

Antibiogram and Biofilm Formation of *Enterococcus faecalis* isolated from women with urinary tract infection in Baqubah city, Iraq

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ABSTRACT

Enterococcus faecalis bacteria positive for Cram dye have a role causing urinary tract infections, and that 15% of urinary tract infections are caused by bacteria *E. faecalis*. The aims of this research are to determine the level of spread of *E. faecalis* and causes urinary tract infections in women, the rates of biofilm formation in local clinical *E. faecalis* isolates and investigate the relationship between multidrug resistances with biofilm forming ability. Between September 2023, and January 2024, 134 urine specimens were obtained from women patients in Baqubah teaching hospital, Al-Batool teaching hospital for maternity in Baqubah city. Twenty-four clinical isolates were diagnosed as *E. faecalis* by a percentage it reached 17.9%, Morphological, microscopical and molecular detection methods were used to diagnose the isolates. The capacity of *E. faecalis* isolates to form biofilm was examined using the micro-titer plate approach, the results demonstrated that all isolate produced biofilm. The method of disc diffusion was used to examine isolates sensitivity to antibiotics approach. The tested isolates exhibited 100% resistance to Erythromycin (E). while the rates of resistance ranged from Fosfomycin (FO), Rifampin (RV), Tetracycline (TE), Doxycycline (DO), Levofloxacin (LEV), Ciprofloxacin (CIP), Nitrofurantoin (F), Chloramphenicol (C), Vancomycin (VA), Ampicillin (AM), Teicoplanin (TEC) 95.83%, 83.33%, 83.33%, 54.16%, 50%, 41.7%, 25%, 20.83%, 16.7%, 12.5%, and 8.33%, respectively

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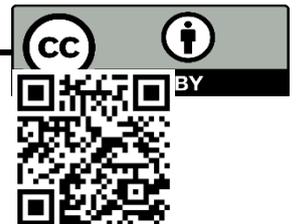
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1. INTRODUCTION

Enterococcus faecalis is a Gram-positive selective anaerobic bacterium that appear as solitary pairs or short chains of cocci under a microscope [1]. Among the various species found, *E. faecalis* and *E. faecium* are the most prevalent species capable of producing illness and posing an antibiotic resistance danger, with *E. faecalis* accounting for the bulk of infections. Enterococci are opportunistic pathogens, with various strains capable of causing community and hospital-acquired infections, particularly in immune weakened hosts. Endocarditis, bacteremia, and urinary tract infections (UTI) are examples of Enterococcal infections [2]. It also affects adolescent women and causes vaginitis, one of the most common venereal diseases [3]. *Enterococcus faecalis* or *Enterococcus faecium* are the most common cause of UTIs infections, followed by *Escherichia coli* [4]. It adheres to a lot of medical equipment in a hospital setting due to the fact that it can develop a biofilm by clinging to diverse surfaces [5]. A community of microorganisms stuck to a surface that offers durability and homeostasis in a changing environment is called a biofilm. Bacterial cells in contrast to their planktonic counterparts Biofilms are more susceptible to antimicrobials and environmental factor [6]. Bacteria's capacity to live in the host is largely dependent on their ability to form biofilms, which is also considered to be a key sign of their pathogenicity and causes dangerous chronic infections [7].

Antimicrobial therapy and innate host defense mechanisms are ineffective against infections caused by *E. faecalis* that are linked with biofilm. This is due to the fact that biofilm is necessary to the pathogenicity of *E. faecalis* [8]. The ability to form biofilms is among the prominent virulence properties of Enterococcus. This ability allows colonization of inert and biological surfaces while protecting against antimicrobial substances and mediating adhesion to host cells. Enterococci are known for their ability to form biofilms. These biofilms are populations of cells irreversibly attached to various biotic and abiotic surfaces and encased in a hydrated matrix of exopolymeric substances, proteins, polysaccharides, and nucleic acids. The biofilm structure provides an optimal microenvironment for growth and facilitates transmission of mobile genetic elements between bacteria [9]. The formation of biofilms by *E. faecalis* and the development of antimicrobial resistance are functionally related. This is due to the fact that the expression of the biofilm phenotype can be influenced by the development of antimicrobial resistance. In addition, the rise of multidrug-resistant (MDR) isolates in human diseases poses a significant challenge to the ability of medical practitioners to provide appropriate antibiotic treatment [10]. *E. faecalis* has intrinsic resistance mechanisms, such as the absence of antibiotic targets, low-affinity targets, impermeability to certain antibiotics, presence of efflux pumps, and lack of uptake mechanisms for various antimicrobials. Intrinsic resistance in *E. faecalis* is typically encoded in the chromosome and not easily transferable between bacteria, MDR in *E. faecalis* can arise from the acquisition of resistance genes through mobile genetic elements like transposons, integrons, and plasmids, as well as chromosomal mutations and antibiotic-modifying enzymes. Resistance genes can be transferred horizontally between closely related bacteria. The ability of *E. faecalis* to form biofilms enhances its resistance to antibiotics, posing challenges in treatment [11]. There has been a significant amount of interest in determining whether or if there is a connection between the biofilm-producing phenotype of bacteria and their multidrug-resistant (MDR) status [12]. Objectives of this work include determining the frequency of MDR phenotypes in *E. faecalis* isolates derived from clinical sources and determining the connection between MDR and the ability to build biofilm [1].

