

“Biofilm Formation Among Clinical Urinary Isolates of Enterococcus Species: A Comparative Evaluation of Tissue Culture Plate, Congo Red Agar and Tube Methods in a Tertiary Care Hospital”.

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Abstract

Background:

Enterococci are important causes of urinary tract infections (UTIs), particularly among hospitalized patients. Their ability to form biofilms contributes to persistence in the urinary tract and may make infections more difficult to treat. Reliable detection of biofilm production is therefore important in clinical microbiology.

Objective

This study aimed to determine the prevalence of biofilm formation among urinary Enterococcus isolates and to compare the performance of the Tissue Culture Plate (TCP), Congo Red Agar (CRA), and Tube

INTRODUCTION

The term “*Enterococcus*” has its origin at the end of the 19th century. Thiercelin described a saprophytic coccus of intestinal origin capable of causing infection. [1] Although more than 60 species in genus *Enterococcus* have been recognized, most common species are *E. faecalis* followed by *E. faecium*. [2] Enterococci are opportunistic pathogens, capable of causing community and hospital acquired infections. [1]

The infection caused by *Enterococcus* are Urinary tract infections, endocarditis, bacteremia especially in patients with intravenous catheter, liver abscess, intra-abdominal abscess, meningitis in neonates, central nervous system infection in adults, rarely osteomyelitis and pulmonary infection. The most common infection caused by Enterococci are urinary tract infections. Enterococcal species are the second most common cause of urinary tract infection. [3, 4] UTIs can be classified as either uncomplicated or complicated. An uncomplicated or simple UTIs can be defined as an infection in the lower urinary tract system in either a male or non-pregnant female patient. Complicated UTIs are associated with atypical organisms, patient considered high risk, pregnancy, comorbidities, immunosuppression or involve in the upper urinary tract. [5]

Enterococci are an opportunistic pathogen that may cause serious infection in immunocompromised individuals, as well as those receiving broad-spectrum antibiotic treatment or those requiring extended hospitalization. It is known for the ability to form biofilms. [6]

Biofilm formation by *Enterococcus* has been observed and studied over the last 40 years. [5] Biofilms are populations of cells irreversibly attached to various biotic and abiotic surfaces and hydrated matrix of exopolymeric substances, proteins, polysaccharides and nucleic acid. The biofilm formation is a multifactorial process that enables evasion of the host defenses and enhances bacterial virulence and antibiotic tolerance. [6, 8]

Biofilm protects Enterococci from host immune response and antibiotics. Biofilm -producing *Enterococcus* cause recurrent, chronic and antibiotic- resistant infections. Based on the national Institute of Health data, 80% of infections are related to biofilm- forming microbes. [5] Although biofilms do form on the anatomical surfaces within the urinary tract, entry into bladder is met with potent innate defenses, including neutrophil influx and epithelial exfoliation which prevent colonization of the surface by the invading microbes. In contrast, catheter surfaces have no inherent defense mechanisms. Presence of biofilms on the intra- luminal surfaces of in-dwelling catheters and urinary stents is a major cause of concern due to increased duration of hospitalization and increased morbidity for the patient. [6, 7]

Ch'ng et al. state that *Enterococcus faecalis* biofilm generation happens in four different stages: initial attachment, maturation, dispersal, and microcolony development. Proteases, glycolipids, and surface adhesins (aggregation material and Enterococcal surface protein) all aid in attachment. *E. faecalis* bacteria proliferate and secrete a biofilm matrix that forms microcolony aggregates after they are firmly adhered. Extracellular matrix components, including extracellular DNA, polysaccharides, lipoteichoic acid, extracellular proteases, modified lipids, and glycoproteins, are then produced as this biofilm matrix evolves. The microcolony experiences environmental stress as it develops and evolves because to local nutritional deficiencies, crowding, hypoxia, and waste accumulation. The ensuing stress reaction causes a change in gene expression from maturation to dispersal, which is typically thought of as the last stage of biofilm formation. During dispersal, the microcolony wall is broken and the biofilm's core liquefies, allowing individual bacteria to break off and start new colonies. Although *E. faecium* and *E. faecalis* have similar biofilm production processes, they are not the same. Despite producing biofilm, *E. faecalis* and *E. faecium* have different defense mechanisms. While *E. faecium* creates a thin film that contains genes for antibiotic resistance, *E. faecalis* produces a thick film that is hard to penetrate. [5, 8]

Biofilm formation plays an important role in the pathogenesis of *Enterococcus* by enabling the organisms to adhere to surfaces and persist within the host. Several virulence-associated factors contribute to this process. These factors can broadly be classified into secreted components, such as cytolysin, gelatinase, and secreted antigens, and surface-associated components, including pili, microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), and aggregation substances (ASs). The development of biofilm is also influenced by quorum sensing, a cell-density-dependent communication process through which bacterial cells coordinate the expression of specific genes involved in biofilm formation and other virulence-related functions [9, 10].

MATERIALS AND METHODS

Methodology:

A laboratory based cross sectional study conducted in the department of microbiology, in a tertiary care hospital total Mysore. Of 133 urine samples were collected from patients suffering from uncomplicated and complicated UTIs. A detailed history with reference to name, age, sex, clinical information including chief complaints, predisposing factors and any previous history of treatment were recorded on a pre designed proforma. Patients were advised to collect clean catch midstream urine in a sterile, dry, wide necked, leak proof container. From catheterized patients, the urine sample was collected after 30 minutes of clamping the catheter and then urine was aspirated under all aseptic precautions by inserting a syringe and needle proximal to the site of clamping. The samples were processed for isolation and identification of Enterococci.

Ethical clearance was obtained by institutional ethics committee (IEC) of JSS Medical College, JSSAHER.

Processing the sample

On receiving the sample, Urine specimens were first examined for their macroscopic characteristics, followed by wet mount microscopy to identify inflammatory cells. The samples were cultured on chromogenic Uri Chrome agar and incubated at 37°C for 24 hours to isolate *Enterococcus* species. Preliminary identification was based on colony morphology and Gram staining, which showed Gram-positive, oval cocci in pairs with a spectacle-like appearance. The isolates were catalase-negative and produced blackening on bile esculin agar. For species-level identification, sugar fermentation tests were used. *E. faecium* ferments arabinose distinguishes it from *E. faecalis* which does not ferment arabinose sugar. Then the Enterococcal isolates were subjected to antibiotic susceptibility testing on Muller Hinton agar by Kirby Bauer's disc diffusion method and interpreted according to CLSI guidelines.[11]

Screening for Enterococcal biofilm production

Biofilm production was evaluated using three different methods: Tissue culture plate method, tube method and the Congo red agar method.

