ANTIBACTERIAL ACTIVITIES OF BIOFILM-POSITIVE UROBACTERIA ISOLATED FROM PATIENTS ATTENDING UROLOGY DEPARTMENT OF A TEACHING HOSPITAL IN OGUN STATE.

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Abstract: Bacteria in the urine (Bacteriuria), if not properly managed by the host can turn from uncomplicated to complicated and thereby cause urinary tract infections. Ability of the urobacteria to produce biofilm may add to the already increase rate of antibiotic resistance thereby causing more challenges to health. The urobacteria from Olabisi Onabanjo Teaching Hospital were studied to know their ability to form biofilm and their reactions to some antibiotics. One hundred and sixty-eight mid-stream human urine produced by consented patients who visited the urology unit during the study were cultured on Cysteine lactose electrolyte-deficient agar and identified using microbiological standard methods. Biofilm test was done using the Congo red agar method, and Kirby-Bauer disc diffusion techniques were used to test their antibacterial reaction. One hundred and fifty-two identified pathogens were *Escherichia coli* (26.32%), *Staphylococcus aureus (*44.08%), *Klebsiella pneumoniae (*19.74%), *Proteus mirabilis (*4.61%), *Pseudomonas aeruginosa (*3.95%), and *Candida albicans* (1.32%). The highest occurrence was observed in the age group 31-40 years and among females. Biofilm formation was observed in 23 bacterial species isolated. The highest inhibitory zones observed against the biofilm-positive bacteria were augmentin (34 mm), ciprofloxacin (32 mm), and meropenem (30 mm) while total resistance was observed in *K. pneumoniae*. The findings revealed that females (young adults) are most affected by bacteriuria, with *S. aureus* being the most prevalent bacteria. Biofilm-positive bacteria were variably resistant to antibiotics tested, but the most sensitive ones like augmentin, ciprofloxacin, and meropenem can still be used for effective treatment in the study area.

Keywords: Antibiotics resistance, Bacteriuria, Biofilm formers, Congo red agar, *Staphylococcus aureus*

Introduction

Uropathogens (bacteria, fungi and viruses), present in urine may be significant or not significant which means that they may or may not cause infection (Zhao *et al*., 2020; Cai, 2021). When infection is been caused, it may be complicated (present with symptoms) but when not complicated, there won't be any symptoms but does not mean the bacteria are not present. The problem is that the bacteria present may turn from uncomplicated and thereby cause infection due to host and pathogen actions. This could be linked with development of virulence factor like biofilm.

Biofilm is known as a group of microorganisms that attaches their cells to surfaces, sticks together, and are protected by extracellular polymeric substances (EPS), which make them recalcitrant to unfavorable environments like exposure to antibiotic (Wen *et al*., 2019; Pompilio *et al*., 2020). Communities of microorganisms in biofilm association benefit from various mechanisms such as host defense mechanisms, nutrient absorption and storage, water retention and desiccation resistance, and physical protection against antibiotics and other antimicrobials. Additionally, biofilm exhibits high levels of extracellular enzymatic activity, adherence to the infection site, and cell aggregation, which coordinate the expression of virulence factors through quorum sensing or cell-to-cell communication (Fleming and Rumbaugh, 2017).

Urobacteria are the bacteria found in the host urinary tract. The urinary tract (UT) refers to the urinary part, namely, the kidney, ureter, bladder, and urethra, involved in the production, transport, storage, and excretion of urine (Ammenti *et al*., 2020). Pathogens of different types like bacteria, fungi and viruses can be found in the urinary tract to cause urinary tract infections (Bassey *et al*., 2024). The uropathogens which are the major cause of UTIs consist of more than a hundred species of over fifty genera dwelling within the urinary tract with bacteria being the most responsible for more than 95% of UTI cases (Neugent *et al*., 2020).

The Gram-negative bacteria is the most prevalent, especially, Enterobacteriaceae like *Proteus mirabilis*, *Escherichia coli*, *Enterobacter* species, *Klebsiella pneumoniae*, that cause 90% of UTIs, while Gram-positive bacteria like *Enterococcus faecalis*, *Staphylococcus aureus* and *Streptococcus* species cause only 10% of cases (Hiro and Shawbow, 2015; Seifu and Gebissa, 2018). However, fungal (e.g. Candida species) and viral (e.g. Cytomegalovirus, Adenovirus) infections may occur (Kim *et al*., 2019, Sunho *et al*., 2021).

