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Comparison of the Analytical Methods Employed in the Detection of Biofilm-Forming Bacterial Pathogens.

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Page | 3396

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ABSTRACT

Bacterial biofilm is a matrix of microorganisms, usually pathogenic that are incorporated in a polysaccharide structure. Biofilms usually confer resistance on bacteria, making their presence in infections detrimental. This study aimed at evaluating the sensitivity of three analytical methods (Tissue Culture plate-TCP, Tube method-TM and Congo Red Agar-CRA) in the detection of biofilms from clinical isolates as well as the antibiogram profile using disc diffusion method. A total of 100 isolated and microbiologically identified bacteria of clinical origin belonging to Staphylococcaceae, Enterobacteriaceae, Pseudomonaceae, Campylobacteriaceae, Moraxellaceae and Streptococcaceae were screened for biofilm formation using the aforementioned analytical methods. Bacteria pathogen showed varying degrees of biofilm formation, of the three methods compared, the TCP was the most sensitive and further classified as, 28 (28%) strong, 20 (20%) moderate and 52 (52%) weak biofilm producers; Tube method detected 16 (16%) strong, 46 (46%) moderate and 38 (38%) weak biofilm producers while Congo red agar detected 14 (14%) strong, 46 (46%) moderate and 40 (40%) weak biofilm producers. The biofilm producers, especially the Gram negative bacteria exhibited multi-drug resistance to antibiotics used against Ciprofloxacin (85%), Amoxicilin (80%), Ceftriaxone (70%), Tobramycin (60%), Amikacin (10%) and Imipenem (10%). Succinctly, the results from this study showed that TCP was found to be most efficient method for biofilm detection. This may be considered as a screening method to detect pathogens for biofilm production, this may assist in better clinical management.

Keywords: Tissue culture plate, Tube method, Congo Red Agar, Biofilms, Resistance

Introduction

Microbial biofilms develop when microorganisms permanently adhere to a water-logged surface which could be inert or non-living tissue and then produces extracellular polymers which facilitates

the adhesion and provide a structural atmosphere. Biofilm-associated microorganisms behave differently from the freely suspended organisms (i.e. planktonic) with respect to rate of growth and

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resistant ability to antimicrobial treatments and therefore pose a public health problem (Rodney, 2001; WHO, 2017).

Biofilm on indwelling medical devices and equipment are usually composed of gram-positive or gram-negative bacteria or yeasts. Bacteria commonly isolated from these devices include the gram-positive *Enterococcus faecalis*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Streptococcus viridians*; and the gram-negative *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, and *Pseudomonas aeruginosa*. These organisms may originate from the skin of patients or health-care workers, tap water to which entry ports are exposed, or other sources in the environment (Rodney, 2001). Biofilms have major medical significance as they decrease the susceptibility to the antimicrobial agents. Even in individuals with competent innate and adaptive immune responses, biofilm based infections are rarely resolved. In fact, tissues adjacent to the biofilm might undergo collateral damage by immune complexes and invading neutrophils (Singh *et al.*, 2000; Yung-Hua and Xiao-Lin, 2016).

Furthermore, the proximity of cells within biofilms can facilitate plasmid exchange and hence enhance the spread of antimicrobial resistance (Watnick and Kotler, 2000; Bispo *et al.*, 2015). Early detection of biofilms is beneficial to address any issue that may lead to fatality or mortality. This study therefore evaluated the sensitivity of three analytical methods used in the detection of biofilm

producers and the antibiogram profile of the biofilm producers.

Methodology

Duration of study: The study was conducted at the Microbiology Department, Pathcare, Nigeria from November 2015 to January 2016.

Selection of isolates: A total of 100 clinical bacterial isolates recovered from wound, urine, catheter, stool and environmental surfaces of the hospital were subjected to the biofilm screening. Bacterial isolates were initially cultured on their respective bacteriological media, identified by standard microbiological procedures (Osho and Fawole, 2001), and further confirmation was done using the API (Biomérieux, France). *Pseudomonas aeruginosa* ATCC 27853 was used as the reference strain of positive biofilm producers.

Biofilm detection: The following methods were used for detecting biofilm production in the clinical isolates.