Tissue culture plate method (TCP) [12, 13, 14]

Tissue culture plate (TCP) method described by Christensen's et al., 1995 is considered as the gold standard method for detection of biofilm formation. *Enterococcus* isolates were tested for their ability to produce biofilms using a quantitative microplate assay. A 0.5 McFarland suspension of the overnight cultures of *Enterococcus* strains were prepared. To each well of 96-well-flat bottom tissue culture plates were filled with 180µl of trypticase soy broth (TSB) + 0.5% glucose were added along with 20µl of 0.5 McFarland suspension of the isolates, and then incubated at 37°C with 5% CO₂ for 24hours. Next, the medium was discarded, and microtiter plates were gently washed three times with 300 µl of sterile phosphate buffer saline (PBS) to remove planktonic cells. Then, 150 µl of 99% methanol were added to each well for 20min to fix biofilm biomass. Later, the methanol was removed, and the plates were left to dry in room temperature and then. 150 µl of 0.1% crystal violet were added to each well for 20min. Excess stains were removed from the plates using sterile distilled water and the plates were located at room temperature for 30mins. The dye bounded to the adherent cells was dissolved using 150µl of 33% glacial acetic acid for each well. The optical density (OD) was measured using ELISA reader (BIORAD 680) at a wavelength of 550nm. The OD values were calculated for all the tested strains and negative controls; the OD cut-off value (ODc) were established.

It is defined as three standard deviations (SDs) above the mean OD of the negative control: ODc = average OD of negative control + (3 x SD of negative control). Final OD value of a tested strain was expressed as average OD value of the strain reduced by ODc value (OD=average OD of a strain-ODc); ODc value were calculated for each microtiter plate separately. When a negative value was obtained, it is presented as zero, while any positive value indicated biofilm production. For interpretation of the results, strains were divided into the following categories.

- Nonbiofilm producer (0) = OD ≤ ODc,
- Weak biofilm producer (+ or 1) = ODc < OD ≤ 2 x ODc
- Moderate biofilm producer (+ + or 2) = 2 x ODc < OD ≤ 4 x ODc
- Strong biofilm producer (+++ or 3), 4 x ODc < OD

Congo red agar method [12, 13, 14]

Freeman et al. (1989) described a simple qualitative method for detecting biofilm formation using the Congo Red Agar (CRA) method. This method utilizes a specialized medium composed of Brain Heart Infusion (BHI) broth with sucrose and Congo red, with the following composition: BHI – 37 g/L, sucrose – 50 g/L, agar – 10 g/L, and Congo red – 0.8 g/L. The Congo red stain was prepared as a concentrated aqueous solution and autoclaved separately at 121°C for 15 minutes. It was then added to the medium after the agar had cooled to 55°C. The plates were inoculated and incubated aerobically at 37°C for 24 hours. Biofilm production was indicated by black colonies with a dry, crystalline appearance. An indeterminate result was indicated by darkening of the colonies without the presence of a dry, crystalline morphology, whereas weak or non-biofilm-producing strains formed pink-colored colonies.

Representative Congo Red Agar results are shown in Fig. 03.

Tube method [12, 13, 14]

This is a qualitative method used for the detection of biofilm as described by Christensen et al., in 1995. 5ml of Trypticase soy broth were inoculated with a loopful of microorganism from overnight culture plates and incubated for 24hours at 37°C. The tubes were decanted and washed with phosphate buffer saline (Ph 7.2) and were allowed to air dry. Tubes were then stained with 0.1% crystal violet for 10min and washed with deionized water. The tubes were left to air dry in inverted position.

The representative appearance of the Tube method is shown in Fig. 02.

The scoring of biofilm formation was performed on the control strains used. Biofilm formation was considered positive when a visible film lined the sides and bottom of each tube. Ring formation at the liquid interface were not indicative of biofilm formation and was interpreted as negative. Tubes were examined and the amount of biofilm formation were scored as strong, moderate, weak and nonbiofilm.

RESULTS

Biofilm formation was evaluated in all 133 urinary *Enterococcus* isolates using three phenotypic methods: the Tissue Culture Plate (TCP) method, Tube Method, and Congo Red Agar (CRA) method. Isolates that exhibited biofilm formation by any of these methods were classified as biofilm producers. Tissue culture method was considered the gold standard phenotypic test for the detection of biofilm. Of the 133 patients with Enterococcal urinary tract infections, 99(74.43%) were infected with *Enterococcus faecalis* and 34(25.56%) were infected with *Enterococcus faecium*.

The distribution of biofilm production by the TCP method is presented in Table 01.

Table 01
Screening of 133 Enterococcal urinary isolates for Biofilm production by TCP method.

BIOFILM CATEGORIZATION	<i>Enterococcus faecalis</i> (n = 99)	<i>Enterococcus faecium</i> (n = 34)	TOTAL (n = 133)
Strong biofilm producers	5 (5.05%)	2 (5.88%)	7 (5.26%)
Moderate biofilm producers	33 (33.33%)	1 (3.12%)	34 (25.56%)
Weak biofilm producers	28 (28.28%)	6 (17.64%)	34 (25.56%)
Non-biofilm producers	33 (33.33%)	25 (73.52%)	58 (43.60%)
Total	99 (74.44%)	34 (25.56%)	133(100%)

Out of 133 Enterococcal urinary isolates, 7 (5.26%) were strong biofilm producers, with 5 isolates (5.05%) being *E. faecalis* and 2 (5.88%) *E. faecium*. Moderate biofilm production was observed in 34 (25.56%) isolates, of which 33 (33.33%) was *E. faecalis* and 1 (3.12%) was *E. faecium*. 34 (25.56%) isolates showed weak biofilm formation, of which 28 were (28.28%) *E. faecalis* and 6 were (17.64%) *E. faecium*. The remaining 58 isolates (43.60%) were non-biofilm producers, consisting of 33 (33.33%) *E. faecalis* and 25 (73.52%) *E. faecium*.

The species-wise distribution of TCP biofilm categories is illustrated in Fig. 04.

The difference in biofilm production between *E. faecalis* and *E. faecium* was statistically significant (Chi-square test, P < 0.001).

The CRA biofilm results are presented in Table 02.

Table 02
Screening of 133 Enterococcal urinary isolates for Biofilm production by CRA method.

BIOFILM CATEGORIZATION	<i>Enterococcus faecalis</i> (n = 99)	<i>Enterococcus faecium</i> (n = 34)	TOTAL (n = 133)
Strong biofilm producers	1 (1.01%)	1 (2.94%)	2 (1.5%)
Moderate biofilm producers	15 (15.15%)	9 (26.47%)	24 (18.05%)
Weak biofilm producers	29 (29.29%)	9 (26.47%)	38 (28.57%)
Non-biofilm producers	54 (54.55%)	15 (44.12%)	69 (51.88%)
Total	99 (74.44%)	34 (25.56%)	133 (100%)

Among the 133 Enterococcal urinary isolates, 2 (1.5%) isolates were strong biofilm producers of which 1 (1.01%) was *E. faecalis* and 1 (2.94%) was *E. faecium*. Moderate biofilm production was observed in 24(18.05%) isolates, of which 15 (15.15%) *E. faecalis* and 9 (26.47%) *E. faecium*. Weak biofilm formation was seen in 38 (28.57%) isolates, with 29 (29.29%) *E. faecalis* and 9 (26.47%) *E. faecium*. The remaining 69 isolates (51.88%) were non-biofilm producers, of which 54 (54.55%) were *E. faecalis* and 15 (44.12%) were *E. faecium*.