2. Experimental Methodology

2.1 Specimen's collection and culture

From September 2023 to January 2024, (134) actual cases of urinary tract infections were compiled from women at two major hospitals in Baqubah city, province of Diyala. Baqubah teaching hospital and Al- Batool teaching hospital for maternity and children, in addition visitors the specialized consulting clinic in Baqubah city. The isolates were initially identified through microscopical and cultural characterization on MacConkey agar, Blood agar and Pfizer selective agar. The plates were incubated aerobically at 37°C for 24 hours; all culture media were procured from Himedia/India.

2.2 Molecular diagnosis based *ddl* gene

Boiling method was used for genomic DNA extraction [13]. The concentrations and purity of the extracted DNA were estimated by Nano drop spectrophotometer at 260 and 280 nm [14]. Specific primer for *ddl* gene was used to identify *E. faecalis* Table 1. PCR was performed via reaction components were prepared by adding 5µl DNA template, 12.5µl of Go Taq® Green master Mix (2X) from Promega, 1µl from each forward and reverse *ddl* primers, and 4.5µl nuclease free water was added for completed volume to 25µl, Table 2 illustrate PCR conditions. PCR products were detected by gel electrophoresis (1.5 % agarose gel stained with ethidium bromide for 1 hr. at 60 V) than visualized by transilluminator.

Table 1. Primer sequence and size of the PCR product

| Primers | Primer sequences (5'— 3') | size (bp) | Reference |
|-----------------------|--|-----------|-----------|
| <i>ddl E.faecalis</i> | F: GGCAGAAGTGAAGAGCACGA R: CATGCGCTGGGATTTGCATT | 775 | [15] |

Table 2. PCR conditions

| Amplified gene | Initial denaturation | No. of cycles | Denaturation | Annealing | Elongation | Final extension |
|---------------------|----------------------|---------------|--------------|------------|------------|-----------------|
| <i>ddl faecalis</i> | 94°C/ 1 min | 35 | 94°C/1 min | 54°C/1 min | 72°C/1 min | 72°C/2min |

2.3 Biofilm detection by Micro titer plate method (MTP)

The production of biofilm of *E. faecalis* cultures were calculating in a micro-titer plate, according to procedures by Kerkeni et al. [16]. ELISA reader at 630 nm were used to observe the optical densities (ODs) of the plates.

2.4 Susceptibility of Antibiotics Examination

Antibiotics sensitivity was investigated toward 12 was using the Bauer-Kirby disc diffusion method, discs obtained from Bioanalyse/Turkey Table 3. The Antimicrobial Susceptibility Test (AST) was carried out on Muller Hinton agar plates (MHA) and the resistance of the isolates was determined according to clinical laboratory standards institute[17].

Table 3. Antibiotics were utilized on isolated samples in the investigation

| Antibiotic families | Antibiotics | Concentrations (μg) |
|---------------------|---------------------|----------------------------------|
| Fluoroquinolones | Ciprofloxacin (CIP) | 5 |
| | Levofloxacin (LEV) | 5 |
| Fosfomycin | Fosfomycin (FO) | 200 |
| Phenicols | Chloramphenicol (C) | 30 |
| Lipoglycopeptides | Teicoplanin (TEC) | 10 |
| Macrolides | Erythromycin (E) | 15 |
| Glycopeptides | Vancomycin (VA) | 30 |
| Penicillin | Ampicillin (AM) | 10 |
| Ansamycins | Rifampin (RV) | 5 |
| Nitrofurans | Nitrofurantion(F) | 300 |
| Tetracycline | Tetracycline (TE) | 30 |
| | Doxycycline (DO) | 30 |

3. RESULTS AND DISCUSSION

3.1. Identification of *E. faecalis*

The results of morphological, microscopic, biochemical, and genetic techniques shown, there were 24(17.9%) isolates of *E. faecalis* among 134 samples (urine) over the investigation period. All *E. faecalis* isolates appeared capacity to produce esculin on Pfizer selective agar, non-lactose fermentation on MacConkey agar medium, and on blood agar medium appeared as gray colonies with variable hemolysis ability. Also, the isolates shown positive results in the *ddl* gene output for diagnostic molecularly Figure 1. Current study shown an increased prevalence of *E. faecalis* were found which causes UTIs in women.

