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Virulence Characteristics and Biofilm Production in Clinical and Environmental Isolates

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Abstract

Microbes that grow in a biofilm are associated with chronic and acute human infections that are highly resistant to antimicrobial agents. Soil, water and clinical samples were collected and 62 isolated organisms were screened for biofilm formation and their virulence characteristics were determined. Sixty two isolates were studied for proteinase production, hemolytic activity, DNase production, serum susceptibility and cytotoxic activity. Out of all the isolates grown overnight 22 (35.48%) were able to hydrolyze casein at 37 ° C. About 26 (41.94%) isolates presented β hemolysis. About 16 (25.81%) isolates showed positive for DNase activity. Cytotoxic activity was presented positive for about 16 (25.81%) isolates and 20 (32.26%) isolates were significantly more resistant to the bactericidal activity of Normal Human Serum. From the total of 62 isolates crystal violet binding assay method detected 45 (72.58%) were biofilm producers. Among 24 pathogenic isolates crystal violet binding assay detected 4 (16.67%) as strong, 13 (54.16%) as moderate and 7 (29.17%) as weak or non- biofilm producers. It was observed higher pathogenicity in biofilm producing bacteria than non- biofilm producers. From the study it was concluded that the majority of the biofilm producers were from clinical isolates.

Keywords: Clinical, environmental samples, virulence characteristics, biofilm assay.

1. Introduction

Microbially derived sessile communities that are characterized by the cells irreversibly attached to a substratum is called as biofilm [1]. They bound in a matrix of extracellular polymeric substances (EPS) [13]. Biofilm are seen ubiquitously, in medical, industrial, food and other natural settings where bacteria exists [8]. A recent study states that approximately 80% of bacterial infections occurring in the human body are biofilm- mediated [5]. They are associated with many medical factors which include indwelling medical devices, peritonitis, upper respiratory tract infections, dental plaque and urogenital infections [29]. Both Gram- positive and Gram- negative bacteria have the ability to produce biofilms. Bacteria commonly involved include, *Escherichia coli, Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Enterococcus faecalis , Proteus mirabilis and Klebsiella pneumoniae* [11]. The three factors which determine i an infection in a host [15,16] are: particular microbe the host contacts, the virulence component of the microbe and resistance of the target organ system of the host. The evaluation of health risk for each microbe concerns the vehicle of delivery, nature of its pathogenesis, the capability of the host response and the microenvironment that activates the virulence factors [15,14,17,18,23,30]. The are many direct ways to assay the number of microbes that challenge the host [28]. It was described that bacterial pathogenesis are associated with several extracellular enzymes and microbial products [15,17,18,23,20] . Some of the enymes include elastase, which dissolves cellular glue; proteinase, that disrupts the body proteins; coagulase, that sequester a microbe; gelatinase, that hydrolyses the body proteins; DNase, which destroys the nucleic of cells; haemolysin, which destroys red blood cells; lipase, that dissolves lipids; and lecithinase which dissolves a cell membranes [18,20,7,21,27]. The methods to

detect biofilm production are: Tube method [10], Piezoelectric Sensors [2], Congo Red Agar method (CRA) [19], Bioluminecsence assay [12], tissue culture plate (TCP) [9] and Fluorescent microscopic examination [32]. It is concluded from the recent study that tissue culture plate method is a more quantitative and effective method for the detection of biofilm producing microorganisms [1]. In this paper we aimed to isolate the pathogenic organisms which serve as most potent biofilm producer for the potential application in hospitals.

2. Materials and methods:

2.1 Isolation and Identification:

A total of 199 isolates from environmental (soil-garden soil, municipal waste soil and water- oil spilling area, kitchen waste water) and clinical sources (blood, pus, sputum, urinary catheter tips and swabs of hospitalized patients) were tested with phenotypic assays (Table 1). The clinical specimens received were mainly from pateints with nosocomial infections. The collected samples were inoculated on appropriate culture media (MacConkey agar, Nutrient agar, King B agar, Hirrella agar and Starch agar) and incubated overnight at 37 ° C. Bacterial isolates were identified by standard microbiological procedures (Gram staining, colony morphology, catalase test, oxidase test and other biochemical tests).

