Biofilm Detection and Antibacterial Susceptibility Pattern of Isolates from In-patients with Urinary Tract Infection in a Tertiary Hospital

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ABSTRACT

A survey for the detection of biofilms amongst uropathogens was conducted in a Medical ward of a Tertiary hospital in Ilorin, Kwara State. This was done to determine the prevalence of biofilm producing uropathogens among patients with CA-UTI (Catheter-Associated Urinary Tract Infection) using MTP (microtitre plate) method. Effect of media variations in the production of biofilms and the antibiogram pattern of the isolates were also determined. Out of the 50 urine samples collected and analyzed, 27 (54.0 %) yielded growth and 28 isolates were identified. *Klebsiella pneumoniae* was the most prevalent (42.9 %), followed by *Staphylococcus aureus* (35.7 %) and *Pseudomonas aeruginosa* was the least (21.4 %). A total of 59. 3 % of the isolates were resistant to various antibiotics used and 39.3 % of the isolates were found to be multiple antibiotic resistant. Of the 28 uropathogens isolated, 21 (75.0 %) were biofilm formers comprising of 17.9 % high biofilm formers and 57.1 % of moderate biofilm formers while 25.0 % did not form biofilm in BHI_{suc}. High biofilm formation was observed in *Staphylococcus aureus*. There was variation in biofilm formation with different media as more isolates (75. 0 %) produced biofilm in BHI_{suc} than in TSB_{glu} (67.9 %). Clinical isolates of *Staphylococcus aureus, Klebsiella pneumoniae* and *Pseudomonas aeruginosa* have potential of biofilm production which could lead to relapse of disease condition and eventually treatment failure in CA-UTI.

Keywords: CA-UTI, Biofilm, Multiple Antibiotic Resistance.

INTRODUCTION

Urinary tract infections (UTIs) represents one of the most common diseases that are encountered in clinical practice. [1] UTIs are the fourth most common type of healthcare infections and can be caused by the use of instrumentation in Urinary Tract. [2] They are characterized by microbial invasion of the genitourinary tract extending from the renal cortex to the urethral meatus [3] and can manifest as symptomatic or asymptomatic bacteriuria [4] especially among hospitalized patients with Catheter-associated UTIs (CA-UTI). [5]

Indwelling urinary tract catheterization (IUTC) is a common intervention protocol frequently required in hospitalized patients. [5] Out of 10 to 20 % of hospitalized patients, between 12 - 16 % of adult hospital in-patients have indwelling urinary catheter at some time during their hospitalization, and each day the indwelling urinary catheter remains, a patient has a 3 - 7 % increased risk of acquiring a catheter-associated urinary tract infection (CA-UTI). [6] *Escherichia coli* are the predominant uropathogen responsible for almost 80 % of UTIs followed by *Staphylococcus*, *Klebsiella*, *Enterobacter*, *Proteus* and *Enterococci* spp.[7] Occurrence and relapse of UTIs by uropathogenic *Escherichia coli* (UPEC) has been related to the ability of pathogenic strains to form biofilm [8]. These pathogens are frequently found in catheter associated UTIs which are acquired exogenously through manipulation of the catheter and drainage device. [9]

Biofilms are microbial communities of surface attached cells embedded in a self-produced extracellular polymeric matrix

usually referred as "cooperative community". [10] Bacterial forming biofilm are bacteria population that are enclosed and packed in a matrix of extracellular polymeric substances and usually difficult to eradicate due to antimicrobial resistant phenotypes conferred by these structures. [11] The proximity of cells within a biofilm can facilitate plasmid exchange which further enhances the spread of antimicrobial resistance. [12] The presence of mucus and glycocalyx prevents penetration of antibiotics and the high level of quorum sensing between cells increases resistance signals [13].

