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Assessment of Production of Biofilm in Urinary Tract Infections in Catheterized Tropical Patients: A Comparative Analysis of Detection Techniques

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Abstract:-Urinary tract infections, which can be acute or chronic, are quite common in the Indian population. But untreated UTIs can result in a persistent illness that requires hospitalisation.The current study aims to compare three distinct methods of detecting uropathogen biofilm generation in tropical catheterized patients. Data from 500 catheterized tropical patients admitted to the Civil Hospital of Mazagaon, were collected, and screened. According to a predetermined process, all patients were questioned, and all relevant healthdata was logged in a preset way for easy extraction and interpretation. Samples were taken for testing to determine the isolates' microbialarrangement and the location of Biofilm generation.The identification of microorganisms and biofilms was done using conventional methods.Three methods viz, the tube adhesion method, Congo-red agar method modified, and the tissue culture plate method - were each used to identify the formation of a biofilm.*E. coli* is the highest prevalent urinary pathogen in the catheterized patients in the current study population of

patients, according to the findings, which accounts for urinary tract infections. The most abundant and effective biofilm producers among these clinical isolates were demonstrated to be the clinical isolates containing resistant strains of *E. coli*. The most reliable techniques for spotting the emergence of biofilms in this patient population were found to be the Tube Adherence Method, Tissue Culture Plate Method, and Modified Congo Red Agar Method, particularly when Resistant *E. coli* was the etiological agent.

Keyword: *Escherichia coli, UTI, Catheterised patients, Uropathogens , Biofilm.*

1. Introduction

Complex microbial populations known as biofilms attach to surfaces and are enclosed in a matrix of extracellular polymeric substances (EPS). These small, seemingly insignificant structures have a big impact on many parts of our life, including human health and environmental systems. Understanding biofilms is essential for addressing issues in healthcare, industry, and beyond [1-3].Biofilms seem to be everywhere, which is one of their most remarkable characteristics. From the slimy film in a sink drain to the plaque on our teeth, they are nearly everywhere. When individual microbial cells adhere to a surface, interact with one another through quorum sensing, and collectively build the EPS matrix that protects them, these robust communities are created. Biofilms benefit significantly from this structural unity, including greater resistance to drugs, host immunological responses, and environmental challenges. Biofilms can affect human health in both positive and harmful ways. In medicine, biofilms are notorious for their role in chronic infections, like those associated with indwelling medical devices or cystic fibrosis lung infections[2, 3]. It is difficult to get rid of them since they can resist the immune system and antibiotics. On the other side, biofilms also have a positive impact on the treatment of wastewater where they aid in the degradation of organic materials and contaminants. They also aid in the development of symbiotic partnerships in the gut microbiota, which benefits digestion and general health. By creating chemotactic particles or pheromones, bacteria within a biofilm communicate with one another during the process known as quorum sensing. The antibacterial resistance of planktonic cells is lower than that of biofilm-dwelling microbes. It requires large antimicrobial concentrations to inactivate organisms growing in biofilms due to the 1,000-fold increase in antibiotic resistance. According to a National Institutes of Health research, biofilms are present in more than 80% of all diseases[4].Biofilms have been linked to a number of disorders, including those brought on by urogenital infections, dental plaque, peritonitis, indwelling medical devices and upper respiratory tract infections. Both Gram-negative and Gram-positive bacteria can create biofilms. Bacteria implicated frequently include *Staphylococcus aureus*, *Enterococcus faecalis*, *Staphylococcus epidermidis*, *Klebsiella pneumoniae*, *Escherichia coli*, *Streptococcus viridans*, *Pseudomonas aeruginosa* and *Proteus mirabilis*[5].There are various methods for identifying the development of biofilms. Fluorescence microscopes, tissue culture plates, the tube adherence method, the Congo Red Agar method, the bioluminescent assay, and piezoelectric sensors are a few of them[6].In order to determine whether or not 500 samples may form biofilms, we analysed them using 3diverseapproaches that could be used in a typical clinical lab set up. A somewhat prevalent clinical issue is urinary tract infections (UTI). According to reports, UTI led to lakhs of hospitalizations, nearly 9 million office visits, and 1.5 million trips to the emergency departments. However, because UTIs are not considered to be diseases, it is challenging to determine their prevalence[7-9].

