colony on the congo red agar plate, which then incubated at 37oC for 24 h. Black colonies indicates highly production of biofilm, red colonies indicates moderately production of biofilm, while light colonies means negative result.

Detecting Hyaluronidase Production

The initial screening for hyaluronidase activity typically involves detecting its ability to hydrolyze hyaluronic acid. This can be done using several methods, including the hydrolysis zone test: a common screening test used in microbiology laboratories to identify bacteria that produce hyaluronidase.

Results and discussion

All the samples were subjected to different culture media. It was found that out of a total of 250 samples, 129 samples (51.6%) were culture positive and 18 of them were Enterococci. The most isolated Enterococci were from urine sample(44.4%) followed by wounds samples(22.2%) as showed in table(1).

A study conducted by Rostamzadeh et al. [12] showed that most of the isolated Enterococci species were collected from urine samples (82.52%), followed by wound samples (7.69%), blood samples (5.59%), and other clinical samples. The results of a similar study conducted by Oskoui and Farrokh. [13] showed that more than 95% of the Enterococci species were isolated from urine samples, and less than 5% from wound, blood and other samples, which is consistent with the results of the current study .

Table(1): Positive Enterococcus growth in clinical specimen culture

Specimens	Enterococcus isolates
Urine	8(%44.4)
Wounds	4(%22.2)
Burns	2(%11.1)
Vaginal swabs	2(%11.1)
Throat swabs	1(%5.6)
Ear swabs	1(%5.6)
Total	18(%100)

Isolation and Identification of Enterococci

Culture Diagnosis

Enterococcus bacteria were identified based on their growth characteristics on various culture media. On azide blood agar, colonies appeared round, slightly

raised, with a smooth surface and white to cream-colored edges (Fig. 1A). This medium is highly suitable for the primary isolation of enterococci from clinical specimens because it contains sodium azide, which inhibits many Gram-negative bacteria while allowing Gram-positive organisms to grow. When cultured on MacConkey agar, enterococcal colonies were observed as small, smooth, round, and pink, reflecting their ability to ferment lactose (Fig. 1B). Findings from the initial isolation confirmed that MacConkey agar acts as a selective medium, generally inhibiting Gram-positive bacteria except for enterococci, which are resistant to bile salts and crystal violet [14]. On mannitol agar, yellow colonies formed as a result of mannitol fermentation, causing the medium to turn yellow (Fig. 1C). Importantly, *Enterococcus faecalis* is one of the few bacterial species capable of growing in high-salt conditions, which helps it survive and outcompete other microbial flora.