2.2 Virulence Assays:

Protease assay:

Nutrient broth with skim milk agar was the substrate. Colonies were inoculated onto the surface of the agar and incubated for 72hrs at 37 ° C. A zone of clearance around the bacterial inoculum was evidence of a positive test [31]

Haemolysin assay:

Isolates were inoculated on Blood Agar plate assay with human defibrinated blood at 5% and incubated at 37 ° C for 24hrs. Haemolysis around the bacterial growth was observed [6].

DNase activity:

DNase agar (HiMedia) supplemented with 0.01% toluidine blue was used as a substrate. Bacterial colonies were inoculated on the surface of the agar and incubated for 72hrs at 37 ° C. Appearance of red colour or the zone of clearance observed after the addition of 0.1% 1N HCl represented a positive test [26].

Serum susceptibility:

Pooled sera of group ‘o’ blood were separated and used immediately. The 62 isolates were challenged against 65% of Normal Human Serum (NHS) in a microcolorimetric assay [25]. Cell culture were transferred to microdilution well containing 100µl of Peptone Glucose Broth. After overnight incubation at 37 ° C, 20µl of each PGB culture was transferred to 200µl of fresh PGB and incubated at 37 ° C for 2-3hrs. Log phase bacteria were then inoculated (20µl) into 100µl PGB containing 65% NHS and 0.5% 0f 1.5µl of stock solution of bromothymol blue. Serum resistance was assayed by visible colour change from green to yellow. Control consisted of PGB with 65% heat inactivated serum 56 ° C for 4-5hrs. The OD at a wavelength of 490nm was determined using a micro ELISA auto reader (Cyber viewer).

Cytotoxicity analysis:

HeLa cancer cell line was grown and maintained in a humidified incubator at 37° C in a 5% CO2 atmosphere. Modified Eagle's medium supplemented with 10% Faetal Bovine Serum, 100unit /ml of penicillin and 100 µg/ml of streptomycin was used for cell culture. Cells were incubated in 96- well plates containing 100µl of the growth medium per well for 24hrs and then treated with various concentrations of 62 isolates dissolved in medium incubated for 48hrs. 20µl of 5mg/ml MTT in phosphate buffered saline were added and the plate was incubated at 37 ° C for 4 h and the absorbance at 590nm was measured by a microplate ELISA reader (Cyber viewer) [22].

2.3 Microtitre plate method of biofilm assay:

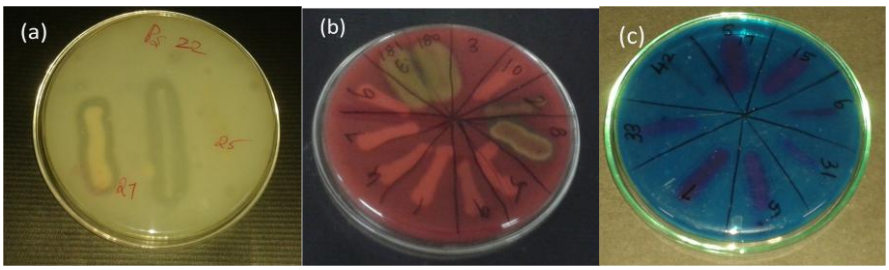
Isolates were grown overnight in Nutrient Broth (HiMedia) at 37 ° C and aliquots of 200µl of diluted bacterial suspension was inoculated in each well of sterile 96 well-polystyrene microtitre plate and incubated for 24hrs at 37 ° C. After the incubation period, the wells were gently aspirated, washed thrice with 200µl phosphate buffered saline (PBS), pH 7.2 to remove planktonic cells. The biofilms were fixed with methanol for 15-30min. The biomass quantification of the biofilms was performed by staining with 100 µl of 1% crystal violet for 15 minutes [4]. The crystal violet was then removed and the wells were rinsed with distilled water to remove non-attached material. Biofilm was quantified by the addition of 200µl of 33% glacial actetic acid to the crystal violet stained cells. The optical density at a wavelength of 570nm was determined using a ELISA auto reader (BioRAd, USA). The OD values were denoted as an index of bacteria adhering to surface and forming biofilms. Isolates were detected as strong OD_{570} >2; medium OD_{570} , 1 to 2; or weak $OD_{570,}$ greater than 0.5 but less than 1 [24,3].

