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Original Article

Biofilm and Extended Spectrum Beta Lactamase Production amongst Uropathogenic *Escherichia Coli* Isolates at the University of Ilorin Teaching Hospital, Nigeria

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ABSTRACT

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Background and Aims: Uropathogenic *Escherichia coli* (UPEC) are considered major reservoir for genes encoding antimicrobial resistance. The mechanism of resistance and persistence of UPEC has been attributed to the production of biofilm and Extended Beta Lactamase (ESBL). This hospital-based prospective study determined how biofilm and ESBL production facilitate antibacterial resistance amongst UPEC isolated from catheter urine of patients attending the University of Ilorin Teaching Hospital, Nigeria.

Materials and Methods: Urine samples from 113 catheterized inpatients and outpatients were analysed. Female subjects accounted for 47 (41.6%) of the study population. Standard microbiological methods and Analytical Profile Index (API) 20E were used for the isolation and identification of UPEC. Tissue culture plate technique was used to demonstrate biofilm production potentials and double-disc synergy test was used to determine ESBL production.

Results: Catheter associated urinary tract infection in this study was 70.8% of samples analysed. Of this, *Escherichia coli*, 44 (55.0%) was the most predominant. UPEC, biofilm and ESBL production amounted to 38.9%, 81.8% and 27.2%, respectively. ESBL production was significantly associated with degree of biofilm formation ($p<0.005$). Both strong and moderate biofilm producers showed the same level of resistance to ceftazidime (31.6%). Moderate biofilm producers were 46.7% resistant to cefriaxone. Resistance to Amoxillin-clauvanate significantly occurred in all grades of biofilm producers ($p>0.05$). Imipenem, however, was the most sensitive with no resistance by the UPEC.

Conclusions: ESBL and biofilm production were associated with antibacterial resistance. The incidence of ESBL production amongst biofilm forming UPEC is of great public health concern.

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Introduction

Urinary tract infection (UTI) is a broad term that encompasses asymptomatic microbial colonization of urine and symptomatic infection with microbial invasion and inflammation of the human urinary tract [1]. A major cause of morbidity and mortality, UTI has an enormous disease burden and represents the most commonly acquired bacterial infections in human accounting for an estimated 25.0-40.0% of nosocomial infections [2]. About 150 million cases are reported globally per annum with significant incidences also being reported in Nigeria, costing the world economy billions of dollars in treatment and work loss [3].

Although every individual is susceptible to UTIs, certain specific subpopulations are more predisposed to UTIs. These include infants, pregnant women, the elderly, patients with spinal cord injuries and/or on urinary catheters, patients with diabetes, multiple sclerosis, impaired immunity and patients with underlying urologic abnormalities. The risk of developing UTI increases significantly with the use of indwelling devices such as catheters and urethral stents or sphincters common with patients suffering from obstructive uropathy [4]. This risk is further increased when any breach in the closed system is encountered such as during emptying the catheter drainage bag or taking urine sample [5].

The presence of a urinary catheter is the most important risk factor for bacteriuria with a daily incidence rate of 3.0-10.0% [6]. Typically, in the absence of antimicrobial therapy, catheterized patients will become colonized with low level bacteriuria which usually progresses to >105 colony forming unit (CFU) within 24 to 48 hours and biofilm begins to form within 72 hours of catheterization [7].

Several published articles have documented various virulence factors that contribute to the ability of uropathogens to cause disease, which includes; fimbrial adhesins, toxins, flagella, auto transporter proteins and iron-acquisition systems [8]. Biofilm formation amongst bacteria cells have been described as an important virulence factor currently estimated to be responsible for over 65.0% of nosocomial infections and 80.0% of all microbial infections [9]. As reported by Donlan, majority of biofilm producing bacteria are from catheterized patients. Urinary catheters are tubular latex or silicone devices, which when inserted, may readily acquire biofilm on the inner or outer surfaces [10]. Uropathogenic *E.coli* strains an important member of the Enterobacteriaceae family present a suitable model for this study as they are consistently reported to be the most common extra-intestinal pathotypes identified in patients with UTIs thereby accounting for about 90.0% of reported cases. These uropathogenic bacteria refer to certain strains that are selected from faecal flora, not by chance or based on prevalence but because of specific virulence factors which aid their persistent, colonization and circumventing host defense to allow invasion of the normally sterile urinary tract [9, 10]. Biofilm formation described as a transition from planktonic living, presents itself as sticky accumulation of small

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colonies of bacteria surrounded by an extracellular polysaccharide matrix in which cells aggregate and adhere in an irreversible manner to various surfaces, including medical devices and injured tissues [11].

