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Research Article

DIFFERENTIATION OF BACTERIAL BIOFILM BY TUBE ADHESION METHOD USING VARIOUS STAINS

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ABSTRACT

Microbial pathogens have a survival capability in a variety of environmental conditions and adhere themselves with different surfaces, thus forms a matrix like structure called biofilm. These biofilms provide unconditional environment for the pathogenic microbes that have barrier to the migration of drugs, thus facilitate the microbes to resist towards various antimicrobials. Thus, this study was designed to analyze comparatively about the biofilm formation among the antimicrobial resistant bacterial strains by test tube adhesion method using seven different stains where crystal violet act as a control. The drug resistant bacterial pathogens were allowed for biofilm production and determine it on the surface of test tubes using crystal violet, methylene blue, nigrosin, safranin, Gram's iodine, leishman, giemsa stains. The results revealed that the observation and recording of biofilm deposit on the test tubes showed better and maximum while using crystal violet followed by methylene blue and safranin. The test bacterial pathogens like *Klebsiella* sp, *Proteus vulgaris* and *S. aureus* showed maximum adhesion and adsorption of the stains.

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INTRODUCTION

The stay of patients in hospitals or home need aseptic environment in order to heal the infection effectively and/or avoid spreading. Fomites play a vital role for this purpose and usage of highly disinfected equipments should be handled. These biomedical device induced infections are the most embarrassed situation in healthcare industry for avoiding additional morbidity and mortality^[1,2]. These indwelling devices, prosthetics, water pipelines, tubes on the endoscopes and on wounds are the major source of infection to be spreaded among the patients both in biotic and abiotic surfaces^[3,4].

Water, a major media for the accumulation and proliferation of various microbial entity on the surface of devices, thus form a stable biofilm formation^[5]. Biofilm, an aggregation of microorganisms evacuated in any surfaces where the suitability and sustainability occurs, it was determined the availability of the nutrients, chemotaxis near the surface, surface adhesion and

presence of surfactants^[6,7,8], thereby, not all microbes can able to produce biofilm^[9]. The awareness related to biofilm formation in the health care environment also to be considered as a major source of various nosocomial infections either directly or indirectly^[10,11,12]. The mechanism of formation of microbial biofilm starts from single colony, micro-colonies, thick macroscopic biofilm matrix and end up with detachment from the surface^[13,14].

The structure and composition of the biofilm was enhanced by the communication between the microbial cells; where these matrix was observed in both live and dead host cells^[15,13]. *Staphylococcus aureus* is considered as a highly virulent pathogen that are capable to resist towards visceral generations of antibiotics; thus it is difficult to treat the biofilm producing *S. Aureus* that are observed in patients with endocarditis and osteomyelitis who are largely depend on medical devices for their survival^[16]. Some other bacterial pathogens including *Pseudomonas aeruginosa*, *Escherichia coli*, *Proteus* sp.,

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Acinetobacter baumannii are also observed to produce trace to thick biofilm in medical devices [17]. The multi-variant microbial occurrence in the biofilm is intrinsically highly resistant to antimicrobial agents compared to a colony. To overcome this issue, the pharmaceutical industries have to be addressed for the synthesis and production of broad spectrum antimicrobials agents with multi-targeted receptors [5,18,19].

In general, the detection of biofilm production itself helps to recognize virulent pathogen, severity of the infection and antimicrobial resistance [20]. Determining the biofilm quantification is tough in the earliest stage due to the complex structure that creates heterogeneous and sometimes transparent biofilms [21,22]. To understand the literature, this study has an objective to detect and differentiate the biofilm forming nature of the bacterial pathogens by tube adhesion method using various stains.

Experimental Section

Specimen Collection and Culturing of Sample

Pus and wound swab samples were collected and kept in the transport media and transferred to the laboratory for microbiological processing by culturing in Nutrient agar, MacConkey Agar, Blood agar and Potato Dextrose Agar (PDA) plates. Processed plates were kept for incubation at 37°C for 24 hours, except PDA plates which was kept at room temperature for 2 to 3 days.

Identification of Microbial Isolates

After appropriate incubation, the colonies were identified using standard microscopic, colony determination and biochemical tests. As a result, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Micrococcus* sp, *Klebsiella* sp and *Proteus vulgaris* were identified. The bacterial isolates were maintained as stab culture and refrigerated at low temperature until use.