The species-wise distribution of CRA biofilm categories is illustrated in Fig. 05.

The difference in biofilm production between *E. faecalis* and *E. faecium* was not statistically significant (Chi-square test, P = 0.294).

The Tube method biofilm results are presented in Table 03.

Table 03
Screening of 133 Enterococcal urinary isolates for Biofilm production by Tube method.

BIOFILM CATEGORIZATION	<i>Enterococcus faecalis</i> (n = 99)	<i>Enterococcus faecium</i> (n = 34)	TOTAL (n = 133)
Strong biofilm producers	3 (3.03%)	1 (2.94%)	4 (3.01%)
Moderate biofilm producers	14 (14.14%)	2 (5.88%)	16 (12.03%)
Weak biofilm producers	36 (36.36%)	5 (14.71%)	41 (30.82%)
Non-biofilm producers	46 (46.46%)	26 (76.47%)	72 (54.13%)
Total	99 (74.44%)	34 (25.56%)	133 (100%)

Among the 133 Enterococcal urinary isolates by tube method, strong biofilm production was observed in 4 (3.01%) isolates, 3(3.03%) *E. faecalis* and 1(2.94%) *E. faecium*. Moderate biofilm production was seen in 16 (12.03%) isolates, with 14 isolates of *E. faecalis* (14.14%) and 2 of *E. faecium* (5.88%). Weak biofilm producers were detected in 41 (30.82%) isolates, of which 36 were *E. faecalis* (36.36%) and 5 were *E. faecium* (14.71%). A total of 72 (54.13%) urinary isolates were non-biofilm producers, with 46 *E. faecalis* (46.46%) and 26 *E. faecium* (76.47%).

The species-wise distribution of Tube method biofilm categories is illustrated in Fig. 06.

The difference in biofilm production between *E. faecalis* and *E. faecium* was statistically significant (Chi-square test, P = 0.002).

A comparison of biofilm categories by TCP, CRA and Tube methods is presented in Table 04.

Table 04
Comparison of biofilm production among *Enterococcus faecalis* and *Enterococcus faecium* by TCP, CRA and tube method

NO. OF URINARY ISOLATES		<i>Enterococcus faecalis</i>			<i>Enterococcus faecium</i>		
BIOFILM PRODUCERS		(n = 99)			(n = 34)		
		TCP	CRA	Tube Method	TCP	CRA	Tube Method
Clinical Isolates n = 133	Strong Biofilm Producers	5 (5.05%)	1 (1.01%)	3 (3.03%)	2 (5.88%)	1 (2.94%)	1 (2.94%)
	Moderate Biofilm Producers	33 (33.33%)	15 (15.15%)	14 (14.14%)	1 (2.94%)	9 (26.47%)	2 (5.88%)
	Weak Biofilm Producers	28 (28.28%)	29 (29.29%)	36 (36.36%)	6 (17.65%)	9 (26.47%)	5 (14.71%)
	Non- Biofilm Producers	33 (33.33%)	54 (54.55%)	46 (46.46%)	25 (73.53%)	15 (44.12%)	26 (76.47%)

Among the 133 Enterococcal urinary isolates, biofilm production was evaluated using three different methods: TCP, CRA, and Tube method. According to the TCP method, strong biofilm producers included 5 *E. faecalis* (5.05%) and 2 *E. faecium* (5.88%), while the CRA method identified only 1 *E. faecalis* (1.01%) and 1 *E. faecium* (2.94%) as strong producers. The Tube method detected strong biofilm production in 3 *E. faecalis* (3.03%) and 1 *E. faecium* (2.94%). Moderate biofilm production was highest by the TCP method, with 33 *E. faecalis* (33.33%) and 1 *E. faecium* (2.94%), followed by the CRA method with 15 *E. faecalis* (15.15%) and 9 *E. faecium* (26.47%), and the Tube method with 14 *E. faecalis* (14.14%) and 2 *E. faecium* (5.88%). Weak biofilm production was most frequently observed by the Tube method, with 36 *E. faecalis* (36.36%) and 5 *E. faecium* (14.71%), followed by CRA 29 *E. faecalis*, 29.29%; 9 *E. faecium*, 26.47% and TCP 28 *E. faecalis*, 28.28%; 6 *E. faecium*, 17.65%. Non-biofilm producers were highest detected by CRA, with 54 *E. faecalis* (54.55%) and 15 *E. faecium* (44.12%), while the TCP method identified 33 *E. faecalis* (33.33%) and 25 *E. faecium* (73.53%) as non-producers, and the Tube method reported 46 *E. faecalis* (46.46%) and 26 *E. faecium* (76.47%) as non-biofilm producers.

The chi-square analysis shows a statistically significant difference between species for TCP and Tube methods, but not for CRA.

The concordance and method-specific detection of biofilm are summarized in Table 05.

Table 05
Detection of Biofilm-Producing Enterococci by Tissue Culture Plate (TCP), Congo Red Agar (CRA) and Tube Method

Enterococcal isolates (n = 133)	Biofilm detected by all three methods		Biofilm detected by any of the two methods			Biofilm detected by single method		
	Positive(n = 31)	Negative(n = 27)	TCP + CRA	TCP + TM	TM + CRA	TCP	CRA	TM
<i>E. faecalis</i>	25 (25.25%)	15(15.15%)	27(27.27%)	53(53.53%)	25(25.25%)	66(66.66%)	45(45.4%)	53(53.53%)
<i>E. faecium</i>	6 (17.64%)	12(35.29%)	6(17.64%)	7(20.58%)	7(20.58%)	9(26.47%)	19(35.3%)	8(23.52%)
Total	31	27	33	60	32	75	64	61

Out of the 133 Enterococcal isolates tested for biofilm production using three phenotypic methods—Tissue Culture Plate (TCP), Congo Red Agar (CRA), and Tube Method (TM) – 31 isolates were identified as biofilm producers by all the three methods, while 27 isolates were negative by all three methods. 33 isolates were positive for biofilm detection by TCP and CRA method, while they were negative by TM. 60 isolates were positive for biofilm detection by TCP and TM method, while they are negative by CRA. 32 isolate was positive for biofilm detection by TM and CRA and negative by TCP. 75 isolates showed biofilm detection by TCP method only and they were negative when detected by CRA and TM. 30 isolates showed biofilm detection by CRA but they were negative by

TM. 61 isolates were detected positive for biofilm production by tube method. It suggests that TCP and CRA methods individually identified more biofilm producers compared to TM, while the TCP and TM combination yielded the highest detection rate.

Biofilm formation according to catheterization status is presented in Table 06.