Most commonly, urinary tract infections happen when bacteria enter the urinary tract through the urethra and start to grow in the bladder [10]. Today's arsenal of therapeutic agents for bacterial infections is limited to antibiotics that were developed specifically to kill or stop the growth of individual bacteria in order for them to survive and protect themselves against various environments, such as antibiotic agents, microorganisms that produce a barrier, and biofilms[11-13]. An intricate collection of microorganisms called a biofilm forms when cells cling to one another and to an abiotic or biotic surface. The majority of infections that affect the urinary system are caused by faeces, but only facultative aerobic species, like *Klebsiella pneumonia* or *Escherichia coli*, have the features required to colonise the urethral tract. Therefore, *E. coli* (52.18%) is the major biofilm-producing bacteria in UTIs, followed by *K. pneumonia* (23.91%), *Proteus species* (13.04%), and *Enterococcus*

species (10.87%) [14, 15]. A biofilm is a collection of microbial cells created by some bacterial species that is encased in a matrix of polysaccharide and protein substances [16, 17]. This offers several benefits, including resistance to antimicrobial agents, nutrition exchange, and genetic material interchange[18]. Chronic infections and nosocomial infections are brought on by biofilms[19]. The genitourinary tract's anatomical features may develop biofilms, which can lead to chronic UTIs. This investigation's goal was to identify the frequency of biofilm utilising sensitive, convenient, and generally applicable techniques in samples taken from patients who had been placed on tropical catheters.The study also aimed to evaluate three detection methods of biofilm production and compare their efficiency.

2. Materials And Methods

2.1 Sample Collection and Microbiological analysis

An examination was conducted on a comprehensive set of five hundred specimens ofurine obtained from hospitalised patents (catheterized)in civil hospital Mazagaon city, all of whom reported suffering from urinary tract infections (UTIs). This analysis involved the examination of Gram smears from non-centrifuged urine samples that exhibited a solitary bacterium per oil immersion field, as well as centrifuged urine samples displaying more than 4cells/HPF (High Power Field). These techniques were employed to detect and diagnose UTIs. Additionally assessed were a number of symptoms such suprapubic soreness, frequency, incontinence, abdominal pain, and dysuria. To confirm a severe infection, a qualitative colony count was performed on the urine sample [8, 9,20]. Routine biochemical assays were used to identify the infections.

2.2 Detection and spotting of Biofilm formation

To spot and detect the development of biofilm, TCP, TAM, and MCRA methods were employed. Methods for detecting biofilms were used to clinical isolates. Five hundred clinical isolates in all were gathered, and they were all assessed.All of the samples were obtained from hospitalised individuals. Standard microbiological techniques were used to identify the isolates (Colonial morphology, motility, Gram staining, catalase test, cytochrome oxidase reaction, biochemical tests). *E. coli* ATCC (American Type Culture Collection) 35218, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 35556, and were utilised as the reference strain of positive biofilm producers. following techniques were used to find biofilms:

2.2.1 Tube Adherence Method (TAM)

Test organisms were added to 10 ml of trypticase soy broth and 1 percent glucose in borosilicate glass tubes that were made from culture plates that had been stored overnight. These tubes were then incubated for 24 hours at 37°C in an aerobic atmosphere. The tubes were emptied, rinsed with a 7.3 pH solution of phosphate-buffered saline and then dried after incubation. After staining, any extra solution was wiped away, and then rinsed with distilled water and subjected to drying upside-down. Figure 1 shows how a visible film covering the tube's walls and bottom signified effective biofilm development. Three grades - Grade 1, Grade 2, and Grade 3 - were used to describe the degree of biofilm development: Grade 1 denoted Weak or No biofilm production, Grade 2 denoted Moderate biofilm production, and Grade 3 denoted High or Strong biofilm production[21].