Antimicrobial Susceptibility test

All the bacterial isolates were tested against specific antibiotics by standard Kirby Bauer disc diffusion method. Further, the antibiotic resistant bacterial strains were selected to determine the biofilm forming nature. The bacterial isolates that are showing resistance towards minimum of three antibiotics were impregnated for analyzing the biofilm forming nature. Also, this study emphasized to compare the biofilm determination using various stains, where crystal violet acted as stain control.

Tube Adhesion Method

The antibiotic resistant bacterial isolates were subjected for tube adhesion method in order to determine the visual biofilm deposits. All the test pathogens were dispensed into the sterile nutrient broth and incubated overnight at 37°C. After incubation, the broths were poured off and the tubes were washed with sterile distilled water. One percent solution of Crystal violet, Methylene blue, Safranin, Gram's Iodine, Leishman, Giemsa and Nigrosin stains were prepared freshly and added into the dried test tubes and allowed to adhere for 15 to 20 minutes. All the stains were washed with sterile distilled water and dried. The dried and stained deposits were considered as the biofilm; where the levels of biofilming were

determined by 1+ to 5+ based on the trace to thick deposition respectively [23].

RESULT AND DISCUSSION

The results of antimicrobial susceptibility pattern of the clinical isolates against a gallery of specific antibiotics showed visceral observation. The mechanism of adhesion of bacteria on the surface of biomedical devices or even in damaged tissues is observed in concealed manner in a hydrated matrix is defined as biofilm. These forms of microbial entities having the ability to resistant against various routine and new generation group of antibiotics [24]. The study reported the biofilm producing bacteria are *E. coli*, *P. aeruginosa*, *B. subtilis*, and *S. aureus* [25], this study also supported the same that isolates including *S.aureus*, *E. coli*, *Klebsiella* sp, *Micrococcus* sp, *Proteus vulgaris* and *P. aeruginosa* producing biofilms.

In this study, the sensitive, intermediate and resistant patterns of antimicrobial nature were observed, but to fulfill the objective, we took the bacterial strains that are resistant to more than three antibiotics. The community and hospital acquired *S.aureus* showed high levels of resistance against antibiotics including Ampicillin (97.8%), tetracycline (80.4%) and gentamycin (69.6%) [26]. A research report recorded that *E. coli* resist against amikacin, genatmycin, cefotaxime, nitrofuratoin, whereas nalidixic, cefotaxime and amikacin resisted by *K.pneumoniae* and *P. vulgaris* [27].

Next to *S. aureus*, the hospital acquired infectious bacteria - *P.aeruginosa* are also evinced with the drug resistant characters by showing non-activeness towards amikacin, genatmycin, cefotaxime and amikacin [28]. We also stated the resistance nature of bacterial isolates was highly resisted against antibiotics, in this gentamycin, cefoxitin and amikacin. The strains of *S. aureus* showed resistant to gentamicin, cefoxitin and ampicillin. Next to *S. aureus*; *E. coli*, *Klebsiella* sp, *Micrococcus* sp, *Proteus vulgaris* and *P. aeruginosa* were identified as antibiotic resistant strains. The detailed antibiotic resistant patterns were depicted in table 1.

Among the biofilm detection procedures, the tube method was highly preferable method for the quantification of biofilm production compared to other classical methods [12]. In the test tube method, the usage of Crystal violet stain for biofilm detection provide visual outlook of the microbial cells that attached on the surfaces by mild to deep coloration [29]. As per the literature to be concerned, the comparative analysis of various methods of biofilm formation are available but determination of effective stain for biofilm detection was not much found. A study highlighted the nanoparticle encapsulated Methylene blue stain was used to eradicate the biofilm suspended cultures of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Acinetobacter* sp [30].