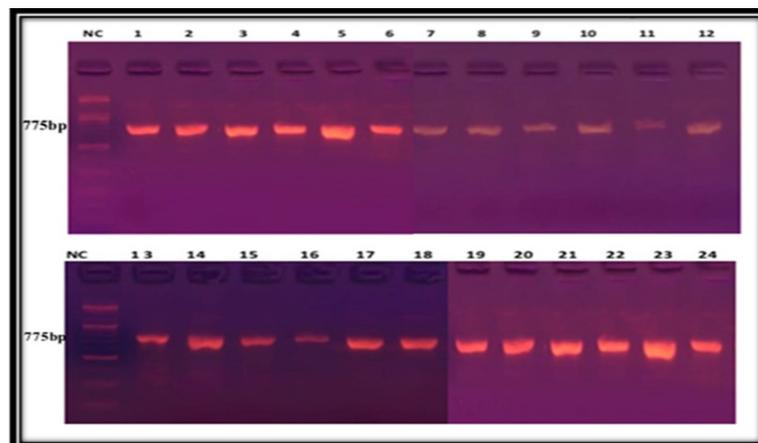


Figure 1. Gel electrophoresis of the *ddl* gene result (775 bp) at a voltage of 60 V, a current of 100 mA, along with an exposure duration of 60 minutes.

3.2. Identification of Biofilm Development

The results indicated that all 24(100%) isolates were biofilm generators to varying extents when compared to the negative control, as shown in Table 4. In addition to our biofilm-producing isolates, 66.7% were classified as strong producers and 33.33% as moderate producers. This result aligns with the work by Hashem et al [18] in Baghdad, which shown that all *E. faecalis* isolates were capable of producing a biofilm. Where was reported 62%, 25% and 13% of isolates as strong, moderate and weak respectively. Discrepancies in results between studies may be attributed to differences in the type of medium utilized, variations in incubation periods (as extended incubation can enhance cellular density of biofilms), or differences in sample size. biofilm contribute In the bacteria defense against the host's immune system, its aggregates nutrients and protects them from phagocytic cells and antibiotic assaults [19].

Table 4. Biofilm production

| Biofilm | Number of isolates | Percentage(%) |
|----------|--------------------|----------------|
| Strong | 16 | 66.7% |
| Moderate | 8 | 33.33% |
| Total | 24 | 100% |

3.3. Antibiogram profile

A schematic illustration of the outcomes from the antibiotic susceptibility test presented in Table 5. In the investigation, the results showed a discernible difference in the way that the bacterial isolates responded to the antibiotics provided.

Table 5. Antibiogram of 12 different antibiotics against *E. faecalis*

| Antibiotics | Susceptibility percentage % | | |
|-----------------|-----------------------------|--------------|-----------|
| | Resistance | Intermediate | Sensitive |
| Erythromycin | 100 | 0 | 0 |
| Fosfomycin | 95.83 | 4.1 | 0 |
| Tetracycline | 83.33 | 12.5 | 4.1 |
| Rifampin | 83.33 | 4.1 | 12.5 |
| Doxycycline | 54.16 | 8.3 | 37.5 |
| Levofloxacin | 50 | 8.3 | 41.7 |
| Ciprofloxacin | 41.7 | 8.3 | 50 |
| Nitrofurantion | 25 | 25 | 50 |
| Chloramphenicol | 20.83 | 33.33 | 45.83 |
| Vancomycin | 16.7 | 50 | 33.33 |
| Ampicillin | 12.5 | 0 | 87.5 |
| Teicoplanin | 8.33 | 20.83 | 70.83 |

The isolates exhibited a resistance Erythromycin rate of 100%, this aligns with the findings of Khalid et al [20] in Duhok, which indicated a resistance Erythromycin rate of 96% by *E. faecalis*. Also, the isolates under study appeared resistance toward Fosfomycin reach 95.83%. The findings do correspond with Rostkowska et al[21]the result, which reached Fosfomycin resistance via *E. faecalis* at 100%.