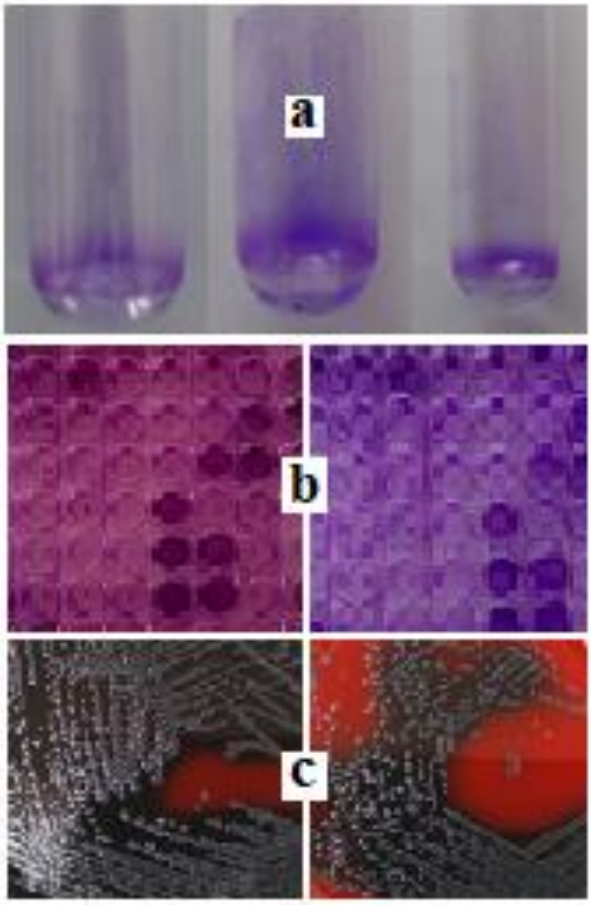

Figure 1:Biofilm production detection by a. TAM,b. TCP andc.CRA method

Tissue culture plate method

The experiment was started by inoculating isolated organisms from freshly made agar plates with 12 mL of soy broth of trypticase that contained one percent solution of glucose.These broths had been then subjected to incubation at a temperature of 37°C for 24 hours. Following this incubation period, a fresh medium was prepared by diluting the cultures at a ratio of 1:100.Subsequently, 200 µL of the diluted cultures were dispensed into each well of sterile 96-well flat-bottom polystyrene tissue culture plates (Sigma-Aldrich, Costar, USA). The control organisms were also subjected to the same procedure of incubation, dilution, and placement into tissue culture plates. Negative control wells contained sterile broth for inoculation.The plates were then incubated once more, this time for 24 hours at 37°C. After the incubation period, the contents of each well were carefully aspirated by gentle tapping. To eliminate free-floating microorganisms, a total of four washes, each with 0.3 mL of a 7.3 pH saline buffer in phosphate, were performed.Any bacterial biofilm that had developed on the well surfaces was preserved using a 2 percent sodium acetate solution and subsequently stained with crystal violet (0.1 percent). Excess staining was removed with deionized water, and the plates were allowed to air-dry. Finally, the optical density (OD) of the stained adherent biofilm was measured at a wavelength of 570 nm using a microELISA (Enzyme Linked Immunosorbent Assay) autoreader (model 680, Biorad, UK). This entire experiment was conducted in triplicate to ensure accuracy and reliability of the results[21].

2.2.3 Congo Red Agar method (CRA)

A previous study [21, 22]introduced a simple qualitative method for detecting biofilm development using Congo Red Agar (CRA) media. To prepare the CRA medium, Oxoid's brain heart infusion broth from the UK was utilized, consisting of 50 g/L of sucrose, 10 g/L of agar No.1, and 8 g/L of Congo Red indicator. The first

step involved creating a concentrated aqueous solution of Congo Red stain, which was autoclaved separately from the other components at 121°C for 15 minutes. Subsequently, the autoclaved brain heart infusion agar with sucrose was mixed with the Congo Red solution at a temperature of 55°C. Test organisms were then inoculated onto five CRA plates, followed by aerobic incubation for 24 hours at 37°C. Biofilm formation was indicated by the presence of black colonies exhibiting a dry, crystalline appearance.To ensure the reliability of the results, the experiment was conducted in triplicate, with three repetitions of each run. This methodology serves as a straightforward and effective means for qualitative biofilm detection using CRA media.

3. Results And Discussion

500 urine samples from patients with UTI who were hospitalised were examined in total. Gram-negative bacteria were found in 69% of the specimens, whereas Gram-positive growth was observed in 31% of the specimens. After *Klebsiella pneumonia*, *E. coli* was the most often isolated organism. The most prevalent isolate of Gram-positive bacteria was *Enterococcus faecalis*. Out of five hundred cases, 81 (16.2%) clinicalisolates demonstrated biofilm development using the TCP method, 68 (13.6%) clinical isolates demonstrated biofilm formation using the Tube Adherence Method, and 60 (12.0%) isolates shown biofilm formation using the MCRA method. *Klebsiella pneumoniae*and *Escherichia coli*produced the most biofilms, followed by *Enterobacter* spp. and *Klebsiella* spp.