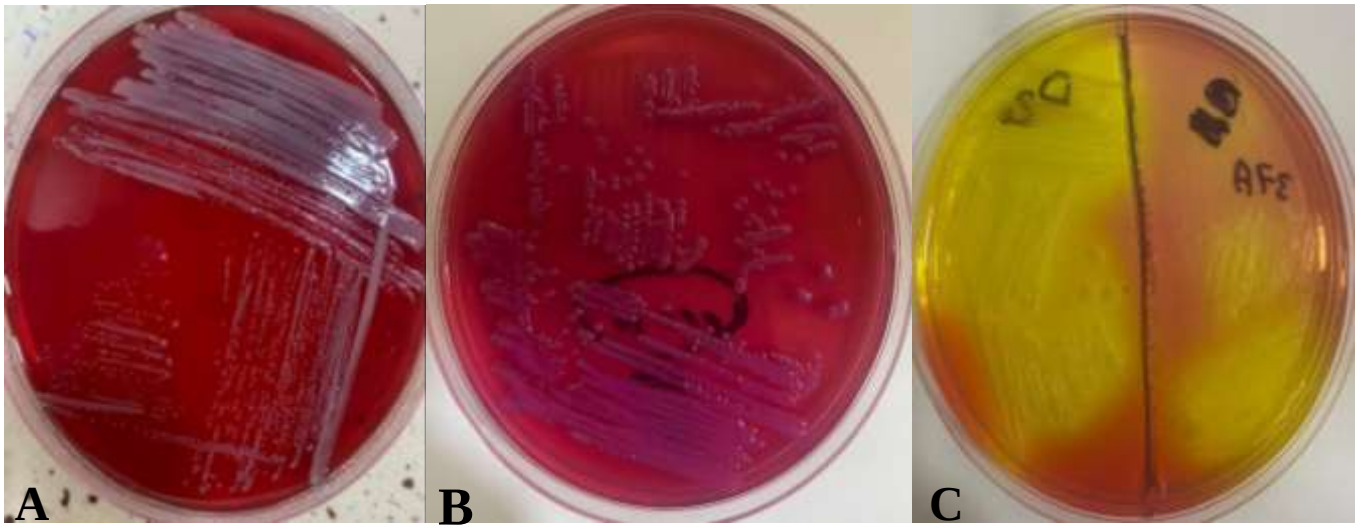
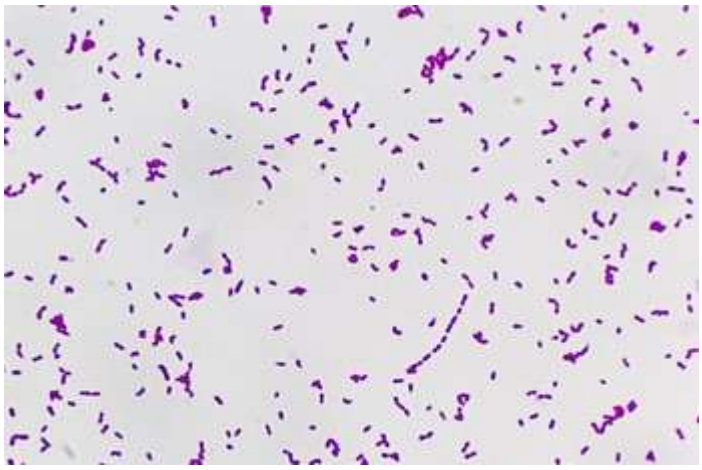


Figure (1): Enterococcal colonies on: A- Blood agar medium B- MacConkey agar C- Mannitol saline medium

Microscopic Diagnosis

Microscopic examination of smears prepared from bacterial colonies grown on solid media and stained using the Gram technique showed that the cells appeared as individual spherical, occasionally as elongated ovals, and were sometimes arranged in pairs or short chains, as illustrated in Figure 2.



Figure(2): Enterococcus colonies under the microscope

Biochemical tests

The bacterial isolates underwent a series of biochemical tests, which demonstrated that they do not produce catalase or oxidase enzymes, as evidenced by the absence of a purple color following the addition of reagent. They also tested negative in the urease assay, indicated by the medium remaining yellow without shifting to red, confirming that these bacteria do not utilize urea due to the absence of urease activity. Furthermore, the isolates were unable to reduce citrate and yielded negative results in the indole test. Conversely, the bacteria tested positive for the PYR assay, as detailed in Table 2.

Table(2): Biochemical tests for Enterococcus

Indole	urease	citrate	PYR	oxidase	Catalase
-	-	-	+	-	-

Figure (3): Biochemical tests for Enterococcus

Diagnosis using the DL-120

Bacterial isolates identified as *Enterococcus* spp. were further analyzed using the DL-120 card system, which contains specific reagents that react with the bacterial suspension to generate positive results, as shown in Figure 4. Following confirmation through this method, the results revealed that 16 isolates belonged to *E. faecalis*, while two isolates were identified as *E. faecium*.

Figure(4): Identification of Enterococcus spp. By using DL-120 strip

Antibiotic susceptibility

Different antibiotic groups and concentrations were used to demonstrate their effect on the Enterococcus spp. isolates (Table 3). Antibiotic resistance was performed using the DL-120 card system.

The results of the current study showed that Enterococci bacteria showed a high resistance to the Rifampicin, Trimethoprim / Sulfamethoxazole, Cefepime, Ceftriaxone and cefuroxime which MIC were (4,0.5/9.5,4,8,8) respectively, while all of them showed high sensitivity to other antibiotic in all clinical sources, as showed in table(3).