Several risk factors are associated with UTIs (especially the uncomplicated ones), which can result in the urinary tract becoming colonized with bacteria. These include anatomy design of the urethra, frequent post-coital delaying of urination, poor habit of wiping from the anus to the vagina or penis after defecation, recurrent UTIs, unsafe sexual activity, vaginal infections, excessive douching in females, use of catheter, diabetes, use of birth control implantation, obesity, genetic susceptibility, wearing occlusive and wet underwear (Seifu and Gebissa, 2018; Cai, 2021). Urinary

tract infection is prevalent worldwide affecting all age groups but females are more vulnerable than males because of their broader and shorter urethra which make it easy for fecal microorganisms to enter their urinary tract (Williams *et al*., 2023).

Historically, antibiotics have been the first medication of choice for treating urinary tract infections (UTIs), and several varieties are still in use today. Moreover, biofilm formation is the major virulence determinant of uropathogens, as it has been recorded that over 80% of human infections are caused by biofilm (Fleming and Rumbaugh, 2017; Dhanalakshmi *et al*., 2018). Thus, factors like gender, age, pregnancy status, and reaction to subsequent use of antibiotics, drug mechanisms of reaction are to be considered in prescribing antibiotics, especially now that the rise in drug resistance has become a pressing global challenge (Patel *et al*., 2021).

Hence, the study's objectives were to ascertain the uropathogens' capacity to form biofilm and also antibiotic sensitivity of the seven antibiotics against biofilm-positive urobacteria isolated from in and outpatients of Olabisi Onabanjo Teaching Hospitals in Southwest, Nigeria during the study period.

Materials and Methods

Study area and study subjects

The study was conducted from November 2021 to June 2022 in Olabisi Onabanjo University Teaching Hospital (OOUTH), Ayegbami, Sagamu Local Government, Ogun State in the Southwestern Nigeria. The study subjects approved for this study were both inpatients and outpatients routinely attending the Urology Department of Olabisi Onabanjo University Teaching Hospital (OOUTH) Sagamu, Ogun State.

Ethical approval

The Olabisi Onabanjo University Teaching Hospital Health Research Ethics Committee (OOUTH-HREC) granted ethical approval for the project with reference number OOUTH/HREC/449/2021AP.

Sample collection and Culturing

One hundred and sixty-eight (168) clean-catched mid-stream urine samples of 10 ml each were collected from consented patients that visited the urology units of OOUTH respectively during the time of study and processed immediately in the urology laboratory. A loop full of urine samples was streaked on already solidified Cysteine lactose electrolyte-deficient (CLED) agar (Himedia, India) plates. Inoculated plates were incubated aerobically at 37°C for 24 hours and bacterial colonies were counted using a colony counter (CC-00 SANSEL, Indian). Distinct colonies from each plate were subcultured on nutrient agar (Oxoid) plates, incubated at 37°C for 24 hours, stocked on agar slants and kept in a refrigerator at 4°C for further analysis (Matthijs, 2018).

Identification and Characterization of Bacterial Isolates

The morphologies of the isolated bacteria were obtained by observing the color, shapes, textures and sizes of their colonies on the CLED agar (Sagar, 2022). Also, the biochemical test of the pure colonies of bacteria obtained were identified according to the standard methods (Cheesbrough, 2010). Bacterial isolates were presumptively identified by catalase reaction, oxidase activity, indole reaction, urease test, citrate test, motility test, methyl-red, hydrogen sulfide production, Voges-proskeur test and sugar fermentation test (glucose, maltose, cellulose, lactose, sucrose, dextrose and fructose test) (Cheesbrough, 2010).