Table 1:Biofilm-producing uropathogenic*Escherichia coli* and the symptom distribution

UTIs Symptoms	**Patients count**	**Number of producers of biofilm**
Burning sensation during urination	256	48
Abdominaldiscomfort and pain	141	33
Fever	147	60
kidney stone / Anuria or tumour	68	24
Frequency	173	63
Trouble urination	85	11
Dysuria	77	48
Haematuria	46	18
Urgency	94	49
Renal stone	76	8
Chi test=14.61df 1, p value < 0.0001, Denoted Significant		

Biofilms present a significant wellbeing concern owing to the augmented resilience of microorganisms within them to antibiotic drugs. This resistance, coupled with the potential for these biofilm-associated organisms to infect individuals with implanted medical devices, underscores the seriousness of the threat they pose. Unlike planktonic bacteria, which can be killed by antimicrobial agents at relatively low concentrations, biofilm bacteria typically require concentrations 1000 to 1500 times higher for effective eradication. This high incidence highlights the persistent challenge posed by biofilms in medical settings, where these microbial communities can thrive and lead to infections that are often difficult to treat. Consequently, combating biofilm-related infections is a crucial aspect of safeguarding public health and improving patient outcomes in the context of medical procedures involving indwelling devices.

Gram negative organisms made up the majority of the 500 specimens that were examined. More than half of the urine samples contained *Escherichia coli*, which was trailed by *K. pneumoniae* and *P. aeruginosa*. Resistant *Escherichia coli* made up 36.40% of the total number of *Escherichia coli* isolates. The results of earlier research in the same field are consistent with the ones we have just seen. *Escherichia coli* and *Klebsiella pneumoniae* were also identified in Indian investigations as the main uro-pathogens. The current study also produced the same outcomes.

Table 2:Microbes found in the isolates and their distribution

Organism	Resistant *Escherichia coli*	*Escherichia coli*	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*	*Enterobacter spp.*	*Staphylococcus aureus*
Isolates	182	241	133	124	194	97
Percent	36.40%	48.20%	26.60%	24.80%	38.80%	19.40%

Table 3: Distribution of biofilm formation by microorganisms in the present study

Organism	Resistant *Escherichia coli*	*Escherichia coli*	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*	*Enterobacter spp.*	*Staphylococcus aureus*
Total Isolates	182	241	133	124	194	97
Biofilm Producers	**41**	-	-	-	-	-
Percent	22.53%	-	-	-	-	-

The highest levels of biofilm development were observed in isolates of resistant *Escherichia coli*. Previous research (Praharaj et al. 2013) identified *Enterococcus* species as the main biofilm generator which indicated 53 percent of the isolates of *Enterococcus* spp. asbiofilm developers. In the current investigation, 36.40% of Resistant *Escherichia coli* isolates and 48.20% of *Escherichia coli* isolates showed evidence of biofilm development. The observed phenomenon can be attributed to the geographical context of the current study. The samples under examination were obtained from patients residing in tropical regions, where Escherichia coli (E. coli) has been consistently identified as the predominant causative agent of urinary tract infections (UTIs). This regional prevalence of E. coli as the primary pathogen responsible for UTIs is a well-documented epidemiological pattern. Consequently, it is not surprising that the study's findings align with this established trend, further underscoring the importance of regional variations in microbial etiology when investigating UTIs.

Table 4:Biofilm detection in uropathogenic infections (*Escherichia coli*clinical isolates) that are drug-resistant utilizing 3phenotypical techniques.

Methods	**Tissue culture plate method**	**Tube adherence method**	**Modified congo red agar method**
Number of biofilm producers	81	68	60
Percentage of biofilm producers	16.2%	13.6%	12.0%

Table 5:Risk factors associated with uropathogenic*Escherichia coli* that produce Biofilm.

Related risk component	Patients with UTIs in total	Producers of biofilms in number
Diabeties	92	38
Immuno suppression	70	33
Hypertension	72	26
LSCS	134	34
prolonged cathertization	397	21
Recurrent UTI	71	11
Chi test=14.11 df 1, p value =0.0007, The result is "Significant" because the *p* value is less than the threshold for significance, which is 0.05 or less.		