Bacterial biofilms have been associated with more than 60 % of nosocomial infections and 80 % of all microbial infections [14] occurring as chronic and persistent infections due to relapse with the increasing the cost of treatment, time of morbidity and burden of infections [15] with serious public health significance, [16] Several reports have described the involvement of a large number of biofilm forming bacteria in the etiology of UTIs in order climes, [8, 9] but there exists paucity of literature in this study location hence, the need to identify and categorize biofilm formers, with their antibiotic resistant ability in order to establish a novel, effective control strategy for biofilm control and ultimately prevent reoccurrence.

MATERIAL AND METHODS

Ethical considerations

Ethical clearance of the study was obtained from the University of Ilorin Teaching Hospital Ethics Committee. The objectives of the study were explained to the patients in English and their mother tongue (Yoruba) and their right to say no to participate in this study was explained to them. Once the patients had agreed to participate in the study they were requested to sign a consent form. To preserve their privacy, the patients were given a code and were referred to by that code. Urine specimen were collected from indwelling catheterized patients within the ward.

Study Population and Sample size

This was a prospective study amongst indwelling catheterized patients of the medical ward from the University of Ilorin Teaching Hospital, Ilorin between April and June, 2017. A total of 50 urine specimen were obtained from patients who met the criteria of study (Developed at least 2 symptoms of urinary tract infection after at least 2 days of indwelling urinary catheters, not on antibiotics a week before the study period and also gave the consent).

Sample collection

Urine specimen were collected aseptically into a sterile universal bottle from the urinary catheters of patients and taken to the Department of Pharmaceutical Microbiology and Biotechnology Laboratory for processing within two (2) hours and when immediate processing was not possible, specimens were promptly refrigerated at 4°C to avoid multiplication of bacteria at room temperature.

Isolation and Identification of Bacteria

Urine samples were cultured on Cysteine Lactose Electrolyte Deficient (CLED) agar using a calibrated sterile platinum wire loop. The plates were incubated at 37°C for 18 hours and colonial morphology was read. Obtained colonies were further sub-cultured onto MacConkey agar and Mannitol Salt agar plates and incubated for another 18 hours. Repeated sub-culturing was carried out to yield pure isolates that were maintained on Nutrient Agar (NA) slant at 4^{o}C until required.

Bacterial pathogens were identified on the basis of gram staining, cultural and morphological features in combination with biochemical characteristics previously described.[17]

Antibiotic Susceptibility Testing of the Isolates

The disc diffusion method of Bauer Kirby was to ascertain the sensitivity of isolates to antibiotics. Antibiotic susceptibility testing of the isolated test organisms, namely *Staphylococcus aureus, Klebsiella pneumoniae* and *Pseudomonas aeruginosa* was carried out using the following antibiotics: amoxicillin/clavulanic acid (AMC - 30µg), ampicillin (AMP- 10 µg), ciprofloxacin (CIP - 5 µg), ceftriaxone (CRO - 30 µg), erythromycin (E -15 µg), gentamicin (CN - 30 µg), imipenem (IPN - 10 µg), nitrofurantoin (F - 300 µg), sulphamethoxazole/trimethoprim (SXT - 25 µg). Antibiotic sensitivity testing was carried out by emulsifying selected isolates in normal saline at a turbidity compared to 0.5 MacFarland standard. Using sterile swabs, suspensions were inoculated on Muller-Hinton agar and incubated at 37 ^{o}C for 18 hrs. The resulting diameter of inhibition was measured in millimetre (mm) and interpreted following standard protocol.[18]

Biofilm assay using microtitre plate adherence method

The microtitre plate method of biofilm assay previously described [19] was used as the method to detect biofilm formation, using two different media: Trypticase Soy Broth with 1 % glucose (TSB_{glu}) and Brain Heart Infusion with 2 % sucrose (BHI_{suc}).

Isolates were grown overnight at 37 °C. The overnight broth cultures were diluted to1in100 with sterile BHI_{suc} and TSB_{glu}. Two hundred microliters (200 µL) of diluted cell suspension were then dispensed aseptically into a 96 wells microtitre plate and incubated at 37°C for 48 h in a stationary position. Sterile broth was set as control to check sterility and non-specific binding of media.