Biofilm Test using a Congo-Red Agar Method

The ability of the isolated bacteria to form biofilm by the production of slime was done using Congo red agar (CRA) method (Ramya, *et al.*, 2023). Brain-heart infusion broth (37 g/L) (Himedia, India) was mixed with sucrose (50 g/L) and agar powder (15 g/L) in 1 liter of distilled water and autoclaved at 121℃ for a duration of 15 minutes. The Congo red dye (0.8 g/L) (Kem Light Laboratories Pvt. Ltd.) was prepared separately in another conical flask. The two prepared solution were allowed to cool (55℃), aseptically added and mixed together. Plates with the combined media were streaked with the test organisms and incubated aerobically at 37°C for 24 hours after which observation was made. *Escherichia coli* ATCC 29522 (non-biofilm producer) was used as control strain.

Determination of the Antibiotic Susceptibility

Kirby-Bauer discs diffusion techniques was used to test the antibacterial sensitivity of the biofilm-producing bacteria against seven commonly used antibiotics for the treatment of UTIs: augmentin (10 µg), azithromycin (15 µg), ciprofloxacin (5 µg), erythromycin (15 µg), meropenem (10 µg), nalidixic acid (30 µg) and trimethoprim/sulfamethoxazole (25 µg). The prepared Mueller-Hinton agar (Himedia, India) plates were labelled in triplicate, inoculated with the 24 hours old bacteria suspension (compared with 0.5 McFarland standard turbidity) by streaking with sterile swab stick and allowed to dry.

Antibiotic discs (Bioanalyse Ltd, Turkey and Oxoid, UK) were strategically placed on the dried agar surface using the disc dispenser and allowed to stand for 10 minutes before incubation. The plates were incubated at 37°C for 24 hours and the zones of inhibition were measured (in millimeter) with a ruler and interpreted according to Clinical and Laboratory Standard Institute (CLSI) interpretative chart (CLSI, 2020; Pompilio *et al.*, 2020). The control groups include the non-biofilm producing bacteria.

Results

The findings of the study according to the stated objectives are presented below in a series of tables. The morphology of the isolated organisms showed different colour, edges, shapes, elevations and sizes (Table 1). Gram reaction of the isolates from the two hospitals revealed both the Gram-positive and Gram-negative bacteria (Table 2).

Table 1: Morphology and Gram Reaction of the Uropathogens

Isolates	Morphology					Gram Reaction	Shapes
	Size	Colour	Edges	Clarity	Elevation		
1	Small	Yellow	Round	Opaque	Raised	Gram negative	Rod
2	Samll	Golden Yellow	Round	Translucent	Raised	Gram positive	Cocci
3	Large	Yellowish white	Round	Opaque	Mucoid	Gram negative	Rod
4	Small	Bluish	Round	Translucent	Raised	Gram negative	Rod
5	Small	Greenish	Round	Opaque	Rough	Gram negative	Rod

Table 2: Biochemical identification of the Uropathogens

	Biochemical test									Sugar Fermentation test							Probable Bacteria
I/No	Cat	Oxi	DNa	Ind	Cit	Ure	H_2S	MT	GP	G	C	M	D	F	S	L	
1	+	-	+	+	-	-	-	-	+	+	-	+	+	+	+	+	*Escherichia coli*
2	+	-	-	-	+	+	-	-	-	+	-	+	+	+	+	-	*Staphylococcus aureus*
3	+	-	-	-	+	+	-	-	+	+	+	+	+	+	+	+	*Klebsiella pneumoniae*
4	+	-	-	-	+	+	+	+	+	+	-	+	+	+	-	-	*Proteus mirabilis*
5	+	-	-	+	+	+	-	-	+	+	-	+	+	+	+	-	*Pseudomonas aeruginosa*

Key: Cat- Catalase, Cit- Citrate, H_2S- Hydrogen Sulphide, Ind- Indole, Oxi- Oxidase, Ure- Urease, MT- Motility Test, GP- Gas Production, G-Glucose, C- Cellulose, M- Maltose, D- Dextrose, F- Fructose, S- Sucrose, L-Lactose, + = Positive, - = Negative

Urine samples from patients who visited Olabisi Onabanjo University Teaching Hospital's urology clinic were used to determine the prevalence of isolates. Results showed that the age group 31-40 years had the highest number of isolates (23.81%), followed by the age groups 21-30 years and 41-50 years with prevalence rates of 17.26% (29); 51-60 years, 21 (12.50%); 61-70 years, 17 (10.12%), while 3 (1.79%) for ≤10 years was the least occurrence (Figure 1). Also subjects from OOUTH showed that 51 (34%) and 101 (66%), out of 152 subjects with significant bacteriuria, were males and females respectively (Figure 2).

