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Occurrence and Antibiotic Susceptibility Profiles of Biofilm-Forming Bacteria Associated with Asymptomatic Bacteriuria Among Students

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ABSTRACT

Background and Objective: Asymptomatic bacteriuria may be associated with bacteria capable of forming biofilms which when formed may increase the rate of morbidity and mortality, cost of treatment and length of hospital stay of the patient. This study, therefore, aimed at determining the biofilm-forming capacity of bacteria associated with ABU as well as their susceptibilities to some selected antibiotics. **Materials and Methods:** Urine samples of 200 healthy students were cultured on Cysteine Lactose Electrolyte Deficient (CLED) agar and incubated appropriately. The associated organisms were isolated and characterized using chromogenic agar. The ability of the isolated bacteria to form biofilm was evaluated using the Congo Red Agar (CRA) technique while their susceptibility profiles to antibiotics were determined by the Kirby-Bauer disc diffusion method. **Results:** These revealed that urine samples of forty students had significant bacterial growth characteristics of ABU. *Staphylococcus aureus* was the predominant Gram-positive bacterium which accounted for 47.5% of the isolated bacteria. Biofilm-forming capacity revealed that 40% of the isolates tended to form biofilm. However, ofloxacin and streptomycin were antibiotics of choice for Gram-negative and Gram-positive isolates, respectively. **Conclusion:** The study concluded that biofilm-forming bacteria with varying susceptibilities to antibiotics are present in urine samples of students with ABU despite their showing no ill health.

KEYWORDS

Asymptomatic, antibiotics, bacteriuria, biofilm, susceptibility, occurrence, profiles

INTRODUCTION

Asymptomatic Bacteriuria (ABU) refers to the presence of a significant number of bacterial species in urine without the usual urinary tract infection signs or symptoms[1]. It is a common clinical finding that affects males and females, the old and the young. However, the frequency varies among separate populations, depending on some predisposing factors such as age, sex, sexual activity and the presence of genitourinary abnormalities[2-7]. It has been reported that ABU increases with age and the incidence of asymptomatic bacteriuria is higher among females than among males[8]. *Escherichia coli* is the predominant bacteria associated with asymptomatic bacteriuria[9]. Other organisms that have been implicated, depending on patient variables, include Enterobacteriaceae, *Pseudomonas aeruginosa*, *Enterococcus* species and group B *Streptococcus. While E. coli* may be associated with urine samples of healthy persons

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P. aeruginosa and other multidrug-resistant polymicrobial flora may be associated with a catheterized patient. However, *Enterococcus* species and Gram-negative bacilli may prevail in the urine samples of men[10,11]. It has been recommended that patients with ABU should not be treated except if there is an associated potential benefit. Notwithstanding, pregnant women should be treated for asymptomatic bacteriuria, especially in the first trimester[12]. The ABU becomes persistent and its treatment becomes more difficult when associated with biofilm-forming uropathogens because reduced susceptibility to antibiotics by biofilms predisposes to the persistence of infections. Some of the potential explanations for the enhanced resistance of biofilms to antibiotics include poor drug penetration, nutritional deficiency, delayed development as an adaptive stress response and the development of persister cells[13]. Although information about ABU abounds in literature, there is a paucity of information about the biofilm-forming capacity of associated bacteria.

This study therefore, aimed at isolating and characterizing bacteria associated with ABU among students, evaluating associated bacteria for their capacity to form a biofilm and determining their susceptibility profiles to antibiotics.

MATERIALS AND METHODS

Study area: This study was conducted at the Department of Pharmaceutical Microbiology, Faculty of Pharmacy, Obafemi Awolowo University, Ile-Ife, Osun State, Nigeria between February, 2021 and January, 2022.

Sample collection: Urine samples from 200 healthy students were collected in sterile universal bottles and cultured within 2 hrs of collection. Volunteers were taught how to properly collect the midstream urine to avoid contamination.

Isolation and characterization of the isolates: A loopful of each urine sample was inoculated on a plate containing CLED agar. The plates were incubated at 37 for 24 hrs. Colonies were taken from the plates that yielded growth for identification and characterization using UTI chromogenic agar. The results were interpreted according to the manufacturing instructions as Blue-*Klebsiella pneumoniae*, yellow-*Staphylococcus aureus* and Burgundy-*Escherichia coli*. Identified bacterial isolates were then transferred onto nutrient agar slopes and kept in the refrigerator at 4 until needed.