After incubation, the content of each well were gently removed by tapping the plates. The wells were then washed four times with 200 µL of phosphate buffer saline (PBS pH 7.2) to remove free-floating bacteria. Biofilms formed by adherent 'sessile' organisms in plate were fixed with 2 % $^{w}/_{v}$ sodium acetate and finally stained with freshly prepared 0.1 % $^{w}/_{v}$ crystal violet. Excess stains were rinsed off thoroughly using deionized water and the plates were allowed to dry. Adherent cells usually formed biofilm on all side wells and were uniformly stained with crystal violet. Optical density (OD) of stained adherent bacteria was determined with micro plate reader (Alere AM2100) at wavelength of 450 nm (OD450 nm). These OD values were considered as indices of bacteria adhering to surfaces and forming biofilms. The biofilm assay was carried out in triplicates and the average as well as the standard deviation was calculated for each isolated organism.

RESULTS

Distribution of uropathogens associated with CA - UTI

Represented in Table 1 is the distribution of the different uropathogens isolated from catheters of patients. Out of the 50 urine samples collected and analyzed 27 (54.0 %) yielded growth and a total of 28 isolates belonging to three (3) different genus constituting 64.3 % of gram negative organisms; *Klebsiella pneumoniae* (42.9 %) and *Pseudomonas aeruginosa* (21.4 %). The only gram positive isolate was *Staphylococcus aureus* (35.7 %).

Antibiotics Susceptibility Pattern of Uropathogens associated with CA-UTI

Summary of the antibiotics susceptibility pattern of uropathogenic bacteria associated with CA-UTI is presented in Table 2. A total of 59.3 % of the isolates showed resistance to various antibiotics used. All the isolates were resistant to ampicillin (100 %) and *S. aureus* was also completely resistant to amoxicillin/clavulanate and imipenem but showed considerable susceptibility to

ciprofloxacin, gentamicin, erythromycin and sulfamethoxazole/trimethoprim. *Pseudomonas aeruginosa* exhibited same resistance (66.7 %) to amoxicillin/clavulanate, imipenem, gentamicin and nitrofurantoin. Both *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* had similar resistance of 83.3 % to ceftriazone. *Klebsiella pneumoniae* and *S. aureus* were susceptible to gentamicin. Generally, 39.3 % of the isolates were found to be multiple antibiotic resistant (Table 3).

Table 1: Percentage Distribu-tion of Uropathogens in CA-UTI Patients.

Uropathogen	Number (%)
K. pneumoniae	12 (42.9)
S. aureus	10 (35.7)
Ps. aeruginosa	6 (21.4)
Total	**28 (100.0)**

Table 2: Percentage (%) Antibiotic Resistance Profile of Isolate Uropathogens (n=28)

Antibiotics	*K. pneumoniae* (12)	*Ps. aeruginosa* (6)	*S. aureus* (10)
AMC	83.3	66.7	100
Ampicillin	100	100	100
Ceftriaxone	83.3	83.3	80
Ciprofloxacin	16.7	50	20
Erythromycin	41.7	33.3	40
Imipenem	58.3	66.7	100
Gentamicin	0	66.7	20
Nitrofurantoin	41.7	66.7	60
SMT	58.3	50	50

AMC = Amoxicillin/clavulanic acid; SXT= Sulfamethoxazole/trimethoprim

Biofilm assay using microtitre plate method

The microtitre plate method (MTP) was used to detect the biofilm forming uropathogens. The mean absorbance (or optical density) values considered as indices of bacteria adhering to surface and forming biofilms are shown in Table 4. Isolates in BHI_{suc} had 75.0 % biofilm production as against 67.9 % when TSB_{glu} was used. Of all the isolates, 17.9 % were high biofilm formers, 57.1 % were moderate biofilm formers while 25.0 % did not form biofilm in BHI_{suc} according MTP Classification. [20] Only the *S. aureus* exhibited high biofilm formation. *K. pneumoniae* (28.6 %) and *Ps. aeruginosa* (17.9 %) were moderate biofilm formers.