Table 06

Biofilm Formation by *Enterococcus* Species in Catheterized and Non-Catheterized Urinary Isolates Using Three Different Methods (TCP, CRA, and Tube Method)

No of Isolates (n = 133)	Biofilm Formation	Catheterized urine sample (n = 54)						Non-catheterized urine sample (n = 79)					
		Enterococcus Faecalis (n = 36)			Enterococcus Faecium (n = 18)			Enterococcus Faecalis (n = 63)			Enterococcus Faecium (n = 16)		
		TCP	CRA	Tube Method	TCP	CRA	Tube Method	TCP	CRA	Tube Method	TCP	CRA	Tube Method
	Strong Biofilm Producers	01 (2.78%)	0 (0%)	0 (0%)	1 (5.5%)	0 (0%)	0 (0%)	4 (6.3%)	1 (1.5%)	3 (4.7%)	1 (6.2%)	1 (6.2%)	1 (6.2%)
	Moderate Biofilm Producers	11 (30.5%)	3 (8.0%)	6 (16.6%)	1 (5.5%)	5 (27.7%)	2 (11.1%)	22 (34.9%)	12 (19%)	8 (12.69%)	0 (0%)	4 (25%)	0 (0%)
	Weak Biofilm Producers	10 (27.7%)	9 (25%)	10 (27.77%)	3 (16.6%)	6 (33.3%)	3 (16.6%)	18 (28.5%)	20 (31.7%)	26 (41.2%)	3 (18.7%)	3 (18.7%)	2 (12.5%)
	Non-Biofilm Producers	14 (38.8%)	24 (66%)	20 (55.55%)	13 (72.2%)	7 (38.8%)	13 (72.2%)	19 (30.1%)	30 (47.6%)	26 (41.26%)	12 (75%)	8 (50%)	13 (81.2%)

Biofilm formation among 133 Enterococcal urinary isolates isolated from catheterised and non-catheterised urine samples was evaluated using three phenotypic methods (TCP, CRA, and Tube method). 54 isolates were obtained from catheterized urine samples and 79 from non-catheterized urine samples. Among the catheterized isolates, *E. faecalis* accounted for 36 isolates and *E. faecium* for 18 isolates, whereas among non-catheterized isolates, 63 were *E. faecalis* and 16 were *E. faecium*. This distribution indicates that *E. faecalis* was the predominant species in both catheterized and non-catheterized groups.

Biofilm detection according to UTI clinical category is presented in Table 07.

Table 07

Biofilm detection in Enterococci isolated from cases of complicated and uncomplicated UTI

Enterococcal Isolates (n = 133)	Complicated UTI (n = 53)			Uncomplicated UTI (n = 80)		
	Biofilm Producers	Nonbiofilm Producers	Total	Biofilm Producers	Nonbiofilm Producers	Total
<i>E. faecalis</i> (n = 99)	15 (15.15%)	18(18.18%)	33(33.33%)	40(40.40%)	26(26.26%)	66(66.66%)
<i>E. faecium</i> (n = 34)	5(14.70%)	15(44.11%)	20(58.82%)	3(8.82%)	11(32.35%)	14(41.17%)
Total	20(37.73%)	33(62.26%)	53(39.84%)	43(53.75%)	37(46.25%)	80(60.15%)

Out of 133 Enterococcal isolates, 53 were obtained from patients with complicated UTIs and 80 from uncomplicated UTIs. Among the complicated UTI group, *E. faecalis* were isolated from 33 cases, of which 15 (15.15%) were biofilm producers and 18 (18.18%) were non-biofilm producers. In contrast, among the 20 *E. faecium* isolates isolated from complicated UTIs, 5 (14.70%) were biofilm producers while 15 (44.11%) were non-biofilm producers. In the uncomplicated UTI group, *E. faecalis* were isolated in 66 cases, with 40 (40.40%) showing biofilm production and 26 (26.26%) being non-producers. Among the 14 *E. faecium* isolates, 3 (8.82%) were biofilm producers and 11 (32.35%) were non-biofilm producers. Overall, biofilm production was observed in 20 (37.73%) Enterococci isolates from complicated UTI cases and 43 (53.75%) from uncomplicated UTI cases, indicating a higher prevalence of biofilm-forming isolates in uncomplicated urinary tract infections.

The distribution of biofilm-producing and non-biofilm-producing isolates by UTI category is illustrated in Fig. 07.

The difference in biofilm production between complicated and uncomplicated UTI groups was not statistically significant (Chi-square test, P = 0.070)

DISCUSSION

Enterococcus species are commonly found as normal inhabitants of the human gastrointestinal tract, but they can also act as opportunistic pathogens and cause serious infections, particularly in hospitalized patients. They are frequently associated with urinary tract infections, with *E. faecalis* and *E. faecium* being the major species isolated from clinical specimens. The increasing use of antibiotics has contributed to the emergence and persistence of enterococcal infections. In addition to antimicrobial resistance, the ability of *Enterococcus* to form biofilms may further support bacterial survival and

persistence during infection. Biofilm production is therefore considered an important factor in the pathogenicity of enterococci, especially in urinary tract infections.

Biofilm formation is an important virulence property of *Enterococcus* species. It enables the bacteria to attach to surfaces and survive for a longer period, especially on urinary catheters and within the urinary tract. Bacteria present in a biofilm are more difficult to eliminate because the biofilm matrix can provide protection against antimicrobial agents and host defence mechanisms. Therefore, biofilm production may play an important role in the persistence and recurrence of Enterococcal urinary tract infections. Detection of biofilm formation among clinical *Enterococcus* isolates can help in understanding their pathogenic potential and ability to persist during infection.

Various methods can be employed to screen for biofilm formation. In the present study, 133 Enterococcal isolates were screened using the Tissue Culture Plate (TCP) method, Congo Red Agar (CRA) method, and Tube Method (TM). Tissue culture method was considered the gold standard phenotypic test for the detection of biofilm. [12, 13, 14]

According to the TCP method, 75 isolates (56.39%) were identified as biofilm producers, while 58 isolates (43.6%) were non-biofilm producers. In comparison, a study by Kala Harika et al. [8] reported that 72 isolates (78.2%) were biofilm producers and 20 isolates (21.7%) were non-biofilm producers. Similarly, a study conducted by Mahmoud Shahveh et al. [15] found that 92% of isolates were biofilm producers, while 8% were non-biofilm producers. According to findings by L. Triveda et al., [12] 71 isolates (65.5%) were identified as biofilm producers, and 57 isolates (44.6%) were categorized as non-biofilm producers. A study by Ruchi A Tayal et al. [16] reported 14 isolates (27%) were biofilm producers,

By the Congo Red Agar (CRA) method, 64 isolates (48.12%) were identified as biofilm producers, whereas 69 isolates (51.87%) were non-biofilm producers in this study. In comparison, a study by Kala Harika et al. [8] reported that 52 isolates were biofilm producers, while 41 isolates were non-biofilm producers. A study conducted by Aya H. Al-Halaby et al. [17] found that 26 isolates (92.8%) were biofilm producers and 2 isolates (7.1%) were non-biofilm producer in Congo red agar method. Similarly, in a study by L. Triveda et al., [12] 26 isolates (20.31%) were biofilm producers, while 102 isolates (79.6%) were non-biofilm producers.

By tube method (TM), 61 isolates (46.36%) were identified as biofilm producers, whereas 72 isolates (54.13%) were non-biofilm producers. This method correlates well with TCP in identifying strong biofilm producers. But it was difficult to differentiate between moderate and weak biofilm producers. In comparison, a study by Kala Harika et al. [8] reported that 37% of isolates as biofilm producers and 63% as nonbiofilm producers in tube method. A study conducted by L. Triveda et al., [12] 38(29.8%) isolates were biofilm producers, whereas 90(70.4%) isolates were non-biofilm producers.