The isolates exhibited resistance to Tetracycline 83.33%, this result aligns with the findings of Khalil et al [22] in Saudi Arabia, which reported a resistance Tetracycline rate of 86%. Also, the resistance to Rifampin was 83.33%, which aligns with the findings of Kahdem [23] in Baghdad, result indicating resistance Rifampin 87%. Resistance to Doxycycline reached 54.16%, this result aligns with the findings of Malik et al[24], which reported a Doxycycline resistance of 48%. Like that Levofloxacin resistance seen here was 50%, which converged with the findings of Georges et al [25], shown a Levofloxacin resistance rate of 54%. The resistance of *E. faecalis* toward Ciprofloxacin reached 41.7%, closely aligning with the 45% Ciprofloxacin resistance reported by Saraswathy et al [26] in India.

The findings resistance to Nitrofurantion indicated that 25% of the isolates exhibited, which the results of closely aligning with the Kahdem study [23]that found 25% of *E. faecalis* resistant. The isolates exhibited resistance toward Chloramphenicol 20.83%, while study of Al-Taie [27] in Baghdad reported a resistance rate of 15%. The resistance toward Vancomycin was 16.7%, also the study of Kahdem [23] reported that Vancomycin resistance at 15%. The resistance to Ampicillin was 12.5%, which aligns with the findings of Salaam et al. [28] where the Ampicillin resistance was 14%. The resistance to Teicoplanin was 8.33%, closely aligning with the Teicoplanin resistance reported by Jalal et al. [29] in Duhok 10%.

3.4. Multi-drug resistance (MDR)

The findings of the present investigation indicated that 23 (95.83%) of the isolates exhibited multiple resistance to between 3 and 7 antibiotics, including (4) isolates demonstrating broad resistance, accounting for (16.7%), while 1 isolate (4.16%) displayed resistance to two antibiotics as exhibited in Table 6 and illustrated in Figure 2. This is an indicator of the risk of widespread multi-drug resistance among *E. faecalis* local isolates, making the challenges of treatment and recovery complex.

Table 6. The antibiotic resistance of *E. faecalis* isolates.

| The antibiotics that resist | No. of resistant isolates % | | Isolates numbers |
|--|-----------------------------|-------|---|
| (2) LEV, E | 1 | 4.1% | E ₂₁ |
| (3) E, FO, TE | 2 | 8.3% | E ₂₂ , E ₂₄ |
| (4) E, FO, TE, RA | 4 | 16.7% | E ₃ , E ₁₈ , E ₁₉ , E ₂₃ |
| (5) E, FO, TE, RA, DO | 1 | 4.1% | E ₄ |
| (6) E, FO, TE, RA, DO, LEV | 8 | 33.3% | E ₂ , E ₆ , E ₇ , E ₈ , E ₁₄ , E ₁₅ , E ₁₆ , E ₁₇ |
| (7) E, FO, TE, RA, DO, LEV, CIP | 4 | 16.7% | E ₁₁ , E ₁₃ , E ₂₀ |
| (8) E, FO, TE, RA, DO, LEV, F, CIP, C | 2 | 8.3% | E ₅ , E ₉ , E ₁₂ |
| (6) LEV, E, FO, CIP, TE, RA | 1 | 4.1% | E ₁ |
| (11) E, FO, TE, RA, DO, LEV, F, CIP, AM, VA, TEC | 1 | 4.1% | E ₁₀ |

Ciprofloxacin(CIP),

Levofloxacin(LEV), Fosfomycin(FO), Chloramphenicol(C), Teicoplanin(TEC), Erythromycin(E), Ampicillin(AM), Vancomycin(VA), Rifampin(RV), Nitrofurantion(F) Tetracycline (TE), Doxycycline(DO).