Antimicrobial susceptibility test: Antimicrobial susceptibility testing was carried out on the isolates using the Kirby-Bauer disc diffusion method and the results were interpreted according to the guidelines of the Clinical and Laboratory Standards Institute[14]. Briefly, 20 mL Mueller-Hinton Agar (MHA) plates (Oxoid, UK) were made following the directions of the manufacturer. Five colonies of the test bacteria were inoculated into 10 mL of sterile distilled water and vigorously agitated with a spin mixer to create the McFarland standard equivalent of 0.5 (A625 nm = 0.09). By visually comparing the suspension's turbidity to that of a 0.5 McFarland standard made, the suspension's turbidity was adjusted to match that of the standard. Using a sterile swab stick, the final bacterial suspension was uniformly applied to the Mueller-Hinton agar surface.

The plates were allowed to sit at room temperature for about 3-5 min after which they were incubated for 20 min. Following these, antibiotic-impregnated discs were aseptically dispensed on the surface of the agar. The plates were kept in the refrigerator for 4 min and then incubated at 35±2°C. After 18 hrs, the zones of inhibition were measured including the diameter of the disc to the nearest millimetre. The results obtained were interpreted as resistant or susceptible based on the zones of inhibition and by the use of the Zone Size Interpretative Chart. *Escherichia coli* ATCC 25922 and *S. aureus* ATCC 25923 were used as control strains.

The antibiotic discs used for the Gram-positive organisms include Ampiclox (30 µg), Rocephin (30 µg), Zinnacef (20 µg), Ciprofloxacin (30 µg), Amoxycillin (30 µg), Erythromycin (10 µg), Gentamycin (30 µg), Septrin (30 µg), Streptomycin (30 µg). For the Gram-negative organisms, the antibiotic discs used include Septrin (30 µg), Chloramphenicol (30 µg), Sparfloxacin (10 µg), Ciprofloxacin (30 µg), Amoxycillin (30 µg), Augmentin (10 µg), Gentamycin (30 µg), Tarivid (10 µg), Streptomycin (30 µg).

Biofilm formation test: This was done using the Congo Red Agar (CRA) method as described by Rajkumar *et al.*[15]. Briefly, Congo red dye (0.8 g L^{-1}) prepared as concentrate was added to sterile Brain Heart Infusion (BHI) agar prepared according to the manufacturer's instruction and supplemented with sucrose (5 g L^{-1}) at 45°C. The CRA plates were allowed to set and a colony of each test bacterial isolate was streaked on the plates. The plates were incubated at 37°C for 24 hrs aerobically after which the plates were examined for the growth of black colonies with a dry crystalline consistency, an indication of biofilm formation.

RESULTS

Out of the 200 students screened for asymptomatic bacteriuria, only 40 (53% females and 47% males) were positive and the distribution of isolated bacterial species is shown in Table 1.

Table 1 shows *S. aureus* as the predominant Gram-positive species (47.5%) and *E. coli* as the predominant Gram-negative species (22.5%) in the samples analyzed for asymptomatic bacteriuria.

Table 2 reveals that among the isolates that tested positive for CRA, *S. aureus* has a higher capacity to form biofilms (68.75%) compared to either *Klebsiella pneumoniae* (18.75%) or *Escherichia coli* (12.5%).

The distributions of antibiotic resistance profiles of CRA-positive and CRA-negative Gram-positive (*S. aureus*) and Gram-negative bacterial species (*E. coli* and *K. pneumoniae*) are shown in Table 3 and 4, respectively. While all the CRA-positive (biofilm forming) and CRA-negative (non-biofilm forming)

Table 1: Percentage distribution of the isolates associated with asymptomatic bacteriuria

Bacterial species	Number in males	Number in females	Total number of isolates	Percentage of isolates
Staphylococcus aureus	10	9	19	47.5
Klebsiella pneumoniae	4	5	9	22.5
Escherichia coli	5	7	12	30.0
Total	19	21	40	100.0

Table 2: Percentage distribution of the CRA-positive bacterial species associated with asymptomatic bacteriuria

Bacterial species	Number of CRA-positive (n = 16)	Percentage	Number of CRA-negative (n = 24)	Percentage
Staphylococcus aureus	11	68.75	8	33.3
Klebsiella pneumoniae	3	18.75	6	25.0
Escherichia coli	2	12.50	10	41.7
Total	16	100.00	24	100.0