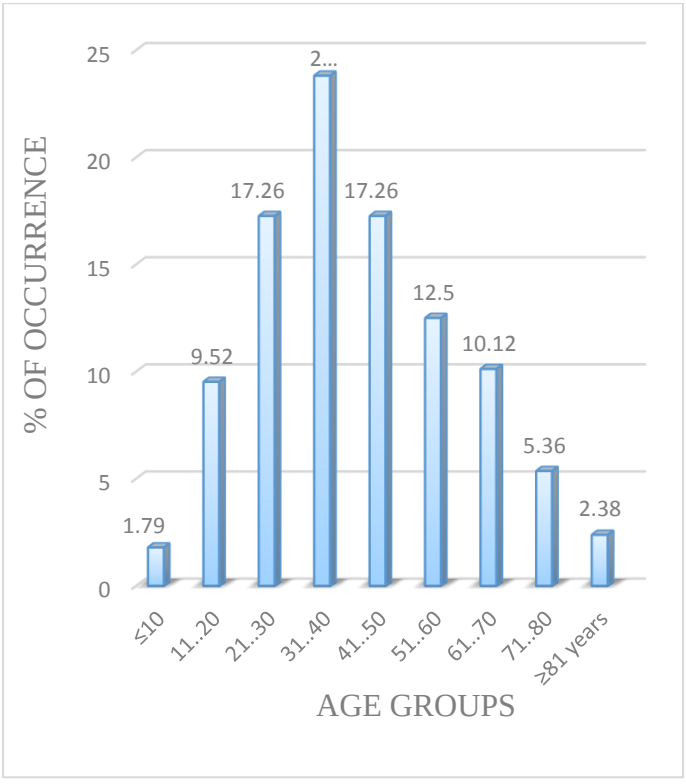

Figure 1: Prevalence of Urobacteria with Respect to Age

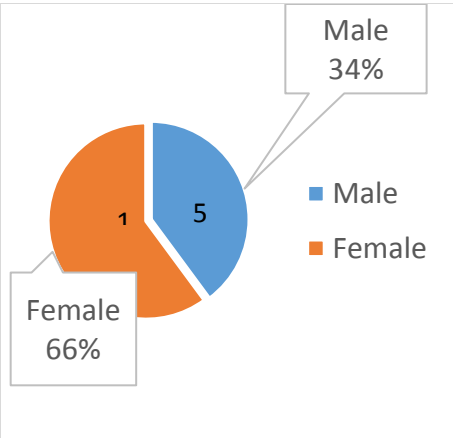

Figure 2: Percentage Occurrence of Bacteriuria with Respect to Gender

The occurrence of biofilm-positive isolates was *Escherichia coli* (8/30), *Staphylococcus aureus* (8/67), *Klebsiella pneumoniae* (5/40), *Proteus mirabilis* (2/7), *Pseudomonas aeruginosa* (0/6) and *Candida albicans* (0/2) were biofilm-positive bacteria (Table 3).

Table 3: Biofilm-Positive Bacteria and Non-Biofilm Producing Isolates

	Total isolates	Biofilm former	Non-biofilm former
Escherichia coli	30	8	22
Klebsiella pneumoniae	40	5	35
Proteus mirabilis	7	2	5
Pseudomonas aeruginosa	6	0	7
Staphylococcus aureus	67	8	59
Candida sp	2	0	2

The seven antibiotics tested showed varied susceptibility (diameter zones) and resistant patterns against all the biofilm-positive bacteria belonging to four genera. Their zones of inhibition in millimeters were grouped into three as sensitive (S), intermediate (I), and resistant (R) (Table 4). Augmentin (34 mm), followed by ciprofloxacin (32 mm), and meropenem (30 mm) showed the highest zones of inhibition against *S. aureus*, *E. coli*, and *S. aureus*, respectively. The lowest zone of inhibition was observed in trimethoprim/sulfamethoxazole (12 mm) against *P. mirabilis* (Table 5).