Table 1 Antimicrobial activity against antibiotics

Bacterial pathogens	Antibiotic resistance pattern
<i>Staphylococcus aureus</i>	Ampicillin, cefoxitin and gentamycin
<i>Escherichia coli</i>	Ampicillin, cefixime and gentamycin
<i>Micrococcus</i> sp.	Erythromycin, streptomycin and chloromphenicol
<i>Klebsiella</i> sp.	Cefoxitin, gentamycin and cefixime
<i>Proteus vulgaris</i>	Gentamycin, nitrofurantoin and cefotaxime
<i>Pseudomonas aeruginosa</i>	Amikacin, ciprofloxacin and gentamycin

All the resistant bacterial strains were tested for the biofilm formation by Crystal violet as standard stain and the same was compared with other stains like Safranin, Gram's Iodine, Methylene blue, Leishman, Giemsa and Nigrosin. This study also determined that the usage of Methylene blue as second line stain next to Crystal violet which was well analyzed in the study where the microbial biofilm have an ability to observe Methylene blue [30].

The maximum biofilm was observed in *Klebsiella* sp, *Proteus vulgaris* and *S. aureus* and that was adhered by stains like Crystal violet followed by Methylene blue and Safranin; whereas Giemsa, Nigrosin and Leishman stains are not absorbed. Low level of adhesion was observed while using Gram's iodine (Figure 1 and Figure 2).

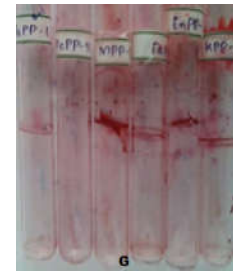


Figure 2 Visual observation of biofilm forming nature of bacterial pathogens

[A-Crystal violet; B-Gram's Iodine; C-Giemsa; D-Leishman; E-Methylene Blue; F-Nigrosin; G-Safranin]

Overall in this study, the biofilm determination was highly observed using Crystal violet, Methylene blue and Safranin when compare with other stains. So, as we conclude after crystal violet, methylene blue may be the preferable alternative stain for biofilm determination. Further, an extensive works are needed to identify the appropriate, easy and expedient method of determining the biofilm using software technology and also analyze the exact implementation of cost effective, eco-friendly stains for this purpose.

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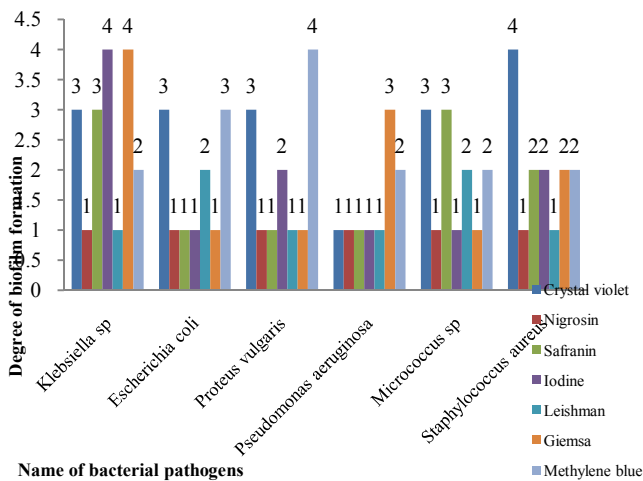
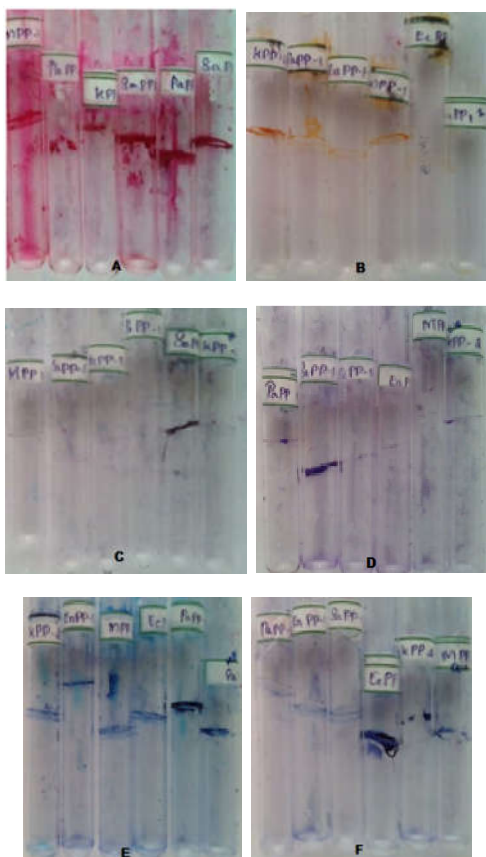


Figure 1 Level of biofilm formation

[1- mild; 2- moderate; 3- high and 4 – very high]



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