Comparison of biofilm production among *Enterococcus faecalis* and *Enterococcus faecium* by TCP, CRA and tube method. In TCP methods we use polystyrene plate of 96 wells for detection of biofilm production as shown in [figure 01] our results showed that 5 (5.05%) isolates of *E. faecalis* were strong biofilm producers, 33(33.33%) isolates as moderate and 28(28.28%) as weak biofilm producers. Close to these results was reported by pooja Nair et al, [18] who find 7(11.1%) isolates of *E. faecalis* as strong biofilm producers, 41(65.07%) isolates as moderate and 10(15.87%) isolates as weak biofilm production. A study conducted by Aya H. Al-Halaby et al, [17] found that 11(39%) isolates of *E. faecalis* detect as strong biofilm producers, 14(50%) isolates as moderate and 3(10%) as weak biofilm producers. The percentage of biofilm formation in *E. faecium* was 2(5.88%) as strong biofilm producers, 1(2.94%) moderate and 6(17.65%) as weak biofilm producers.

In Congo red agar method all bacterial isolates were grown on Congo red agar plates to detect biofilm production as shown in (Fig. 03) our results showed that one (1.01%) isolate of *E. faecalis* were strong biofilm producer, 15(15.5%) isolates were moderate and 29(29.29%) were weak biofilm production, while one (2.94%) isolate of *E. faecium* were strong biofilm producers, 9(26.47%) were moderate and 9(26.47%) were weak biofilm producers. In comparison, a study by T A Dhanalakshmi et al., [14] reported that 40(32.6%) isolates were strong biofilm producers, 47(37.9%) were moderate and 37(29.8%) isolates were weak biofilm producers.

In the Tube Method, 3 (3.03%) isolates of *E. faecalis* were identified as strong biofilm producers, 14 (14.14%) as moderate, and 36 (36.3%) as weak biofilm producers. Among *E. faecium* isolates, one (2.94%) was a strong biofilm producer, 2 (5.8%) were moderate, and 5 (14.7%) were weak biofilm producers. A study conducted by L. Triveda et al. [12] reported that 14 (11%) isolates were strong biofilm producers, 14 (11%) were moderate, and 10 (7.8%) were weak biofilm producers. Similarly, in a study by Kala Harika et al., [8] 13 (14%) isolates were strong biofilm producers, 21 (23%) were moderate, and 33 (36%) were weak biofilm producers.

In the study, the tube method correlates with the Tissue culture Plate method compare to CRA method. The Tissue culture plate method was found to be most sensitive, accurate and reliable screening method for detection of biofilm production. As a quantitative method, the TCP method is considered the gold standard method for biofilm detection. [12, 13, 14]

In the present study, 31 of the 133 Enterococcus isolates were positive for biofilm formation by all three methods, whereas 27 isolates were negative by all three methods. Biofilm detection by combinations of two methods also showed considerable variation. The TCP and TM combination detected biofilm formation in 60 isolates, while 33 isolates were positive by TCP and CRA and 32 by CRA and TM. Among isolates detected by only one method, CRA identified 64 isolates, and TM identified 61, whereas TCP identified 75 isolates. These findings demonstrate that the three phenotypic methods do not provide identical results and that differences in methodology can influence the classification of an isolate as a biofilm producer. The present findings are comparable with the observations of Triveda and Gomathi et al, [12] who evaluated 128 clinical Enterococcus isolates using TCP, TM and CRA. They reported that 55.5% of isolates were biofilm positive by TCP and concluded that TCP was a more reliable and sensitive method than the tube and Congo Red Agar methods. They also observed that the tube method correlated reasonably well for strong biofilm producers but had difficulty distinguishing weak producers from non-producers. This observation is consistent with the present study, where differences were particularly evident in the classification of weak and moderate biofilm-producing isolates. The findings also agree with the study by Khalil et al.,[13] who investigated Enterococcus isolates from urinary tract

infections using Congo Red Agar and a microtiter plate method. They reported biofilm production in 76.6% of isolates by CRA and 100% by the microtiter plate method, demonstrating that the microtiter plate approach detected a greater proportion of biofilm-producing isolates. Although the prevalence observed in their study was higher than in the present study, the methodological difference was similar the quantitative microtiter-based method identified more biofilm-positive isolates than the qualitative CRA method.

Therefore, the present findings support the concept that phenotypic biofilm detection should preferably include a quantitative or semi-quantitative method such as TCP, particularly when the objective is to differentiate weak, moderate and strong biofilm production.

In the study, Enterococcal isolates isolated from catheterized and non-catheterized urine samples were compared for biofilm production. 40.6% were isolated from Catheterized sample, while 59.4% were non-catheterized sample. A study conducted by Lok Bahadur Shrestha et al.[19], found that 25% of catheterized associated UTI. Similarly, in a study conducted by Ruchi a Tayal et al.,[16] found that 30.5% isolates from catheterized urine sample.

Among catheterized samples, moderate and weak biofilm producing *E. faecalis* constituted 30.5% and 27.7% respectively while strong producers constituted only 2.78%. In non-catheterized samples, 6.3% *E. faecalis* showed strong and 34.9% moderate biofilm formation. *E. faecium* exhibited limited biofilm production, with the majority of isolates categorized as non-biofilm producers.

Increased Biofilm production was observed among *E. faecalis* than in *E. faecium* in all methods.

A total of 53 Enterococci isolates were obtained from patients with complicated UTIs, and 80 from uncomplicated UTIs. Biofilm formation was notably higher in isolates isolated from uncomplicated UTIs (63.7%) compared to complicated UTIs (45.2%).

Among species, *E. faecalis* demonstrated a significantly higher ability to form biofilms than *E. faecium*. In complicated UTI, 57.5% of *E. faecalis* isolates were biofilm producers, while 25% of *E. faecium* isolates were biofilm producers. Similarly, in uncomplicated UTIs, *E. faecalis* (71.2%) biofilm producers, whereas *E. faecium* showed lower biofilm formation in 28.5%.

The present study highlights a higher prevalence of biofilm formation among *Enterococcus* isolates isolated from uncomplicated urinary tract infections (UTIs). *E. faecalis* demonstrated significantly greater biofilm-forming ability than *E. faecium*.

Conclusion

The present study highlights the significance of biofilm formation among urinary clinical isolates of *Enterococcus* species in a tertiary care hospital. Among the 133 Enterococcal isolates included in the study, *E. faecalis* was the predominant species, followed by *E. faecium*. Biofilm production was observed among a considerable proportion of the isolates, indicating that biofilm formation may contribute to the persistence of Enterococcal urinary tract infections. Among the three phenotypic methods evaluated, the Tissue Culture Plate (TCP) method demonstrated greater detection of biofilm-producing isolates and showed better overall performance compared with the Congo Red Agar (CRA) and Tube Method (TM). This supports the usefulness of the TCP method as a reliable quantitative approach for assessing biofilm formation. The differences observed between the three methods also indicate that the choice of screening method can influence the detection of biofilm-producing isolates, particularly those with weaker levels of biofilm production.