Table 4: Biofilm screening for isolated Uropathogens

Isolates	Media	Biofilm Formation (OD_{450nm})			Total
		High (> 0.240)	Moderate (0.120 – 0. 240)	Non (< 0.120)	
S. aureus	**(BHI_s)**	5	3	2	10
	(TSB_g)	0	7	3	10
Ps. aeruginosa	**(BHI_s)**	0	5	1	6
	(TSB_g)	0	4	2	6
K. pneumoniae	**(BHI_s)**	0	8	4	12

BHI_s- Brain Heart Infusion supplemented with 2 % sucrose;TSB_g- Trypticase Soy Broth supplemented with 1 % glucose OD-Optical density (absorbance value).

DISCUSSION

Patients with in- dwelling catheter are at higher risk of CA-UTI. Also, biofilm has a very significant role to play in urinary tract infections. Biofilm is so prevalent on urinary catheters because it conveys a survival advantage to the microorganisms and for this same reason, urinary catheter biofilm is difficult to eradicate.[21] In this study, *Klebsiella pneumoniae, Pseudomonas aeruginosa and Staphylococcus aureus* were associated with CA-UTI. Similar studies [7, 21] had reported the isolation of *Klebsiella pneumoniae, Pseudomonas aeruginosa and Staphylococcus aureus* in addition to *Escherichia coli* as the predominant biofilm-forming uropathogens. All the isolates were resistant to ampicillin, consistent with earlier findings of [22] as well as the report of. [23] The high level of susceptibility to gentamicin observed in this study was similar to previous report [24] clearly stating that 100 % sensitivity to gentamycin was observed in their own study. Some *P. aeruginosa* and *Staphylococcus aureus* isolates were resistant to all the eight (8) classes of antibiotics used in this study with MAR Index values of 1.0 which shows absolute resistance, which corroborates the reported [25] ability of biofilms to confer up to 1000-fold resistance to antibiotics compared with planktonic cells due to several mechanisms. Antimicrobial agents are more active against biofilms implicated in pyelonephritis than against those on catheters. [26] This may be as a result of the synergistic effect of antimicrobial agents and host defenses. [27] which of course is not found on the inanimate catheter. Generally, in this study 39.3 % of the isolates were multiple antibiotic resistant which might be due to their high (75.0 %) biofilm forming ability.

Although it is a known fact that *S. aureus, K. pneumoniae* and *Ps. aeruginosa* are biofilm formers, supplementation of TSB and BHI with different sugars increases biofilm formation significantly. [28] Composition of the growth medium has been documented to influence the ability of bacteria to produce biofilm in-vitro. The addition of glucose to standard TSB medium has been reported to enhance biofilm formation. [28, 29] From previous study, preference has been shown towards BHI more than TSB although some strains of *Staphylococcus, Vibrio* and *Pseudomonas* species reportedly produce greater biofilm qualities in TSB while others do so in BHI. [30, 31]

The present study compared the two growth media and observed that they yielded positive but variable results which were in concordance with the documented reports [20] showing variations in the biofilm forming ability among the organisms in the different media. However, a slightly higher number of *S. aureus* strains grown on BHI_s demonstrated high adherence ability to the wells of the microtitre plates indicating that, although both BHI and TSB media could support the growth of *S. aureus* biofilm in vitro, some strains and more inclined to produce stronger biofilm on BHI than on TSB. These findings are in agreement with other studies, [28, 30] which found that enrichment of TSB medium with glucose increased the biofilm forming ability of *Staphylococci.* Adherence of *P. aeruginosa* was only moderate and this was especially noticed on BHI as well. There was equal moderate adherence of *K. pneumoniae* to the walls of the microtitre plates when grown on both media.

CONCLUSION

This study showed correlation between biofilm formation among uropathogens and multiple antibiotic resistance. Therefore, to effectively manage and treat CA-UTI, rapid biofilm screening methods should complement other microbiological processes. The high biofilm formation could lead to relapse of disease condition and eventually treatment failure in CA-UTI.

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