Table 4: Antibiotic Sensitivity Pattern of Biofilm-Positive Bacterial from OOUTH According to CLSI Chart

Antibiotics		***Escherichia coli* (n=8)**			***Klebsiella Pneumoniae* (n=5)**			***Proteus mirabilis* (n=2)**			***Staphylococcus aureus* (n=8)**		
Response scale		**R**	**I**	**S**	**R**	**I**	**S**	**R**	**I**	**S**	**R**	**I**	**S**
Augmentin (10 µg)	**CLSI values (mm)**	≤ 13	14-16	≥17	≤ 13	14-16	≥17	≤ 13	14-16	≥17	≤ 28	-	≥ 29
	Response rate	2	-	6	3	-	2	2	-	-	1	-	7
Azithromycin (15 µg)	**CLSI values (mm)**	≤ 12	-	≥13	≤ 12	-	≥13	≤ 12	-	≥13	≤ 13	14-17	≥18
	Response rate	1	-	7	5	-	0	0	-	2	0	-	8
Ciprofloxacn (5 µg)	**CLSI values (mm)**	≤ 21	22-25	≥ 26	≤ 21	22-25	≥ 26	≤ 21	22-25	≥ 26	≤ 15	16-20	≥ 21
	Response rate	4	2	2	3	0	2	0	2	0	1	0	7
Erythromycin (15 µg)	**CLSI values (mm)**	≤ 12	-	≥ 13	≤ 12	-	≥ 13	≤ 12	-	≥ 13	≤ 13	14-22	≥ 23
	Response rate	2	-	6	3	-	2	0	-	2	4	-	4
Meropenem (10 µg)	**CLSI values (mm)**	≤ 15	16-18	≥ 19	≤ 15	16-18	≥ 19	≤ 15	16-18	≥ 19	≤ 15	16-18	≥ 19
	Response rate	6	-	2	3	-	2	1	-	1	-	-	8
Nalidixic acid (30 µg)	**CLSI values (mm)**	≤ 13	14-18	≥ 19	≤ 13	14-18	≥ 19	≤ 13	14-18	≥ 19	≤ 15	16-20	≥ 21
	Response rate	-	1	7	3	-	2	-	-	2	-	-	8
Trimethoprim/ Sulfamethoxazole (25 µg)	**CLSI values (mm)**	≤ 10	11-15	≥ 16	≤ 10	11-15	≥ 16	≤ 10	11-15	≥ 16	≤ 10	11-15	≥ 16
	Response rate	4	-	4	3	-	2	-	1	1	1	-	7

Table 5: Zones of inhibition of Antibiotics against Biofilm-Positive Bacteria from UTH

UTH BACTERIAL ISOLATES	Range	ANTIBIOTICS DIAMETER ZONES OF INHIBITION (mm)						
		AUG (10 µg)	AZT (15 µg)	CIP (5 µg)	ERY (15 µg)	MER (10 µg)	NA (30 µg)	TMP/ SMZ (25 µg)
Escherichia coli	Highest	24	18	30	20	28	24	25
	Lowest	15	13	23	15	21	14	18
Klebsiella pneumoniae	Highest	25	R	26	22	27	22	20
	Lowest	23	R	26	17	23	20	18
Proteus mirabilis	Highest	27	18	27	19	26	R	22
	Lowest	16	13	23	14	20	R	12
Staphylococcus aureus	Highest	32	22	20	26	29	25	23
	Lowest	29	18	20	23	17	25	18

Discussion

The worldwide weigh down of antimicrobial resistance is a general public health emergency. Antimicrobial resistance is a significant public health urgent situation that has been mentioned as a shadow pandemic by researchers and a pinnacle collective health threat by the World Health Organization (Antimicrobial Resistance Collaborators, 2022).