3. Results:

3.1 Virulence test results:

Table 2 presents the percentage of isolates possessing positive virulence characteristics for each environmental and clinical source. Of the 62 isolates tested 29 (46.78%) showed virulence effect. Among the 29 pathogens 24 (120.83%) were observed as biofilm producers. Out of 29 pathogens majority of the isolates were showed positive for haemolysis 26(111.54%) (**Figure 1b**) and about 22 (75.86%) hydrolysis casein (**Figure 1a**). About 20 (68.96%) isolates were significantly more resistant to the bactericidal activity of Normal Human Serum (**Figure 1d**). DNase production was observed in 16 (55.17%) isolates (**Figure 1c**).

Figure 1:



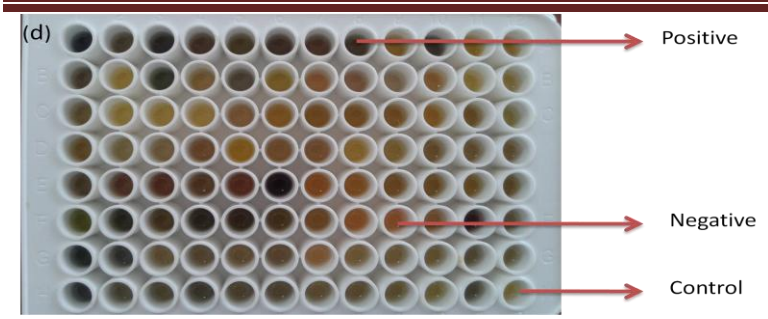


Figure 1: a) A zone of clearance showing positive for protease assay (*Pseudomonas aeruginosa*) b) β-Haemolysin zone formation (*Pseudomonas aeruginosa and serratia marcescens*) c) Pink zone formation representing DNase activity (*Serratia marcescens*) .d) Serum susceptibility assay presenting positive(*Pseudomonas aeruginosa and serratia marscenes)* and negative isolates (*Acinetobacter baumanii , Serratia marcescens*)

Cytotoxicity test results:

All the 62 bacterial isolates were examined for its ability to damage HeLa cells. **Table- 2** presents the results of these cytotoxicity tests.

Table 2: Virulence characteristics of bacteria isolated from Environmental and Clinical Sources.

No. & Percentage of Positive Isolates		
Characteristics	**Environmental Isolates(30)**	**Clinical Isolates (32)**
Haemolysin	8 (26.66)	18 (56.25)
Protease	7 (23.33)	15 (46.88)
DNase	4 (13.33)	12(37.50)
Serum susceptibility	5 (16.66)	15 (50.00)
Cytotoxicity	5 (16.66)	11 (34.37)

3.2 Biofilm producers:

Among 62 isolates tissue culture plate method detected 45 (72.58%) isolates as positive. Out of 45, it was quantified 4 isolates (8.88%) as strong, 28 (62.22%) as moderate and 13 (28.88%) as weak biofilm producers (**Figure 2**) (**Table 3**). The majority of the organisms associated with biofilm production were, followed by *Pseudomonas aeruginosa, Serratia marcescens* and *Acinetobacter baumanii.* The majority of the biofilm producers 55.56% (25/45) were from clinical sources and 44.44% (20/45) were evaluated as the biofilm producers isolated from environmental samples. Among 24 pathogenic isolates biofilm production was highly noticed in clinical isolates 75.00% (18/24) and environmental it was 25.00% (6/24). Out of 4 strong biofilm producing pathogenic isolates *Pseudomonas aeruginosa* was observed as the major biofilm producer 75.00% (3/4).

Figure 2: Quantitative assay for biofilm formation on microtitre plate showing weak, strong and moderate isolates.