Biofilm formation was observed in both catheterized and non-catheterized urinary isolates, demonstrating that although urinary catheters provide an important surface for bacterial attachment and biofilm development, biofilm formation is not restricted to catheter-associated infections. The occurrence of biofilm production among non-catheterized isolates further emphasizes the potential role of this virulence mechanism in urinary tract infection.

The study also demonstrated variation in biofilm production between *E. faecalis* and *E. faecium*, with *E. faecalis* showing a greater proportion of biofilm-producing isolates in several of the evaluated groups. Biofilm production was also detected among isolates from both complicated and uncomplicated urinary tract infections, indicating that biofilm formation may occur across different clinical presentations of Enterococcal UTI.

Overall, the findings emphasize that biofilm formation is an important phenotypic characteristic of urinary *Enterococcus* isolates and should be considered in the microbiological evaluation of persistent or recurrent urinary tract infections. The study also supports the use of a reliable quantitative method such as TCP for screening and characterization of biofilm production. Further studies incorporating molecular detection of biofilm-associated virulence genes, particularly esp., may provide additional insight into the genetic basis of biofilm formation and its relationship with antimicrobial resistance and clinical outcomes.

Declarations

1. **Ethical Statement:** Ethical clearance for the study was obtained from the Institutional Ethics Committee (IEC) of JSS Medical College, JSS Academy of Higher Education and Research (JSSAHER), Mysuru (IEC approval/certificate reference: JSSMC/IEC/18122023/21 NCT/2023-24). Ethical principles, including protection of patient confidentiality and privacy, were maintained throughout the study.

2. **Clinical trial:** Not applicable

3. **Conflict of Interests:** The authors declare no conflict of interest.

4. **Consent:** Informed consent was obtained from the participants as per the requirements of the Institutional Ethics Committee. this case report.

5. **Funding statement:** Nil

6. **Availability of Data and Materials:** All the data obtained during the study were fully

analysed and included in the manuscript

7. **Competing Interests:** The authors declare no conflict of interest.

8. **Author Contribution:** S.G.S. provided guidance throughout the study, supervised the work, and assisted with data analysis and interpretation. S.S.N. conducted the study, performed the laboratory work, collected and analysed the data, interpreted the results, and wrote the main manuscript text. M.R.R. contributed to study supervision and revised the manuscript. S.M. review of the manuscript. All authors reviewed and approved the final manuscript.

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Figures

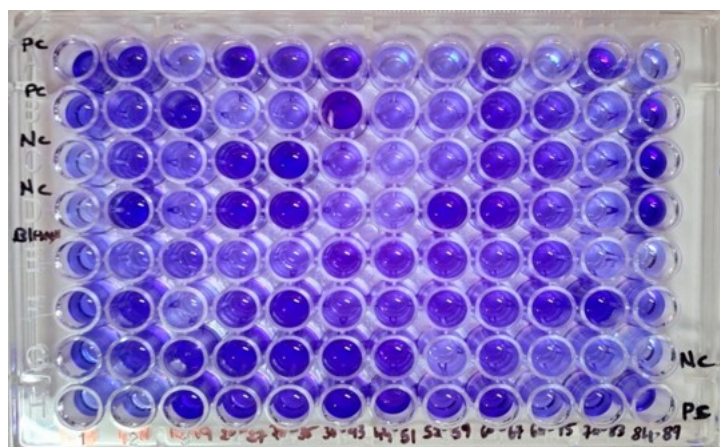


Figure 1

Screening of Biofilm Producers by Tissue culture plate method.

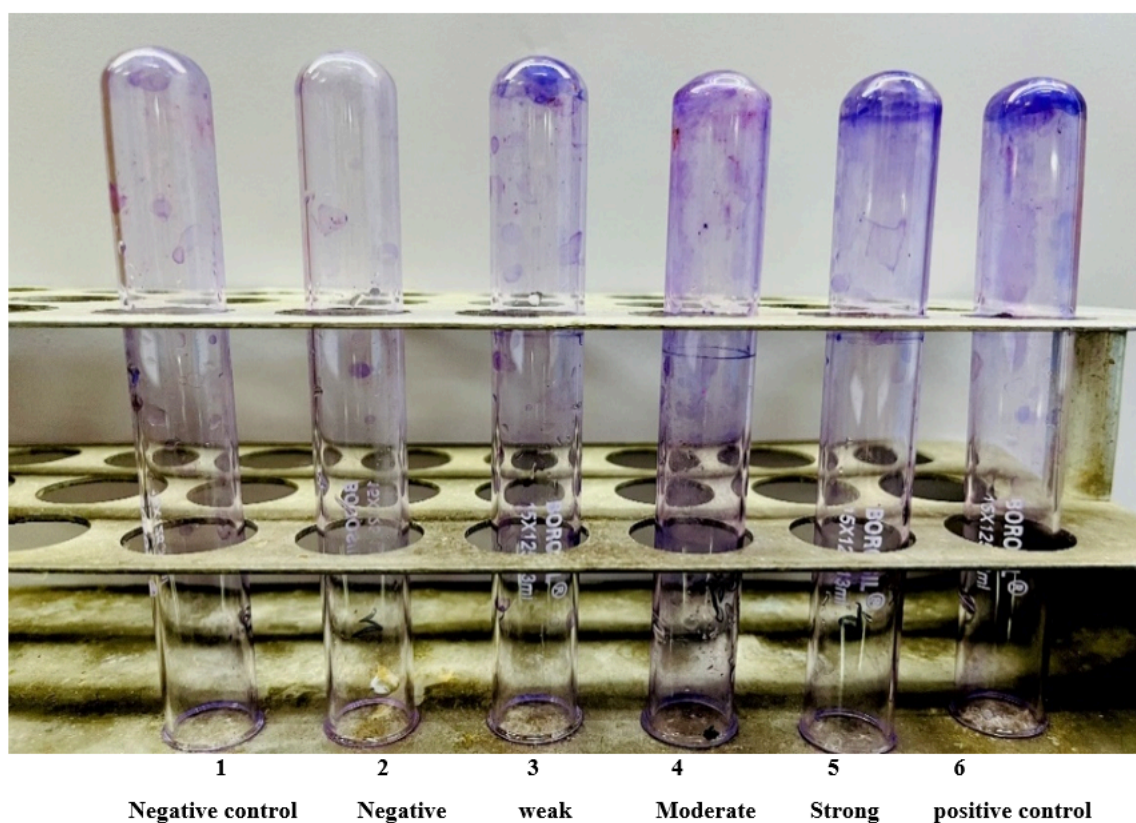
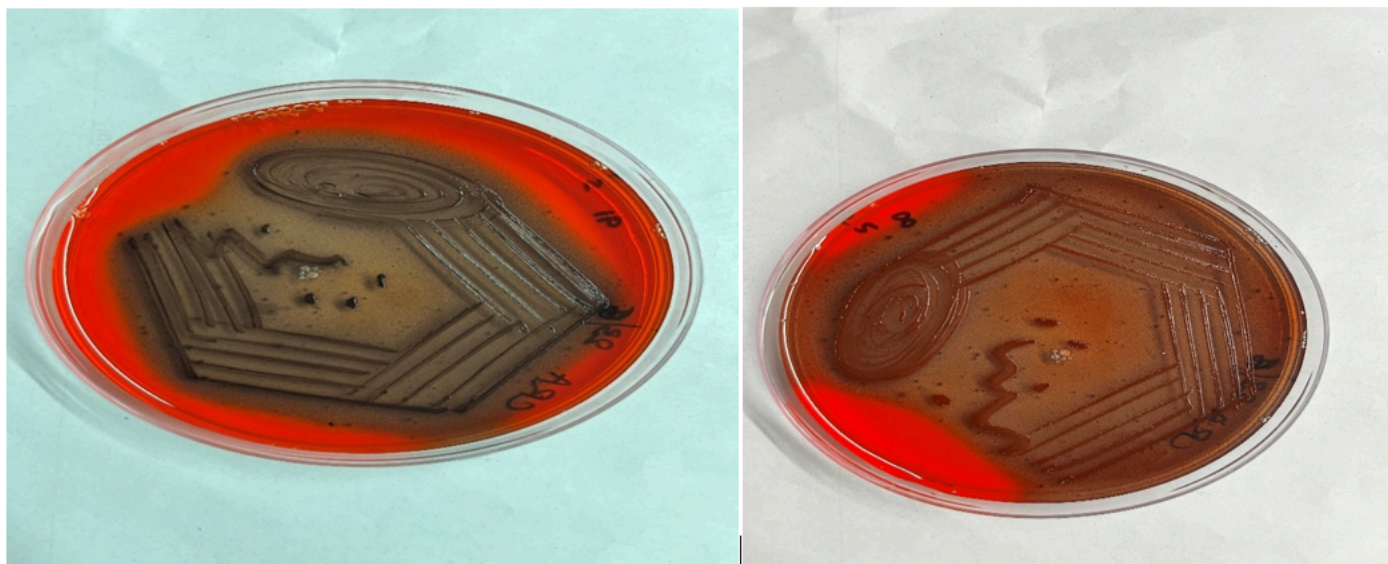


