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Assessment of biofilm formation and antibiofilm agent efficacy against Uropathogens: antibiotic susceptibility and molecular identification

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Abstract

The formation of biofilms by uropathogens is a major concern in urinary tract infections (UTIs), contributing to antibiotic resistance and chronic infections. This study aimed to assess the biofilm-forming ability of uropathogens and evaluate the efficacy of antibiofilm agents in combating these infections. We isolated and identifying uropathogens from urine samples using standard microbiological methods and confirmed by bacterial identification through molecular techniques. The ability of the selected isolates to produce biofilms was primarily confirmed using crystal violet assay. Antibiotic susceptibility profiles were determined by the disc diffusion method. Antibiofilm agents were tested for them to reduce biofilm formation. Our results may be indicated that significant variability in biofilm formation among uropathogens, in contrast some of the strains exhibited strong biofilm production. This study emphasizes the need for alternative strategies in the management of biofilm-associated UTIs, antibiotic susceptibility testing and molecular identification, and the development of effective antibiofilm agents to combat these persistent urinary infections.

Keywords: Antibiotic susceptibility, Biofilm, Uropathogens, Antibiofilm agent

Introduction

A serious public health concern, urinary tract infections (UTIs) are frequent bacterial infections (Zhou *et al.,* 2023). Lower respiratory tract infections account for 24% of nosocomial infections in developing nations, making UTIs the second most common nosocomial infection (Tandogdu and Wagenlehner 2016). Numerous microorganisms,

including as viruses, fungi, and bacteria, are frequently implicated in UTIs. However, the most frequent cause of this type of infection is bacteria (Stamm and Norrby 2001). Although *S. aureus, P. mirabilis, K. pneumonia*, and *P. aeruginosa* are prevalent bacteria, the most common gram-positive bacteria found in UTIs are Enterococci and Coagulase Negative Staphylococci (Saroj *et al.,* 2012). This difference emphasizes how difficult it is to treat uropathogens that are resistant to crucial antibiotics such carbapenems, ß-lactams, aminoglycosides, polyketides, fluoroquinolones, and sulfonamides (Jafri *et al.,* 2014; Marialouis and Santhanam 2016; Khonsari *et al.,* 2021; Ameshe *et al.,* 2022). In order to fight these recurring urinary tract infections, the current study aimed to isolate and identify the pathogenic bacteria, antibiotic resistance, biofilm formation, and the creation of efficient antibiofilm agents.

Materials and Methods

Sample Collection

Three urine samples were collected from locally available lab and neighborhood people. The People are having diabetic, cholesterol and blood pressure diseases. The samples are designated as U1, U2 and U3. (Figure 1).

Bacterial screening

Initially all the urine samples (1 loop culture) are simultaneously streaked on Nutrient agar, Blood agar and MacConkey agar. The plates were incubated at 37^{o}C for 24-48 hours.

Biochemical characterization

Biochemical characterization is the process of identifying microorganisms based on their metabolic and enzymatic activities. It involves a series of biochemical tests that detect specific enzymes, metabolic pathways, and substrate utilization patterns unique to different bacteria. Biochemical characterization is a fundamental step in microbial identification, especially in clinical diagnostics and research projects.

Indole test: Indole production is a characteristic of certain bacteria that degrade the amino acid tryptophan. To test for indole production, bacteria were grown in a tryptophan-rich medium and then inoculated into SIM (Sulfide Indole Motility) medium. After incubating at 37°C for 24 hours, Kovac's reagent was added to the culture. The appearance of a red-colored product indicates a positive reaction, confirming the presence of indole-producing bacteria.

Methyl red test: The Methyl Red (MR) test was used to detect changes in pH levels, indicating acid production by microorganisms. The test exploits the ability of certain bacteria to ferment glucose, producing mixed acids and lowering the pH of the medium. To perform the test, MR-VP medium was prepared, inoculated with a loopful of the bacterial culture, and incubated at 37°C for 24 hours. Following incubation, 5 drops of Methyl Red solution were added, and the tube was observed for a color change, which would indicate a pH drop below 4.5, signifying a positive result.

Voges Proskauer (VP) test: The Voges-Proskauer (VP) test was conducted to detect the production of acetoin (butylene glycol) by microorganisms that ferment glucose to pyruvate. The test involved inoculating the culture into MR-VP medium, incubating it at 37°C for 24 hours, and then adding Barritt's reagent (VP reagent). The tube was observed for a color change, which would indicate a positive result, confirming the production of acetoin.