Table 3: Percentage distribution of antibiotic resistance profiles of CRA-positive *Staphylococcus aureus* isolates associated with asymptomatic bacteriuria

Antibiotics	CRA-positive *S. aureus* (n = 11)	CRA-negative *S. aureus* (n = 8)
Gentamycin	27	-
Ampiclox	100	100.0
Zinacef	100	100.0
Amoxicillin	100	100.0
Rocephin	100	100.0
Ciprofloxacin	18	25.0
Streptomycin	9	-
Septrin	36	37.5
Erythromycin	73	87.5

Table 4: Percentage distribution of antibiotic resistance profiles of CRA-positive Gram-negative bacterial species associated with asymptomatic bacteriuria

Antibiotics	CRA-positive *E. coli* (n = 2)	CRA-negative *E. coli* (n = 10)	CRA-positive *K. pneumoniae* (n = 3)	CRA-negative *K. pneumoniae* (n = 6)
Gentamycin	-	30	33.3	33.3
Septrin	-	10	-	33.3
Ofloxacin	-	-	-	16.7
Chloramphenicol	50	30	66.7	33.3
Ciprofloxacin	-	-	33.3	50.0
Streptomycin	-	40	66.7	66.7
Augmentin	50	30	66.7	83.3
Amoxicillin	-	-	33.0	33.3
Sparfloxacin	-	-	-	33.3

E. coli were susceptible to ofloxacin, ciprofloxacin, amoxicillin and sparfloxacin, all the CRA-positive *K. pneumoniae* species were susceptible to septrin, sparfloxacin and ofloxacin. The CRA-negative *K. pneumoniae*, however, displayed equal resistance of 33.3% each to gentamycin, septrin, chloramphenicol, sparfloxacin and amoxicillin. On the other hand, both CRA-positive and CRA-negative *S. aureus* shows equal resistance to ampiclox, zinacef, amoxicillin and rocephin.

DISCUSSION

In this study, out of the 200 students screened for ABU, 40 had significant bacteriuria. The percentage of prevalence was lower (20%) compared to the 77% prevalence that was earlier reported[16]. The low level of prevalence observed in this study may be attributed to increased immune competence among students as the study was conducted during the covid 19 pandemic when virtually everybody was on one form of immune booster or the other. The study also revealed that more female students had AB than males. This finding was in agreement with the reports of some previous researchers[16-20]. A higher prevalence of ABU in females than in males can be attributed to the proximity of the female urethral meatus to the anus, shorter urethra and sexual intercourse which predispose females to infections more than males[21,22].

Although the concept that the urine of healthy people is sterile[23] has been invalidated through the discovery of bacterial communities in the female bladder[24-32], isolation of potential pathogens from the urine samples of healthy students as found in this study further invalidates the once generally accepted "sterile urine" paradigm. However, despite the obvious differences in the diagnosis of asymptomatic bacteriuria as exemplified by Nicolle *et al.*[33], it remains non-discriminatory with varying degrees of incidence to age and sex, as found in this study.

Several organisms have been reported to be associated with ABU. Although *E. coli* has been reported to be the most common pathogen associated with both symptomatic and asymptomatic bacteriuria, pathogens such as *Staphylococcus aureus*, *Proteus mirabilis*, *Staphylococcus saprophyticus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Enterococcus* spp., among others, have also been involved[34-36]. This implied that prevalent pathogens vary from and among populations. In this study, *S. aureus* is the predominant bacterial isolate. This was consistent with our previous report[16] and in agreement with other previous reports[37-40]. However, the prevalence of *S. aureus* in males than females in this study may be attributed to the ability of *S. aureus* to tolerate high pH and temperature variations which encourages its capacity to colonize different areas of the body such as the urinary tract[41]. Prevalence of two enteric bacteria, *E. coli* and *K. pneumoniae*, in females than males as observed in this study may be attributed to poor personal hygiene on the part of female students which encourages the transfer of these organisms from the anus to the urethral, bearing in mind the proximity of these organs in females.