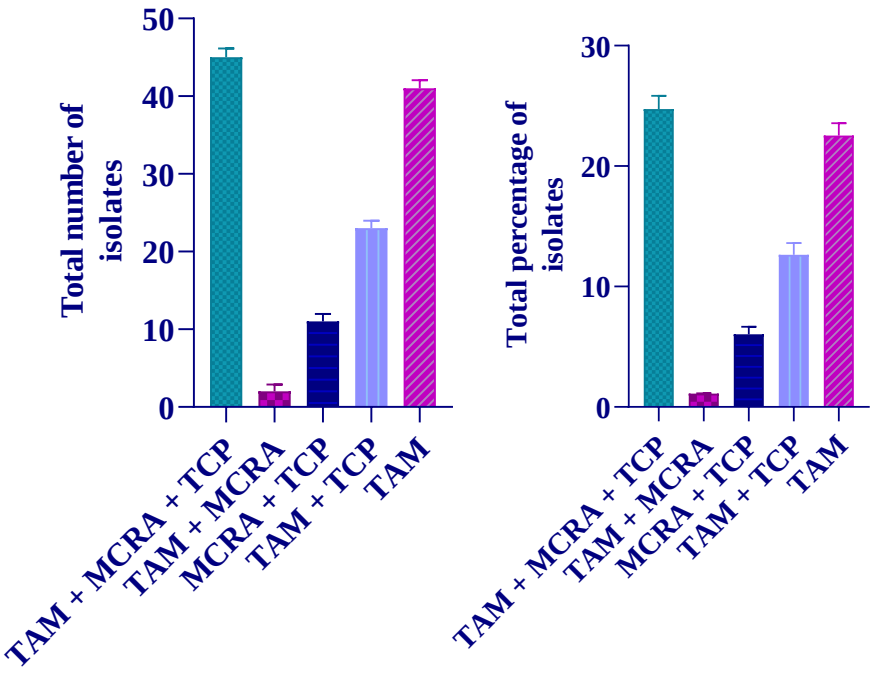

Figure 2:Rate of Biofilm Production in the Modified Congo Red Agar Method (MCRA), the Tube Adherence Method (TAM), and the Tissue Culture Plate Method (TCP) (Total Number of Positive Strains, N=182)

The conventional approach, TCP method, identified 81 Biofilm makers out of 500 samples. The predominant microorganism associated with the formation of biofilms was *Escherichia coli* (*E. coli*), comprising the majority of the organisms involved in this process. It was followed in prevalence by *Klebsiella pneumoniae* (*K. pneumoniae*), *Staphylococcus aureus* (*S. aureus*), and *Pseudomonas aeruginosa* (*P. aeruginosa*). These findings are in accordance with existing reports that highlight *E. coli* and *Staphylococcus epidermidis* (*S. epidermidis*) as notable biofilm producers, particularly on Foley's urinary catheters. Remarkably, these biofilms tended to develop predominantly in immunocompromised patients who were also susceptible to several antibiotics, including meropenem, aztreonam, vancomycin, and linezolid. This observation underscores the challenge posed

by biofilm-related infections in vulnerable patient populations, where these resilient microbial communities thrive and become resistant to multiple antimicrobial agents. Understanding the specific microbial profiles and antibiotic susceptibility patterns associated with biofilm formation is essential for devising effective strategies to combat such infections, particularly in immunocompromised individuals. According to reports, *S. epidermidis*, which is mostly sensitive to linezolid and vancomycin, is the cause of significant Biofilm development in patients with intravenous catheters. There were 68 capable biofilm producers according to TAM. The MCRA method yielded somewhat different results, with 60 isolates showing colonies that were black in colour and seemed to be crystalline (Table 4 and Figure 2).