Table(3): Antibiotic susceptibility of Enterococci

Antibiotics	Minimum inhibitory concentration	Sensitivity
Levofloxacin	2	Sensitive
Nitrofuratoin	16	Sensitive
Penicillin G	1	Sensitive
Ampicillin	0.25	Sensitive

Vancomycin	0.5	Sensitive
Linezolid	2	Sensitive
Daptomycin	1	Sensitive
Erythromycin	0.5	Sensitive
Doxycycline	4	Sensitive
Rifampicin	4	Resistance
Teicoplanin	1	Sensitive
Gentamycin	500	Sensitive
Streptomycin	1000	Sensitive
Trimethoprim / Sulfamethoxazole	0.5/9.5	Resistance
Cefepime	4	Resistance
Ceftriaxone	8	Resistance
cefuroxime	8	Resistance
Tigecycline	0.12	Sensitive

The results obtained by Shareef and Abdullah [15] revealed that the enterococci showed resistance to Ceftriaxone and Cefotaxime. George et al., [16] found that the enterococci showed sensitivity to Ampicillin and Nitrofurantoin. Shahi and his group [17] also found that the enterococci were resistance to Gentamycin while sensitivite to Chloramphenicol and Nitrofurantoin. Ghalavand and his group [18] also found that the sensitivity rate to Vancomycin was high. The results of this study differed from what was reported in the study of Ibrahim and his group [19], as the bacteria were sensitive to Cefotaxime, Ceftriaxone, Ampicillin, and Nitrofurantoin, while the showed resistant to Vancomycin. The results of this study also conflict with what was reached by Saeidi and his group [20], who found a resistance of enterococci to Gentamicin, Chloramphenicol and Nitrofurantoin.

Antibiotic treatment of infections caused by enterococci is problematic due to their intrinsic low susceptibility to many frequently used antibiotics, including aminoglycosides and cephalosporins [21]. Furthermore, acquired resistance is easily acquired and transfers diverse resistance genes to clinically relevant bacteria, thus limiting therapeutic options in the treatment of enterococcal infections [22].

The results of this study showed that Enterococcus bacteria showed high resistance to beta-lactam antibiotics because it has Intrinsic resistance to

cephalosporins[23] . This may be due to several reasons, including the association of the resistance of Enterococcus bacteria to beta-lactam antibiotics (B) lactams in general with their production of beta-lactamase enzymes (β-lactamases), overexpression of penicillin-binding proteins (PBPS) or modifications thereof, which leads to the production of PBPS with less affinity for beta-lactam antibiotics[24].

Detection of Virulence Factors

The assessment of virulence factors in the bacterial isolates revealed that all *Enterococcus* strains exhibited gelatin-degrading activity, indicating the production of the gelatinase enzyme, as illustrated in Figure 5. These findings align with the study by Didem and Kuştimur [25], which reported that all *Enterococcus* isolates expressed gelatinase activity. Similarly, Hasan et al. [26] observed gelatinase production in 85% of their isolates. In contrast, other research reported lower prevalence rates, with one study detecting gelatinase in only 45% of *Enterococcus* isolates [27], while another found the enzyme present in just 27% of isolates [28].

Figure (5): Detection of Gelatinase-producing isolates on Gelatinase agar

In this study, no positive results were observed for the production of the enzymes proteinase and hyaluronidase, as depicted in Figure 6. These findings differ from those reported by Golińska et al. [29], who found that some isolates did produce hyaluronidase. Hyaluronidase is a protein associated with *Enterococcus faecalis* and plays a role in promoting toxin production by the bacteria, thereby increasing

tissue damage and inflammation, which significantly contributes to the pathogenicity and progression of *E. faecalis* infections [30].