Antimicrobial resistance development associated with biofilm producing uropathogens have been linked to upsurge in treatment failures which pose a threat for patients confined to the use of indwelling catheter or undergoing urological surgery in general, and men subjected to prostate biopsy in particular [12]. The emergence of antibiotic resistance in the management of urinary tract infection is a serious public health issue, particularly in the developing world where apart from high level of poverty, ignorance and poor hygienic practices, there is also high prevalence of fake or adulterated drugs of questionable quality in circulation [12, 13].

In a recent study, biofilm producing bacteria had higher antimicrobial resistance than that of nonbiofilm producers to amoxicillin-clavulanate and ciprofloxacin, respectively Moreover, multidrug resistance was observed more among biofilm producing isolates than their counterparts to amoxicillin and cephalexin. This higher antimicrobial resistance among biofilm producing bacteria may emerge from increased properties of efflux mechanism. In addition, it may be also associated with higher plasmid transfer, modified target genes, and metabolic pathway that allow for resistance to antimicrobial agents. Furthermore, it was also reported that about two-third of the biofilm producing bacteria are multidrug resistant to at least three or more antimicrobial agents [13].

Global data shows increasing antibiotic resistance among the uropathogens. The resistance is mostly encountered with the antibiotics frequently used for the treatment of these infections and it varies among communities depending on their prescription pattern [14]. This study is therefore designed to determine the prevalence of biofilm producing UPEC in catheter urine at University of Ilorin Teaching Hospital and to establish association between antibiotic susceptibility pattern and biofilm production.

Materials and Methods

This study was conducted at the Department of Medical Microbiology and Parasitology of the University of Ilorin Teaching hospital (UITH), Ilorin. UITH belongs to the second generation of Teaching Hospitals in Nigeria. It is a tertiary health care centre and the only referral centre in Kwara State with a capacity of over 450 beds and an average of 10,000 to 12,000 annual admissions of in-patient and out-patient's visits respectively in the last five years as captured by the Department of Health Information Management, UITH, in Ilorin, 2015. It is located in the North central region of Nigeria. The Hospital provides quality health care services to the neighboring states like Oyo, Kogi, Niger, Osun, and Ekiti states.

This was is a hospital based prospective study.

The study population comprises catheterized patients, both outpatient and inpatients of all age groups and gender at clinics /units of the hospital such as General Outpatient Department (GOPD), Urology clinic, Male and Female Surgery wards, Accident and Emergency unit.

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Random sampling technique was employed for the selection of patients who had met the inclusion criteria including: patients with indwelling catheter ≥48 hrs calendar days; and not on any antibiotics within the last 72 hrs before recruitments. Patients with indwelling catheter < 24 hrs and on antibiotics within 72 hrs of recruitments were excluded from this research. Ethical approval was sought from the University of Ilorin Teaching Hospital Ethical Review Committee.

Analytical laboratory procedures

catheter urine sample collection and transportation

Urine sampling from patient with an indwelling urinary catheter was obtained from a sampling port using aseptic technique. Where there was no sampling port, the drain tubing was detached from the catheter bag and about 10-15 ml urine was allowed to drain aseptically into a sterile receptacle. Collected urine in the catheter bag was not considered. All sample collection was carried out with the assistance of an assigned doctor. Samples collected were promptly transported to the medical microbiology and Parasitology department for analysis. However the samples that were unavoidably delayed were refrigerated at 2-8˚C for not more than 4 hrs.

Culture and isolation

A modified semi-quantitative culture technique was used. Standard calibrated bacteriological loop (to determine CFU) was used to aseptically transfer 0.001 ml of a well-mixed urine sample on appropriately well labeled culture of Cysteine Lactose Electrolyte Deficient (CLED) agar (Biomarker) and 5.0% sheep blood agar (Biomarker) media. All culture media were prepared according to the manufacturer's specifications. Incubation was at 37˚C for 18-24 hrs aerobically.

Characteristic of isolates

E.coli appears on CLED as Large, elevated opaque yellow lactose fermenting colonies with a slightly deeper yellow center. *E.coli* appears on blood agar as large, opaque, sticky and colorless, ± narrow clear hemolysis zone.