Congo Red Agar Method (CRA)

The Congo red Agar method was carried out using the procedure described by Freeman *et al.* (1989). Congo Red Agar (CRA) medium was prepared with brain heart infusion broth (37 mg/l) (Oxoid, UK), Sucrose (5 mg/ml), agar number 1 (10 mg/ml) (Oxoid, UK), and Congo Red dye (0.8 mg/l) (Oxoid, UK). The Congo red stain was prepared as a concentrated aqueous solution and autoclaved at 121°C for 15 minutes. Then it was added to the autoclaved brain heart infusion agar with sucrose at 55 °C. CRA plates were inoculated

with test organisms and incubated at 37°C for 24 hours aerobically. Black colonies with a dry crystalline consistency indicated biofilm production while pink colonies were recorded as non-producers.

Tube Method

The tube method was carried out using the procedure described by Christensen *et al.* (1982). A loopful of test organisms was inoculated in 10 ml of Trypticase soy broth with 1% glucose in test tubes. The tubes were inoculated at 37 °C for 24 hours. After incubation, tubes were decanted and washed with phosphate buffer saline (pH 7.3) and dried. Tubes were stained with crystal violet (0.1%). Excess stain was washed with deionized water. Tubes were dried in inverted position. The scoring for tube method was according to the results of the control strains. Biofilms formation was considered positive when a visible film lined the wall and the bottom of the tube. The amount of biofilm formed was scored as weak/non, moderate and strong.

Tissue Culture Plate Method (TCP)

The tissue culture plate method was carried out using the procedure described by Christensen *et al.*, 1995, is considered the gold standard for biofilm detection (Marthur *et al.*, 2006). Organisms isolated from fresh agar plates were inoculated in 10ml of trypticase soy broth with 1% glucose. Broths were incubated at 37°C for 24hours. The cultures were then diluted 1:100 with a fresh medium. Individual wells sterile 96 well-flat

bottom polystyrene tissue culture treated plates (Sigma-Aldrich, costar, USA) were filled with 200µL of the diluted cultures. The control organisms were also incubated, diluted and added to tissue culture plate. Negative control wells contained inoculated sterile broth. The plates were incubated at 37°C for 24hours. After incubation, contents of each well were removed by gentle tapping. The wells were washed with 0.2ml of phosphate buffer saline (PH 7.2). This removed free floating bacteria. After washing it was fixed with methanol for 15 minutes. Then the wells were decanted and stained by Crystal violet (0.1%). Excess stain was removed by using deionized water and plates were kept for drying. The optical density (OD) of the stained adherent biofilm was obtained by using micro ELISA autoreader (model 680, Biorad, UK) at wavelength 570nm.

Antimicrobial Susceptibility Test (AST)

The antibiotic susceptibility testing by a standardized disc method as described by Bauer *et al.* (1965) was employed. The antibiotics used for this study had been prepared into kit containing multiple discs, each with small discs impregnated with different types of antibiotics. The zones of inhibition were interpreted using the CLSI (2016) interpretive criteria.

Results

A total number of one hundred (100) clinical isolates obtained between November 2015 and January, 2016 were screened for biofilm production. The frequency of the isolates (Fig 1) are: *Escherichia coli* (20%), Coagulase Negative *Staphylococcus aureus* (10%), *Pseudomonas fluorescens* (10%), *Enterococcus faecalis* (8%), *Klebsiella pneumoniae* (8%), Methicillin Resistant *Staphylococcus aureus* (6%), *Campylobacter* sp. (6%), β Hemolysis *Streptococcus* (6%), *Enterococcus cloacae* (4%), *Raoultella ornitholitica* (4%), *Kluyvera* sp. (4%), *Actinobacter baumannii* (4%), *Pseudomonas luteola* (4%), *Morgella morgani* (4%), *Enterococcus faecium* (2%). These isolates constitute 34% and 66% Gram positive and negative bacteria, respectively (Fig 2). Tissue Culture Plate method detected 28 (28%) strong, 20 (20%) moderate and 52 (52%) weak/non biofilm producers; Tube method detected 16 (16%) strong, 46 (46%) moderate and 38 (38%) weak/non biofilm producers while Congo red agar detected 14 (14%) strong, 46 (46%) moderate and 40 (40%) weak/non biofilm producers (Fig 3). The TCP method exhibited the highest specificity and sensitivity. Also, Tube Culture plate (TCP) and Tube Method (TM) were positive for the positive control strain *Pseudomonas aeruginosa* (ATCC 27853), while it was negative for Congo

red agar method. The bacterial isolates exhibited high level of multi-drug resistance. The resistance pattern for Gram positive bacteria and biofilm producers as follows: Cotrimoxazole (100%), Penicillin (90%), Erythromycin (75%), Rifampicin (50%), Linezolid (0%) and Teicoplanin (0%) (Table 1). The resistance pattern of Gram negative biofilm producers is: Ciprofloxacin (85%), Amoxicilin (80%), Ceftriaxone (70%), Tobramycin (60%), Amikacin (10%) and Imipenem (10%) (Table 2).