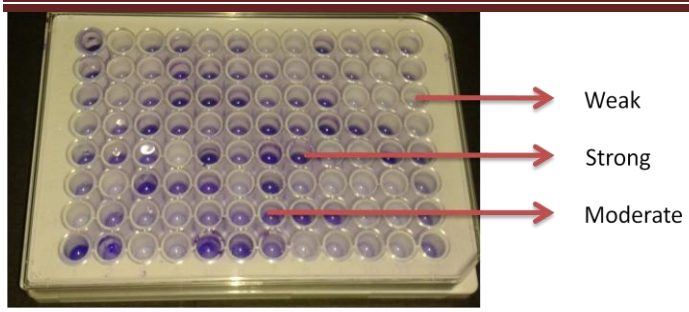


Table 3: Quantification of Biofilm formation among Environmental and Clinical Isolates

			No. & % of Positive Isolates		
Sources	**Tested**	**Positive**	**Weak**	**Moderate**	**Strong**
Environmental	30	20 (66.67%)	8 (40.00%)	11(55.00%)	1(5.00%)
Clinical	32	25 (78.13%)	5 (20.00%)	17(68.00%)	3 (12.00%)
Total	62	45 (72.58%)	13(28.88%)	28 (62.22%)	4 (8.88%)

Table 3: Biofilm production of pathogenic isolate in clinical and environment sources

Organism	**Biofilm Production**	**Sources**	
		Environment	**Clinical**
Pseudomonas aeruginosa	Moderate		Pus
Serratia marcescens	Strong	Kitchen waste water	
Pseudomonas aeruginosa	Weak		Blood
Pseudomonas aeruginosa	Moderate	Municipality waste soil	
Pseudomonas aeruginosa	Weak	Municipality waste soil	
Pseudomonas aeruginosa	Moderate		Blood
Pseudomonas aeruginosa	Strong		Sputum
Pseudomonas aeruginosa	Weak		Urinary catheter
Pseudomonas aeruginosa	Moderate		Hospital Swab
Serratia marcescens	Weak	Municipality waste soil	
Serratia marcescens	Moderate		Urinary Catheter
Serratia marcescens	Moderate		Urinary Catheter
Pseudomonas aeruginosa	Moderate	Oil spilling area	
Pseudomonas aeruginosa	Moderate		Intravenous Catheter

Pseudomonas aeruginosa	Moderate		Blood
Pseudomonas aeruginosa	Moderate		Hospital Swab
Pseudomonas aeruginosa	Moderate		Hospital Swab
Pseudomonas aeruginosa	Strong		Sputum
Pseudomonas aeruginosa	Moderate		Pus
Acinetobacter baumanii	Weak	Oil spilling area	
Organism	**Biofilm Production**	**Sources**	
		Environment	**Clinical**
Pseudomanas aeruginosa	Strong		Sputum
Serratia marcescens	Weak		Hospital Swab
Serratia marcescens	Moderate		Urinary Catheter
Acinetobacter baumanii	Weak		Hospital Swab

4. Discussion:

The result of this study offers an explanation for the increased incidence of the virulence effects in clinical strains than the bacteria isolated from environmental sources. The clinical strains showed evidence of more conserved virulence-associated mechanisms, such as haemolysin, protease, cytotoxicity, serum susceptibility and biofilm production [31,6,22,4]. Further underscoring the difference between the environmental and clinical strains, the comparison of this population indicates that the environmental group produces significantly less biofilm than the clinical group. Biofilm producing bacteria plays a major role in causing many recalcitrant infections, hence they are notoriously difficult to eradicate [1]. In this study we examined 62 isolates (clinical and environmental sources) by microtitre plate method for their [4,24,3] ability to form biofilms and analyzed that the majority of biofilm producing bacteria were from clinical sources (pus, sputum, urinary catheter, blood, swab). *Pseudomonas aeruginosa* is concluded as the most potent biofilm producing strain isolated from clinical source.

We have compared the environmental and clinical sources to completely analyse the origin of biofilm effective producer. From our study we conclude that biofilm production is highly observed in clinical isolates than the environmental microbes. Among the clinical sources the isolates from sputum and urinary catheter are observed as the strong biofilm producers. We also observed the high percentage of biofilm production in pathogens than from non- virulent microbes.

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