Bacterial biochemical testing

A Commercially available phenotypic qualitative miniaturized systems API 20E (Bio Merieux, France) for *Enterobacteriaceae* identification was used for the physiological or biochemical confirmatory identification of UPEC isolates.

Biofilm assay using tissue culture plate (TCP) method

A phenotypic quantitative TCP Method first developed and described by Christensen et al [13]. and considered the gold standard for biofilm detection was used for the detection of biofilm production in all UPEC isolates.

Antibiotic susceptibility testing

This was performed according to the Clinical and Laboratory Standards Institute (CLSI), 2007 guidelines. *In vitro* antibiotic susceptibility testing of established biofilm producing UPEC isolated was carried out by modified Kirby-Bauer disc diffusion method on Muller-Hinton agar plates. The following antibiotics were used; Amoxicillin-clavulanate (10 µg) Cefriazone (30 µg), Ceftazidime (30 µg), Ciprofloxacin (5 µg), Gentamicin (10 µg), Nitrofurantoin (300 mg), and Imipenem (10 µg).

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Extended spectrum beta lactamase (ESBL) detection

The double disk synergy test (DDST) using third generation cephalosporins (3 GS) which includes ceftazidime (30 µg) and ceftriaxone (30 µg) antibiotic discs alongside amoxicillin-clavulanate (10 µg) disc were used for the detection of ESBL production amongst UPEC isolates. This was done by placing the antibiotic disk at distance of 20 mm from each other (center to center). Following incubation at 37˚C aerobically for 18-24 hrs, a positive synergistic effect showed inhibition zone between disks. A >5 mm increase in a zone diameter for either antimicrobial agent was tested in combination with clavulanic acid versus its zone which when tested alone is taken as a positive ESBL production.

Statistical analysis

Data were analysed using statistical apcakage for social sciences (SPSS) version 21, California, USA. Redukts were presented as frequencies and percentages. Chi-square test was used to determine association between ESBL, biofilm production and antibacterial resistance. A p value less than 0.05 at 95% confidence interval was considered significant.

Results

Catheter urine samples from one hundred and thirteen patients comprising both inpatients and outpatients who has met the inclusion criteria set out for this study were collected within the month of April-June, 2016. Catheter associated urinary tract infection in this study was 70.8% of samples analysed. Of this, *Escherichia coli*, 44 (55.0%) proved to be the most predominant pathogen. This study revealed UPEC, biofilm and ESBL production being 38.9%, 81.8% (Fig. 1) and 27.2%, respectively. ESBL production was significantly associated with degree levels of biofilm formation ($p<0.005$) (Fig. 2). Both strong and moderate biofilm producers showed the same level of resistance to ceftazidime of 31.6%. Moderate biofilm producers were 46.7% resistant to cefriaxone. Resistance to Amoxillin-clauvanate significantly occurred in all grades of biofilm producers ($p>0.05$). Imipenem however was the most sensitive with no resistance (Table 1). There was a statistical significant association between ESBL production and degree of biofilm formation. Highest incidence was observed in strong biofilm producers (100.0%) (Fig. 2). Resistance patterns and degree of biofilm formation is shown in figure 3.

Discussion

This study involved the enrollment and evaluation of 113 patients with indwelling urinary catheter, within the period of April and June, 2016. These patients comprised both outpatients and inpatients. The prevalence of CA-UTI in the study area was (70.8%), 55.0% of which was caused by UPEC isolates. Prevalence of biofilm production potential by UPEC isolates was 31.8%. UPEC accounted for 44 (55.0%) of the total uropathogens isolated in this study. In consonance with this study, Yakubu [15]; Ahmed et al. [16] and Niveditha et al. [17] reported similar findings where UPEC was observed as the most prevalent uropathogen despite variation in the prevalence rates of 24.14%, 31.7% and 71.0% respectively.

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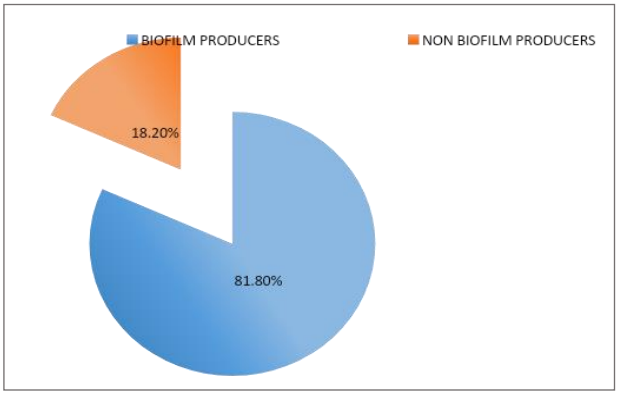

Fig. 1. Biofilm assay using tissue culture plate technique shows the frequency of biofilm production amongst Uropathogenic *E. coli* isolates as 81.8%.