Citrate utilization test: The Simmons' citrate test was performed to determine if microorganisms could utilize citrate as a sole carbon source for energy in the absence of glucose or lactose. The test involved streaking the cultures onto Simmon's citrate agar slants, incubating them at 37°C for 24 hours, and observing for a color change, which would indicate a positive result, confirming the organism's ability to metabolize citrate.

Triple sugar iron test: The Triple Sugar Iron (TSI) test procedure involves inoculating a bacterial isolate into a TSI agar slant, followed by incubation at 37°C for 24-48 hours. The slant is then observed for color changes, indicating sugar fermentation, and the production of hydrogen sulfide gas, which causes blackening of the medium. The test assesses the ability of bacteria to ferment glucose, lactose, and sucrose, providing valuable information for identification and differentiation of enteric bacteria.

Catalase activity test: The catalase test detects the presence of catalase enzyme in aerobic microorganisms, which breaks down hydrogen peroxide into water and oxygen. When a few drops of hydrogen peroxide are added to the culture, the release of oxygen gas is indicated by the formation of white bubbles, confirming a positive result.

In addition, the selected isolates were streaked on different selective chromogenic media such as Mannitol salt agar and UTI agar. After that, genus of the selected isolates was confirmed by Gram staining method.

Determination of biofilm production

The tube method is a simple qualitative assay used to evaluate bacterial biofilm formation. In this technique, bacterial isolates are inoculated in tryptic soy broth (TSB) broth (with or without glucose) and incubated at 37°C for 24 to 120 hours. After incubation, the broth is discarded, and the tubes are washed with tris buffer. Biofilms formed on the inner walls are stained with 0.1% crystal violet, followed by rinsing and drying. The presence of a stained layer and ring formation indicates biofilm formation, categorized as weak, moderate and or strong based on visual intensity.

Antibiotic susceptibility profile

The disc diffusion method is a standardized technique used to assess the antibiotic susceptibility of bacterial isolates. This method follows Clinical and Laboratory Standards Institute (CLSI) guidelines to determine bacterial resistance or susceptibility to antibiotics. Bacterial isolates are inoculated in LB broth and incubated for 24 hours. After that the selected isolates were spread on agar plates using a sterile cotton swab. Antibiotic discs are placed on the agar surface and incubated at 35-37°C for 16-24 hours. After incubation, the zones of inhibition around the discs are measured and compared to CLSI breakpoints to classify the bacteria as susceptible (S), intermediate (I) and or resistant (R).

Results

Urine samples were collected from Palavanatham neighbourhood, Virudhunagar neighbourhood and locally available lab of Virudhunagar. The samples were designated as U1, U2 and U3 (**Figure 1**). The samples U1 and U2 samples collected people having diabetic and cholesterol with blood pressure (**Table 1**).

Table 1: sample collection

Characteristics / Sample name	U1	U2	U3
Age	64	75	32
Disease	Diabetic	Blood pressure, Cholesterol	Normal

Figure 1: Collection of Urine samples

Bacterial screening was done with different media such as nutrient agar, MacConkey agar and blood agar plates (**Figure 2, Table 2**).

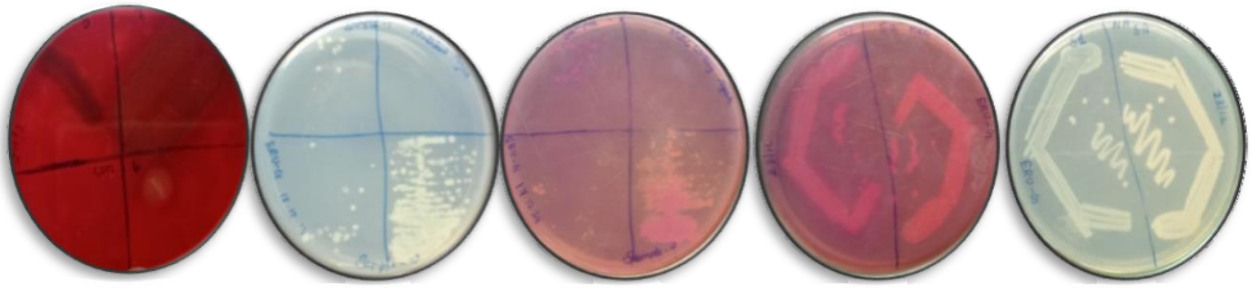

Figure 2: Bacterial screening with different agar plates

Table 2: Selection of organisms from different agar plates

S.No.	Agar media	Samples	Name of isolates
1.	MacConkey agar	U1	SG1
2.	MacConkey agar	U2	C2
3.	Nutrient agar	U1	SG3
4.	Nutrient agar	U2	C4
5.	Nutrient agar	U3	LA5
6.	Nutrient agar	U3	LA6