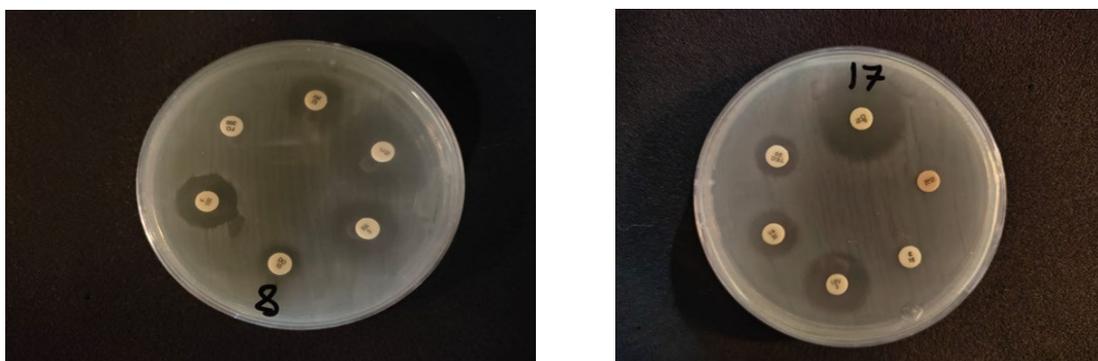


Figure 2. Assessing the Antibiotic against *E. faecalis*

3.5. Dendrogram analysis

The dendrogram in Figure 3 illustrates *E. faecalis* antibiotic sensitivity profile in this investigation, revealing two main groups, 1 and 2, with a similarity rate of 37%. The initial group comprised one isolate, isolate 21, which exhibited resistance to the antibiotics FO, TE, RA, LEV and CIP while demonstrating sensitivity to the antibiotics VA, AM, F, DO, TEC, and C. The second main group had 23 isolates categorized into three groups: group A contained 3 isolates, group B contained 18 isolates, and group C included 2 isolates. The similarity ratio among the three groups was 62%. Group A was divided into two subgroups, A1 and A2, which shown 78% similarity. The first subgroup, A1, comprised isolates 12 and 15, which had comparable resistance to antibiotics E, FO, RA, F, DO and C, although their sensitivity to other antibiotics differed. VA, TEC, CIP, TE, LEV; the second group A2 had one isolate, number 7, exhibiting resistance to E, C, FO, F, and VA. Group B was sub divided into two subgroups, B1 and B2, with a similarity rate of 67%.

Subgroup B1 comprised four clones and seven single isolates, each exhibiting a distinct resistance pattern: 4, 5, 6, 9, 17, 18, and 20. The first clone comprised isolates 13 and 11, exhibiting the resistance pattern E, FO, TE, RA, DO, CIP, LEV. The second clone consisted of three isolates 1, 12 and 14 demonstrating resistance solely to antibiotic E. The third clone included isolates 22 and 24, which displayed the resistance pattern E, FO, TE. The fourth clone encompassed three isolates, 3, 19, and 23, each exhibiting the resistance pattern E, FO, RA, TE, while their sensitivity varied. The four clones to antibiotics LEV, TEC, DO, C, F, AM, VA, CIP. Subgroup B2 included only one isolate 8 that carry resistance to antibiotics RA, CIP, F, C, AM, TEC and sensitive to antibiotics E, FO, TE, DO, LEV, VA. Group C was divided into other sub group C1 and C2 the similarity between them was 90%. Subgroup C1 included one single isolate (10) carrying the pattern of resistance to antibiotics TEC, DO. Subgroup C2 included one isolates (16) with antibiotics resistance pattern; E, FO, RA, TE, LEV, CIP. The results of the current study showed a great diversity among the local isolates of *E. faecalis* that cause urinary tract infection to women in terms of their resistance to antibiotics, which is due to the presence of several pathogenic strains of this bacterium that are common in society and health institutions.

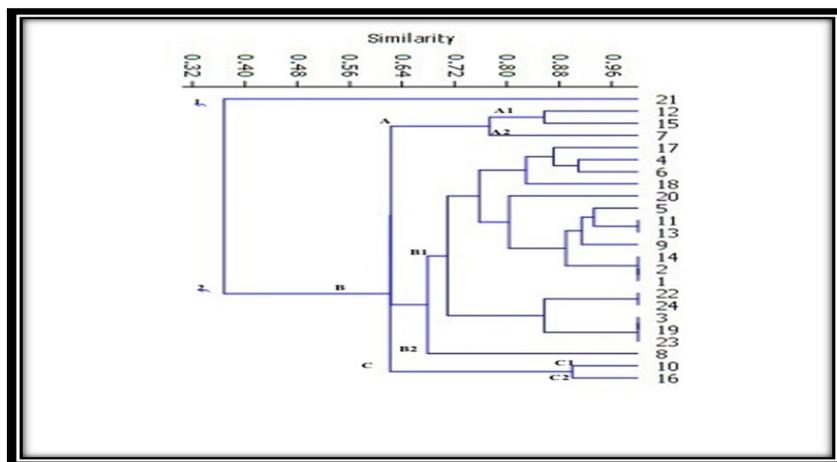


Figure 3. Dendrogram for *E. faecalis* antibiotics resistance pattern

4. CONCLUSION

E. faecalis local isolates showed high resistance toward the Erythromycin, Fosfomycin, Tetracycline and Rifampin while the most effective antibiotics studied against these bacteria was Teicoplanin, Ampicillin and Vancomycin as most of *E. faecalis* isolated from UTIs were sensitive to these antibiotics. The study showed that there is a strong association between the resistance of isolates to antibiotics and their production of the biofilm, as isolates was multi-drug resistant and shown ability to biofilm producers at relatively high levels.

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