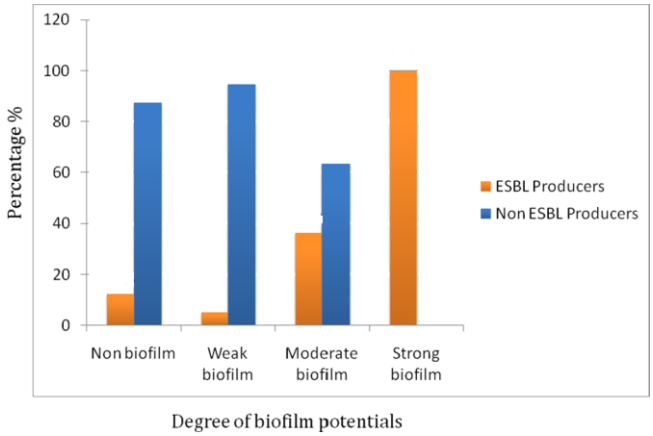

Fig. 2. Degree of biofilm formation among ESBL *E. coli*. ESBL= Extended Beta-lactamase.

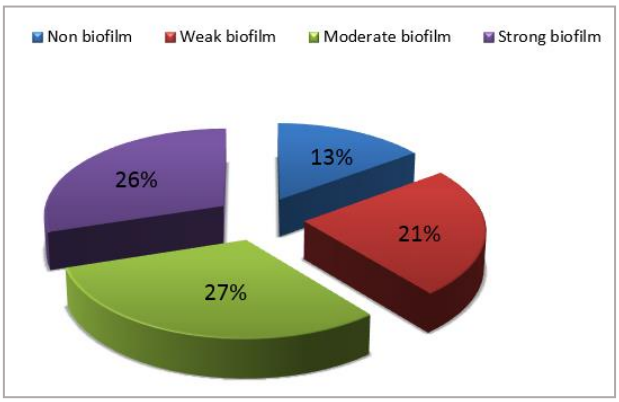

Fig. 3. Resistance patterns and degree of biofilm formation

Table 1. Antibiotic susceptibility pattern of Uropathogenic *E. coli* isolates

Antibiotics	Sensitive (%)	Intermediate (%)	Resistance (%)
Amoxicillin-clauvanate	13 (29.54)	0 (0.0)	31 (70.45)
Cefriazone	29 (65.9)	0 (0.0)	15 (30.09)
Ceftazidime	25 (56.81)	0 (0.0)	19 (43.18)
Ciprofloxacin	22 (50.0)	1 (2.3)	21 (47.72)
Gentamicin	6 (13.64)	0 (0.0)	38 (86.36)
Nitrofurantoin	31 (70.45)	0(0.0)	13(29.54)
Imipenem	41 (93.2)	3(6.8)	0(0.0)

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These variations may partly be explained by the differences in study populations and also in the inclusion criteria used by centers in selecting urine samples for culture [18]. Antibiotic intake prior to presentation at the hospital may also be a key factor for bacterial yield.

The persistence of UPEC as a major ethological agent in UTI and as observed in this study and other similar works can be attributed to the expression of a variety of virulence factors, which include adhesins (e.g., type 1 and P fimbriae) and toxins (e.g., haemolysin), cytotoxic necrotizing factor, fimbriae, aerobactin-mediated iron uptake, K1 capsular polysaccharide and biofilm formation [17, 18].

Biofilm assay using the TCP method showed the frequency of biofilm formation of UPEC isolates in this study as 81.8%. This finding was consistent with other studies such as Fattahi, (2015) who reported a 92.0% biofilm production by uropathogens from UTI. Another study carried out by Ponnusamy et al., [19] showed that among 100 *E. coli* strains, 72 (72.0%) strains displayed a biofilm positive phenotype. The occurrence of biofilm in this study was observed to be more common in female subjects accounting for 24 (66.7%). This was observed to be in accordance to documented studies where recurrent UTI was common in females 74.0%, which were attributed to biofilm producing strains [18].