Six isolates in all were chosen and utilized for further studies. Four of them were identified as Gram-negative bacteria based on the Gram staining method and the growth of MacConkey agar, while the other two isolates were identified as Gram-positive bacteria. On blood agar plates, strains SG1, C2, and C4 showed signs of β-hemolysis. The VP test yielded negative results for every strain. The isolates produced yellow and pink colonies (SG1, SG3), yellow and white colonies (C2, C4, LA5), and yellow colonies (LA6) in Mannitol salt agar

plates, respectively. Isolates SG1, SG3, C2, and C4 tested positive for catalase, while isolates LA5 and LA6 tested negative. With the exception of isolate C2, which displayed an acid slant and an alkaline butt, all of the isolates on Triple Sugar Iron Agar plates displayed an alkaline slant and butt. With the exception of isolate C_4, all isolates exhibit H_2S generation, and there is no gas production. It displayed the creation of H_2S. The biochemical results are mentioned in table1.

Biofilm production

In this study, biofilm formation was analyzed by tube method. Based on analysis, the isolates SG1, C2 and C4 exhibits high biofilm producer and isolate SG3 act as moderate biofilm producer. In other side, the remaining isolates does not produce any biofilm was confirmed by tube method (Figure 3).

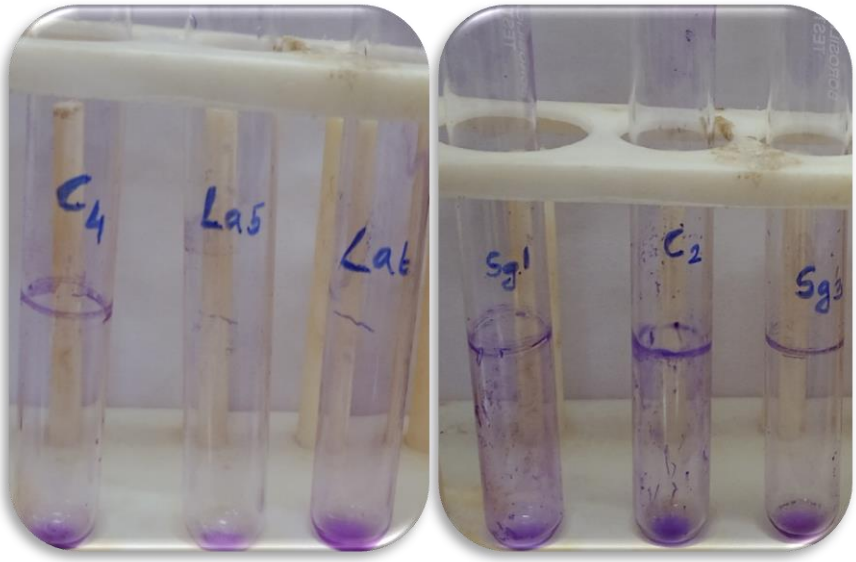

Figure 3: Biofilm production confirmed by crystal violet tube method.

Future aspects

- Antibiotic sensitivity test for remaining isolates.
- To determine the efficacy of antibiofilm against biofilm formation.
- 16S RNA Sequencing of bacterial isolates.

Table 3: Biochemical characteristics

S. No.	Isolates / Characteristics	SG1	SG3	C2	C4	LA5	LA6
1	Nutrient agar	+	+	+	+	+	+
2	MacConkey agar	+	+	+	+	–	–
3	Blood agar	β-hemolysis	–	β-hemolysis	β-hemolysis	–	–
4	Mannitol salt agar	Yellow & Pink	Yellow & Pink	Yellow & White	Yellow & Pink	Yellowish Pink	Yellow
5	UTI agar	Colourless	White	Purple	Greenish blue	Blue to purple	Blue
6	Catalase test	+	+	+	+	–	–
7	Indole	–	+	+	–	+	–
	Methyl red	–	+	+	–	–	+
	VP	–	–	–	–	–	–
	Citrate Utilization	–	–	+	+	–	–
8	*TSI agar* Acid or Alkaline H_2S Production Gas production	Alkaline/ Alkaline – –	Alkaline/ Alkaline – –	Acid/ alkaline – –	Alkaline/ Alkaline + –	Alkaline/ Alkaline – –	Alkaline/ Alkaline – –
9	Gram stain	G^-	G^-	G^-	G^-	G^+	G^+

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