Bacteria are the most prevalence pathogens in human urine. The morphology of the isolated organisms showed different color, edges, shapes, elevations and sizes. Gram reaction of the isolates from Olabisi Onabanjo University Teaching Hospital (OOUTH) revealed the presence of both the Gram-positive and Gram-negative bacteria with six genera. Out of the 152/168 urine pathogens isolated, the percentage occurrence is *Staphylococcus aureus* 44.08% (67), *Escherichia coli* 26.32% (40), *Klebsiella pneumoniae* 19.74% (30), *Proteus mirabilis* 4.61% (7), *Pseudomonas aeruginosa* 3.95% (6) and *Candida albicans* 1.32% (2). The finding of *Staphylococcus aureus* as the most prevalent Gram-positive bacterium agrees with the work of Onyedibia *et al*. (2021), and Osungunna *et al*. (2022) whose findings revealed *Staph. aureus as the most prevalent*. Ndako *et al*. (2019) also reported a higher percentage of *Staph. aureus* that doubled that of *E. coli* which may be due to their higher chances of migrating from the gastrointestinal tract to the urinary tract, host age, catheter use, and extended hospital stay (Ndako *et al*., 2019). In contrast, Maione *et al.* (2023) reported *Enterococcus faecalis* as the most prevalent Gram-positive bacterium.

In this study, it was observed that the age group 31-40 years had the highest number of isolates 40 (23.81%), followed by the age groups 21-30 years and 41-50 years with prevalence rates of 17.26% (29); 51-60 years, 21 (12.50%); 61-70 years, 17 (10.12%), while 3 (1.79%) for ≤10 years was the least occurrence (Figure 1). This was attributed to the fact that they are the sexually exposed age group (Tan and Chlebicki, 2016).

Subjects from OOUTH showed that 51 (34%) and 101 (66%), out of 152 subjects with significant bacteriuria, were males and females respectively (Figure 2)). This translates that female subjects had the highest occurrence. This finding is in accordance with previous findings (Hiro and Shawbow, 2015; Williams *et al*., 2023). The higher prevalence observed in females than males may be due to the different anatomical make-up of the urethra which is shorter in females compared to males (Folliero *et al*., 2020; Williams *et al*., 2023).

Out of 152 uropathogens screened for biofilm formation ability, only 23 bacteria had their colonies turned from white to black on congo red medium, which was indicative that they produce biofilm (Biofilm-positive). *Escherichia coli* (8/30), *Staphylococcus aureus* (8/67), *Klebsiella pneumoniae* (5/40), *Proteus mirabilis* (2/7), *Pseudomonas aeruginosa* (0/6) and *Candida albicans* (0/2) were biofilm-positive bacteria. Biofilm has been reported as one of the virulence factors used by bacteria to thwart the efficacy of the antibacterial agents (Mlugu *et al*., 2023).

All the biofilm-positive bacteria from OOUTH were sensitive to erythromycin, meropenem, nalidixic acid and trimethoprim/sulfamethoxazole. Exception is the *K. pneumoniae* which were totally resistant to azithromycin, and *P. mirabilis* which were totally resistant to augmentin and ciprofloxacin. Augmentin (34 mm), followed by ciprofloxacin (32 mm), and meropenem (30 mm) showed the highest zones of inhibition against *S. aureus*, *E. coli*, and *S. aureus*, respectively. The lowest zones of inhibition were observed in trimethoprim/sulfamethoxazole (12 mm) against *P. mirabilis*. Woldemariam *et al.* (2019) reported a high prevalence of common uropathogens resistant to the commonly available antibiotics. Microbial resistance to antibiotics is gradually increasing, thereby increasing money spent on treatment. The multidrug-resistant ability was linked with their ability to use biofilm production as one of their virulent factors, which helped them disrupt the antibiotic's target site, alter the molecules present, and destroy the already bound antibiotics. Moreover, the ability of uropathogens to form biofilm is one of the virulence factors that make them recalcitrant to treatment (Seifi *et al*., 2016). Zhao *et al*. (2020) and Javed *et al*. (2021) reported a high correlation between biofilm production and antibiotic resistance in UPEC.

Conclusion

In summary, this study presents bacteria as the most prevalent microorganisms in urine of both OOUTH subjects. Furthermore, the Gram-negative bacteria, especially the Enterobacteriaceae, are more prevalent in urine than the Gram-positive bacteria. Females that are young adults and sexually active individuals were the most affected age group compared to their male counterparts. The most prevalent bacteria from OOUTH were *Escherichia coli* and *Staphylococcus aureus*. The ability of the uropathogens to form biofilm was seen in to one-fifth of the uropathogens isolates.

The antibiotics highest inhibitory zones observed against the biofilm-positive bacteria were augmentin, ciprofloxacin, and meropenem, respectively, hence, they can be used for effective treatment in the study area.

Conflict of interest: Nil

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