Pathogenesis of device-associated infections relates to bacteria that attach to and grow on surfaces and produce extracellular polymers thus facilitating adhesion and providing a structural matrix. As documented by Hittinahalli et al. [20], availability of key nutrients, chemo-taxis towards surface, motility of bacteria, surface adhesins and presence of surface bacteria are some contributory factors which influence biofilm formation. Furthermore, it has also been found that biofilm-producing UPEC strains show a significantly greater type 1 fimbriae expression than non-biofilm-producing strains [21].

UPEC isolates showed the highest susceptibility to antibiotics such as Imipenem 93.2% followed closely by nitrofuratoin with 70.45%. The resistance pattern of UPEC isolates to the different antimicrobials agents employed in this study shows that among the antibiotics tested, resistance to gentamicin was the highest (86.36%) while no resistance to Imipenem was recorded (0.0%). Resistance to antimicrobial agents is well documented and has been noted at first use of antibiotics which currently has become a worldwide problem [22]. This study revealed a higher antibiotic resistance development to the commonly used antibiotics for prophylaxis and for empirical therapy for UTI such as gentamicin and nitrofuratoin respectively. This may be due to indiscriminate consumption of these drugs, self-medication, and transfer of antibiotics resistant between isolates. The antibacterial resistance pattern in this study is in line with that of Hryniewicz et al., [23] who reported an increased antibacterial resistance among

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urinary tract pathogens to the conventional and first-line antibiotics.

Occurrence of biofilm producing UPEC across gender was observed to be higher within the female subjects #29 (65.9%); however there was no significant association in terms of biofilm production ($p>0.05$). Similar findings were observed by Mittal et al. [24] where biofilm formation was two times more common in female accounting for 12 (67.0%) as compared to males 6 (33.0%).

The *in vitro* antibiotic resistance pattern across the four levels biofilm potentials of UPEC showed that while strong and non-biofilm producer demonstrated the highest and lowest antibiotic resistance to nitrofurantoin , i.e., 46.2% and 7.7% respectively, the highest resistance to gentamicin was observed amongst weak biofilm 39.5% ($p=0.495$). Antimicrobial susceptibility pattern of ciprofloxacin showed that weak, moderate and strong biofilm producers had the same resistance rate of 28.9% ($p<0.05$). Both strong and moderate biofilm producers showed the same level of resistance to ceftazidime , that is, 31.6%, ($p>0.05$).

Moderate biofilm producers were observed to show antibiotic resistance of 46.7% to cefriazone. Resistance to Amoxillin-clauvanate occurred across all grades of biofilm production with weak biofilm producer having the highest (35.5%). Imipenem however was broadly the most sensitive drug in this study with zero resistance reported. The low resistance observed to imipenem may be associated with least drug abuse, probably because of its high cost of procurement and not been commonly available for purchase as compared to others.

In comparison with other studies in different parts of the world including Nigeria, resistance rates to different drugs have been reported over time. This is therefore imperative to pay attention to local resistance pattern as these will have significant impact on patient care. The clinical implication of this finding is that prudent empiric treatment will be challenging as no single common drug can conveniently be recommended for use. This mandates the need for antimicrobial chemotherapy based on urine culture for all suspected UTIs to properly guide definitive therapy.

Antibiotic resistance pattern amongst biofilm producing UPEC in this study was higher in strong (26.0%) and moderate (27.0%) biofilm producers, respectively. This high antibiotic resistance amongst biofilm producers may be that the drug concentration obtained is insufficient in certain areas of the film or due to reinforced structural support that bacteria recieve by virtue of the biofilm. This makes them metabolically inactive thus resistant to many antimicrobial agents. The incidence of ESBL production of 27.2% amongst biofilm forming UPEC isolates in this study is a double edge tragedy of public health concern which requires the prompt attention by health care providers and policy makers. The highest occurrence of ESBL was 100.0% amongst the strong biofilm producers. The high incidence of ESBL reported in this study could be an indication of selective drug pressure because of the common use of cephalosporin and other antibiotics in our region [24].

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Conclusion

ESBL and biofilm production is associated with resistance to antibiotics. The incidence of ESBL production amongst biofilm forming UPEC isolates in this study is of great public health concern which requires prompt attention.

Conflict of Interest

None.

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