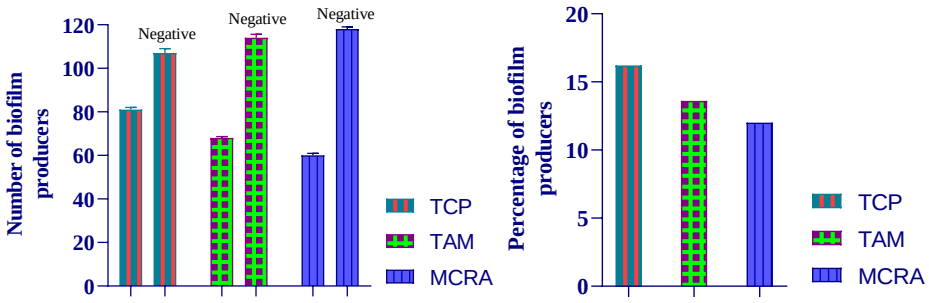

Figure 3: Comparison of the Diagnostic Effectiveness of the Techniques for Biofilm Formation Detection.

Statistical analysis

Data collection was carried out meticulously in triplicate, as required, to ensure accuracy and reliability. Subsequently, a thorough statistical analysis was conducted to assess the significance of the findings. To determine the statistical significance of the data, a variety of tests were employed, including the One-Way Analysis of Variance (ANOVA), the Chi-Square Test, and Fisher's Exact Test. These statistical methods were chosen based on the specific requirements of the data and research objectives, ensuring that the results were robust and trustworthy.

4. Discussion

Biofilm-developingmicrobes or bacteria are accountable for numerous stubborn contagions, and eliminating them poses a considerable challenge. These bacteria exhibit antibiotic resistance through various mechanisms, such as hindered antibiotic penetration within the biofilm, slowed growth rates, and the production of resistance genes[9, 23-25]. Detecting biofilms is crucial for effective management and treatment. Multiple techniques are available for biofilm detection. These methods enable researchers and healthcare professionals to identify and assess biofilm presence, aiding in the development of appropriate strategies for combating biofilm-related infections. In the current investigation, the capacity to produce biofilms of 500 isolates was assessed using three screening techniques. There were 81 isolates that formed biofilms using the TCP technique (16.2 percent). We used the TCP approach, which involves adding 1% glucose to trypticase soy broth. Sugar addition aids in the development of biofilm. A total of 68 (13.6%) isolates were identified by the tube adherence technique as biofilm producers. In the context of identifying high biofilm producers, this approach showed strong agreement with the Tissue Culture Plate (TCP) method. However, a notable challenge arose as the results were inconsistently interpreted by different observers, making it difficult to distinguish between moderate, weak, and non-biofilm producers accurately. Based on insights from previous research investigations, it is challenging to recommend the Tube Adherence Method (TAM) as a universally applicable screening test for isolates that produce biofilm. These findings highlight the variability in results obtained through different biofilm detection methods and emphasize the importance of selecting an appropriate approach based on the specific requirements of the study or clinical situation.They demonstrated that TAM is superior to MCRA at detecting biofilms. Less effective correlation was seen between the MCRA approach and the other methods and parameters. In the

current study, when comparing the TCP technique to both the TAM and the MCRA technique, it was observed that the TCP technique exhibited a higher sensitivity in detecting the formation of biofilms by *Escherichia coli*. This suggested that when compared to the other two techniques, TAM and MCRA, the TCP Method was more successful at detecting the growth of *E. coli* biofilms.

5. Conclusion

Our study has shown that the TCP approach is a quantitative and trustworthy way to find bacteria that produce biofilms. The Tissue Culture Plate (TCP) Method emerges as a practical and versatile screening approach for identifying biofilm-producing bacteria in laboratory settings when compared to the Tube Adherence Method (TAM) and the Modified Congo Red Agar Method (MCRA). In conclusion, biofilms play a substantial role in the recurrence of urinary tract infections (UTIs), contributing to heightened patient morbidity, prolonged hospitalization periods, and increased financial burdens. Addressing biofilm-related infections is essential for improving patient outcomes and reducing healthcare costs. Rarely has the production of biofilms by the whole spectrum of uro-pathogens been investigated in uncomplicated UTI. Further research is imperative to gain a deeper understanding of how biofilms influence the progression of simple urinary tract infections (UTIs) into chronic UTIs, particularly when the infection is only partially cleared. One promising approach that offers both good reproducibility and specificity in studying biofilm formation is the Tissue Culture Plate Method (TCP). Investigating the role of biofilms in the persistence and recurrence of UTIs can provide valuable insights into the development of more effective treatments and preventive measures for chronic UTIs, ultimately improving patient care and outcomes.

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6. Reference

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