Table 1: Resistance pattern of the Gram positive bacteria

Antibiotics	Number of resistant isolates	Resistance in %
Cotrimoxazole	34	100
Penicillin	31	90
Erythromycin	26	75
Rifampicin	17	50
Linezolid	0	0
Teicoplanin	0	0

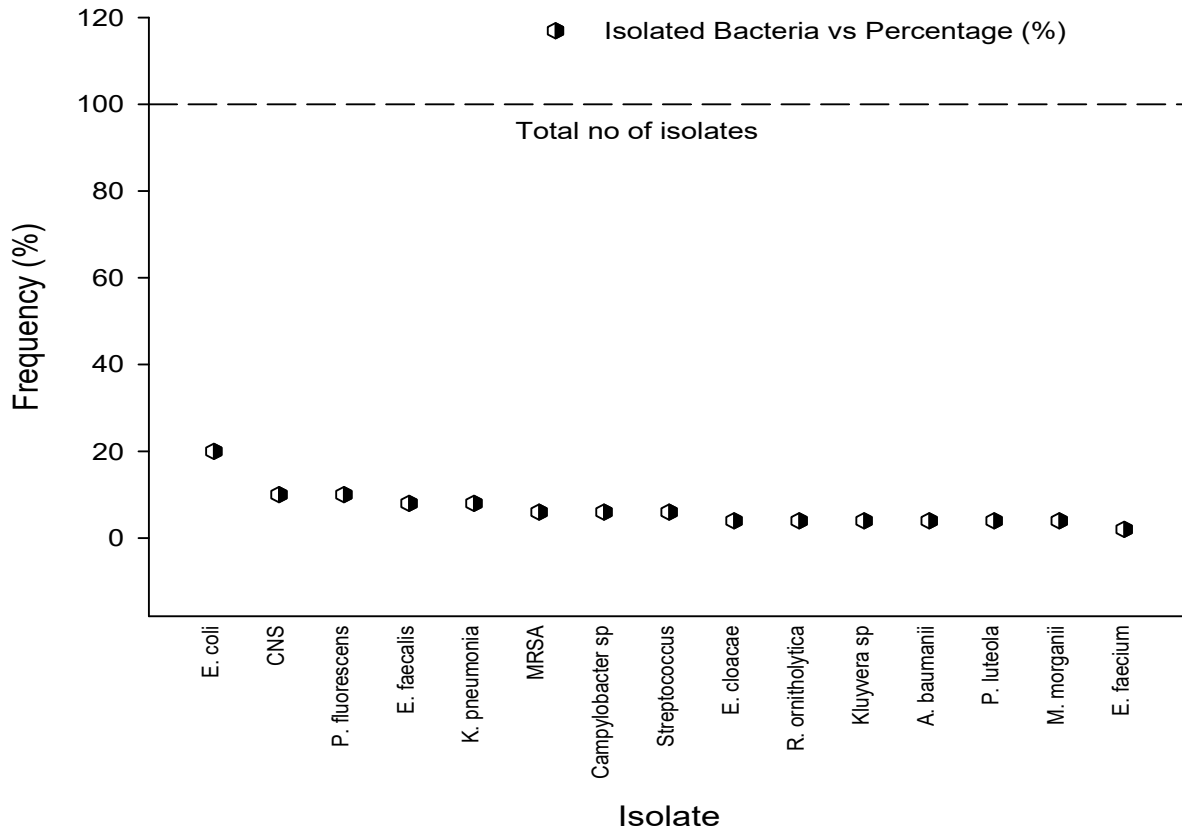


Figure 1: Frequency of the bacterial isolates

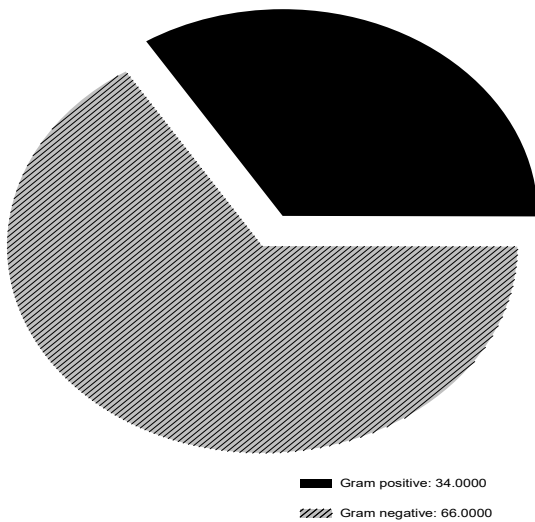


Figure 2: Distribution of the bacteria per Gram reaction

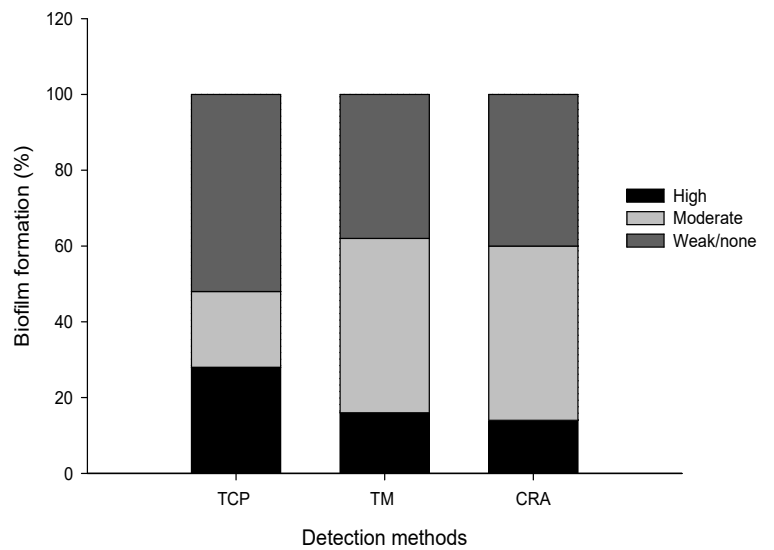


Figure 3: Sensitivity of the three methods in detecting biofilm formation

Table 2: Resistance pattern of the Gram negative bacteria

Antibiotics	Number of resistant isolates	Resistance in %
Ciprofloxacin	56	85
Amoxicillin	53	80
Ceftriaxone	46	70
Tobramycin	40	60
Amikacin	7	10
Imipenem	7	10

Discussion

Biofilm producing bacteria are responsible for many infections which are considered as recalcitrant and are notoriously difficult to treat and eradicate. Restricted penetration of antibiotic into biofilm, decreased growth rate and expression of resistance genes are some of the methods by which antibiotic resistance are exhibited (Kim, 2001). The bacteria used in this study represent both gram positive and negative usually implicated as etiological agents of severe infections as well as nosocomial agents. There was however, a higher number of gram negative bacteria. The production of exopolysaccharides by this group of bacteria could be linked to their high frequency of occurrence in biofilms. In the study, it was discovered *E coli* was the most prevalent isolates (20%), while other isolates ranged from 2-10%. This correlates with result obtained by Rodney (2001), that reported similar organisms used in this study as being mostly isolated from catheter and hospital-devices biofilms (Eliot *et al.*, 1997).

In the TCP method, the number of isolates showing biofilm formation was 48% and non or weak