Figure 2

Screening of Biofilm Producers by Tube method.



a) Strong biofilm producer on CRA

b) Moderate biofilm producer on CR

Figure 3

Screening of Biofilm Producers by Congo Red Agar method.

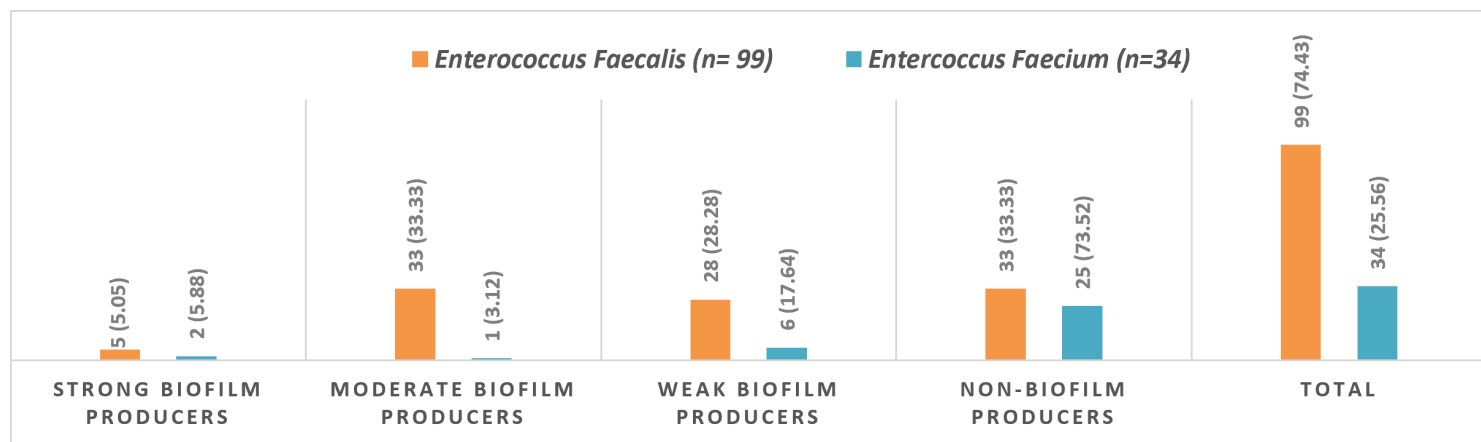


Figure 4

Screening of 133 Enterococcal urinary isolates for Biofilm production by TCP method

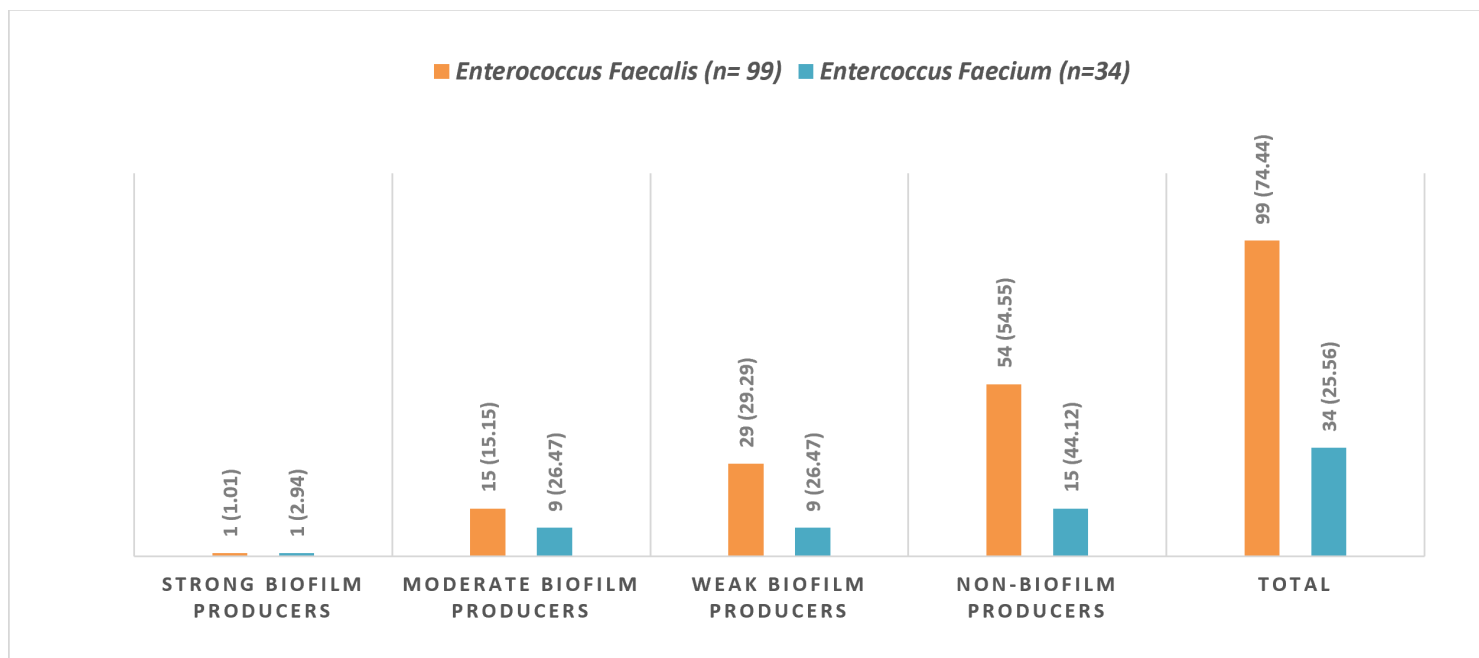


Figure 5

Screening of 133 Enterococcal urinary isolates for Biofilm production by CRA method.