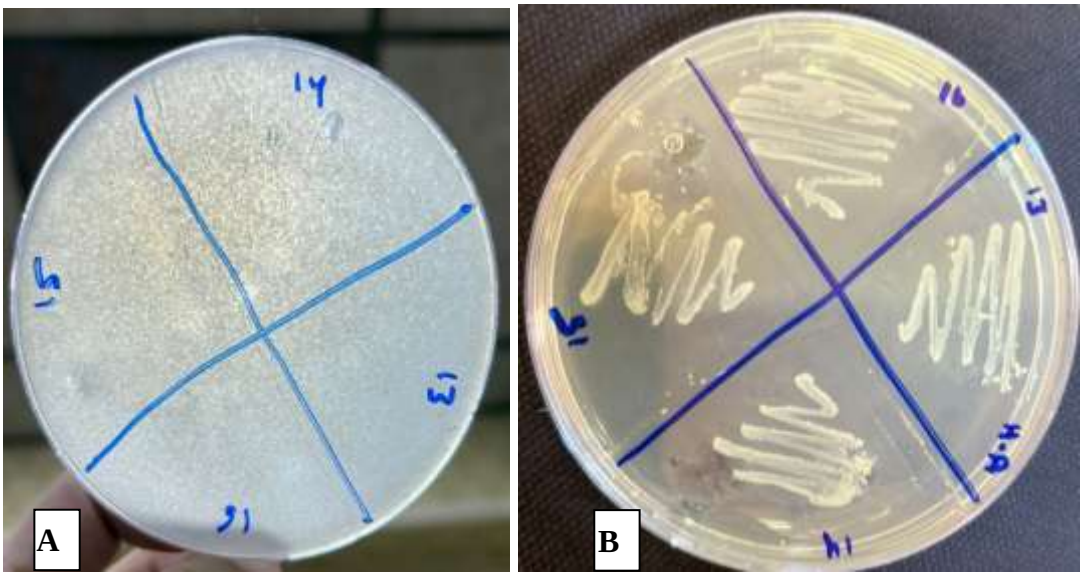


Figure (6): A- Testing the proteinase enzyme on milk medium B- Testing the hyaluronidase enzyme

In this study, among the 18 Enterococci isolates assessed for biofilm production via Congo red agar (CRA), 14 isolates (77.8%) exhibited a positive result as showed in figure(7), demonstrating their capacity for biofilm formation, whereas 4 isolates (22.2%) were negative. The elevated proportion of biofilm-positive isolates indicates that biofilm development is a prevalent characteristic among the studied Enterococci strains. Biofilm formation is a significant virulence determinant in Enterococci, as it facilitates bacterial survival, antibiotic resistance, and evasion of host immunological defences[31]. The Congo red agar method, while qualitative, serves as a straightforward and economical screening approach for detecting biofilm producers by colony morphology and colour alterations resulting from exopolysaccharide synthesis. The significant correlation between biofilm production and pathogenicity in clinical contexts underscores the relevance of our findings[32]. The existence of four non-biofilm-forming isolates may result from genetic variances, environmental factors, or constraints in the sensitivity of the CRA technique. These isolates may retain additional virulence pathways that enhance their clinical significance[33].

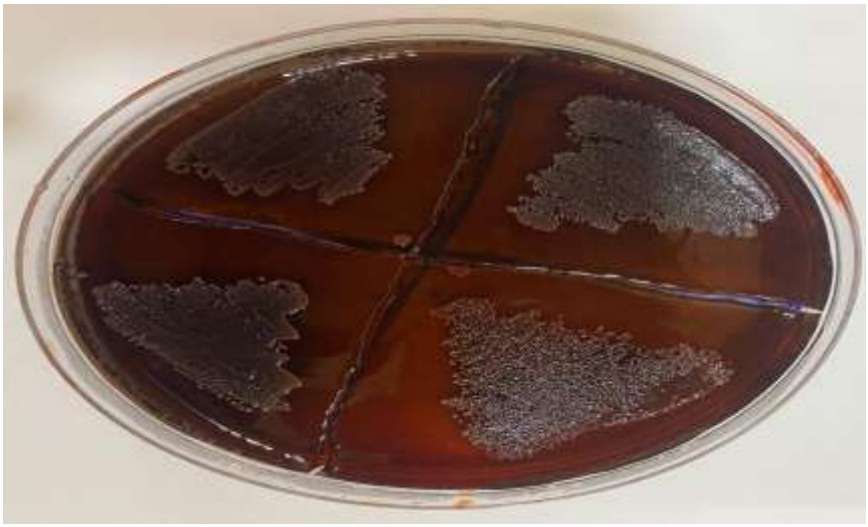


Figure (7): Biofilm production on Congo red agar

Conclusion

The Enterococci were mostly isolated from urine then from wounds samples. The isolates were high resistance to the third-generation beta-lactam cephalosporin antibiotics. Gelatinase and biofilm production were most prevalent between Enterococcus spp.

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