biofilm producers were 52%. Data from the study of Mathur *et al.* (2006) also showed that out of 152 isolates tested, the number of biofilm producers identified by TCP method was 53.9%, and non-biofilm producers were 46%. The Tube method detected 62% biofilm producers and 38 non-biofilm producers while CRA detected 60% biofilm producers and 40% non-biofilm producers. Although the results of sensitivity for TM and CRA seem to be higher, it was observed that there were lots of false-positive results which amplified weak/non-biofilm producers as moderate. Overall, TCP detected strongest biofilm producers. Hence, TM and CRA may not be desirable as a general screening technique for identifying biofilm producers. Notably, the TCP and TM methods showed positive biofilm production against the control strain while the CRA failed to detect biofilm production. The order of sensitivity and specificity of these methods according to these results is TCP > TM > CRA.

The bacterial isolates used in this study were resistant to one or more classes of antibiotics including the carbapenems regarded as last resort. The Gram positive bacteria were all susceptible to

Linezolid and Teicoplanin. The Gram negative bacteria on the other hand showed no complete susceptibility to any of the antibiotics although they exhibited varied percentage of resistance to the different antibiotics. This affirms the existing literatures that biofilm production confers high resistance on bacteria and prolongs their survival.

Conclusively, TCP is a more reliable quantitative technique for the detection of biofilm producers among clinical isolates. Tube method may also be considered in rapid screening of biofilm producers. Hence, measures for the early detection of biofilm producers in patients and hospital environments should be considered. This will reduce the rate of fatality or morality of infections that are seemingly untreatable due to presence of biofilms.

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