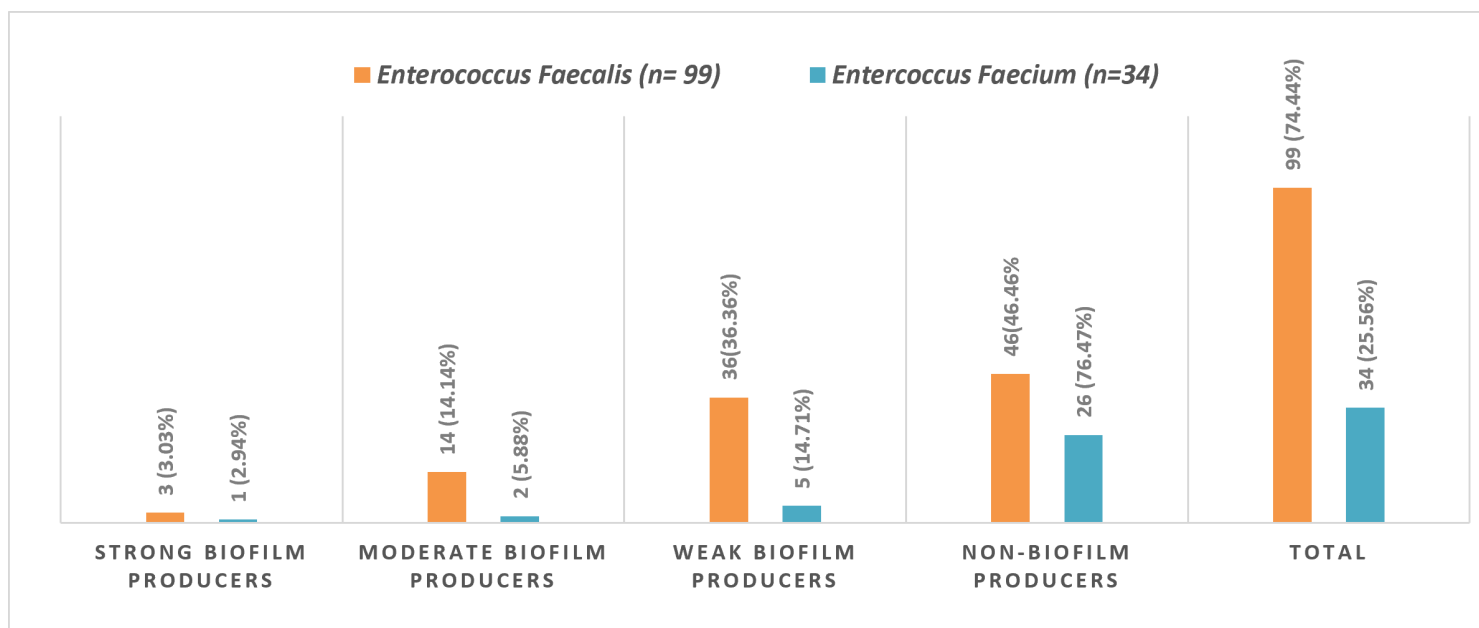


Figure 6

Screening of 133 Enterococcal urinary isolates for Biofilm production by Tube method.

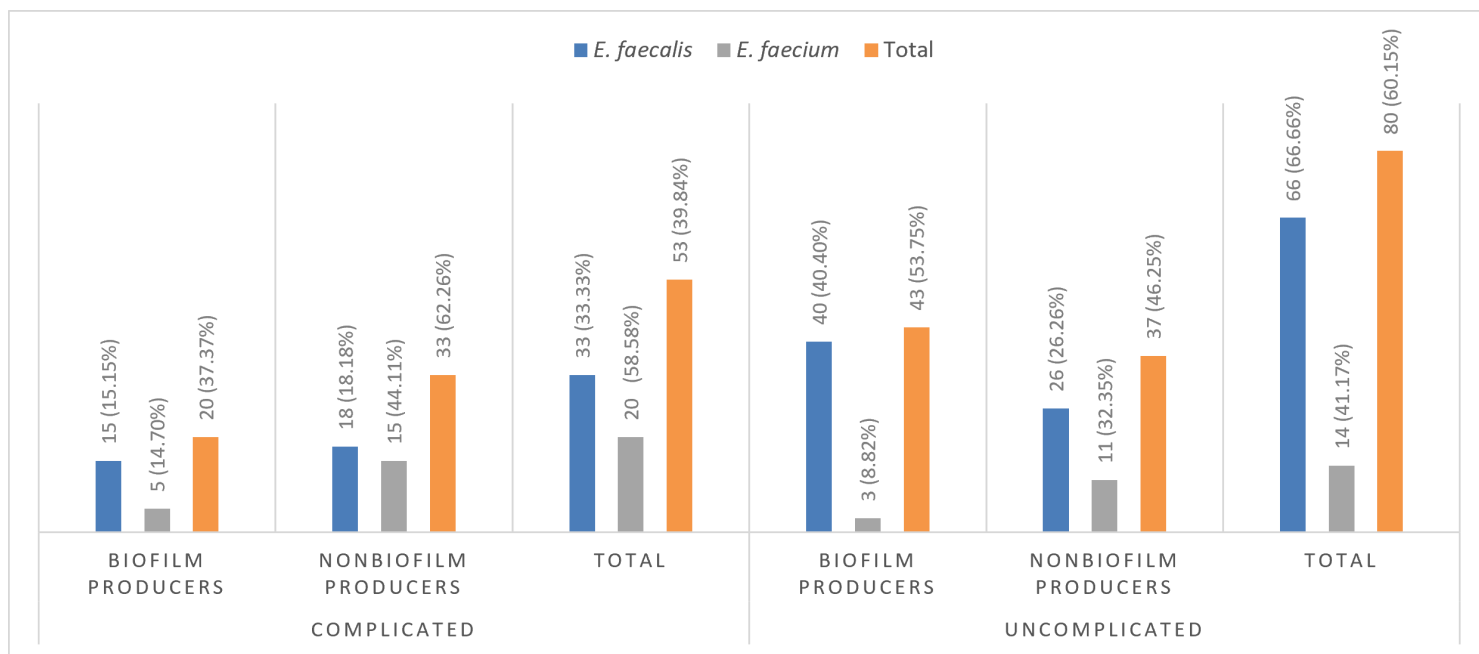


Figure 7

Biofilm detection in Enterococci isolated from cases of complicated and uncomplicated UTI