Apart from variations in their prevalence, bacteria species associated with AB vary in their virulence. One such virulence factor common to uropathogens is their ability to adhere to uroepithelial cells which prevents them from urinary lavage and allows for multiplication and tissue invasion[42]. Suffice it to say that adhesion is a prerequisite for the pathogenesis of urinary tract infection as well as biofilm formation. The formation of biofilm is responsible for persistent and chronic infections. Biofilms are characterized as communities of surface-attached cells and are embedded in a self-produced extracellular polymeric matrix. They are usually less susceptible to antimicrobials and are therefore difficult to treat. Their reduced susceptibility can result in increased morbidity and mortality, increased cost of treatment and increased length of hospital stay. In this study, 40% of the total isolates showed the capacity to form biofilms with *S. aureus* as the predominant biofilm former. The distribution was exemplified in Table 2. The ability of *S. aureus* to form biofilm can be regulated by the expression of Polysaccharide Intracellular Adhesion (PIA), which mediates cell-to-cell adhesion[43].

Despite that treatment has been recommended only for patients with asymptomatic bacteriuria that will benefit from treatment, bacteria associated with ABU continue to express varying susceptibilities to antibiotics. It has been reported that biofilm can be up to 1000-fold more resistant to antibiotics than planktonic cells[44]. In this study, all the *S. aureus* (CRA-positive and CRA-negative) were resistant to ampiclox, zinacef, amoxicillin and rocephin, all β-lactam antibiotics and least resistant to streptomycin, 5.3% (Table 3). However, the gram-positive isolates are least resistant to streptomycin is consistent with our earlier report, although at a lower percentage[16]. Resistance to β-lactam antibiotics can be mediated through three main mechanisms which include (1) Enzymatic degradation by β-lactamases, (2) Target modification of the Penicillin-Binding Proteins (PBPs) resulting in a lack of β-lactam binding and (3) Regulation of β-lactam entry and efflux[45]. Streptomycin is an aminoglycoside and resistance to it can be mediated by a variety of mechanisms, including the following: (1) Modification and inactivation of the aminoglycosides by enzymes, (2) Enhanced efflux, (3) Reduction in permeability to the antibiotic and (4) Modifications of the 30S ribosomal subunit in such a way that prevent aminoglycosides from binding to it[46]. On the other hand, both CRA-positive and CRA-negative *E. coli* were susceptible to Ofloxacin, ciprofloxacin, sparfloxacin and amoxicillin while all CRA-positive *K. pneumoniae* were susceptible to septrin, ofloxacin and sparfloxacin. The CRA-negative *K. pneumoniae* displayed the least resistance to ofloxacin. Ofloxacin as the drug of choice against gram-negative bacteria in this study is in agreement with our previous report[16] (Table 4). Resistance to ofloxacin, a second-generation fluoroquinolone, can be through one or a combination of target-site gene mutations, increased production of Multidrug-Resistance (MDR) efflux pumps, modifying enzymes and/or target-protection proteins[47].

Since it has been established in this study that some bacterial isolates associated with AB can form biofilms, it, therefore, implies that the tendency for AB to transit to symptomatic UTI is high and may persist longer than necessary if not properly and promptly treated. Persistence may be associated with an increased cost of treatment and/or morbidity and mortality, among others.

The patient presenting with AB shows no sign or symptom of urinary tract infections, it, therefore, means routine laboratory tests to establish its existence will be a necessity. This should be followed by proper and prompt treatment with appropriate antibiotic(s).

This study is limited to both male and female students of a tertiary institution in southwestern Nigeria who had not experienced urinary tract infections before or without any symptom/sign that may be attributed to UTIs. Also, the students have not been on any antibiotic for at least six months before the sample collection. The paucity of the fund has limited the number of participants in this study.

CONCLUSION

The study concluded that ABU is prevalent among students of tertiary institutions despite their not showing symptoms or signs of urinary tract infections. However, about 40% of the bacteria associated with ABU, majorly *Staphylococcus aureus*, form biofilms which were susceptible to ofloxacin and streptomycin. Biofilm formation by uropathogens may result in persistent and chronic urinary tract infections.

SIGNIFICANCE STATEMENT

This study uncovers the ability of some bacterial isolates associated with asymptomatic bacteriuria among students to form a biofilm, which inadvertently influences their susceptibility to antibiotics. This will help researchers to provide an answer as to whether AB should be treated with antibiotics or not, having established an association between biofilm formation and persistence of infections as well as the reduced susceptibility to antibiotics by biofilms.

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