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Detection of Biofilm formation and Antibiotic Resistance Profiles in Bacterial Isolates from Urinary Specimens of Female Students at a Tertiary Institution

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ABSTRACT

This study focused on assessing biofilm formation and antibiotic susceptibility of uropathogens in female students at Federal University Otuoke, Bayelsa State, Nigeria. A total of Ninety (90) urine specimens were collected and analyzed using conventional microbiological methods. Antibiotic susceptibility testing was conducted by standard laboratory guidelines. Biofilm production was determined using phenotypic methods (congo red agar method). The antibiotic resistance profile was via plasmid profile analysis. The data obtained was subjected to an Analysis of Variance (ANOVA). Out of 90 urine specimens, 25(27.78%) exhibited significant bacteriuria. Notably, *Citrobacter* spp, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Enterobacter* spp, *Proteus mirabilis*, and *Staphylococcus aureus* were identified as prominent uropathogens. The Gram-negative and Gram-positive bacteria isolates showed a significant difference ($P<0.05$) in antibiotic susceptibility. *Citrobacter* spp 5(62.5%) was susceptible to ofloxacin and nitrofurantoin. *Enterobacter* spp 2(50%) was susceptible to levofloxacin while resistant to most of the antibiotics. *P. aeruginosa* exhibited resistance to all antibiotics. *K. pneumoniae* was highly sensitive to Oflaxacin and cefixime with 2(50%) respectively, while *P. mirabilis* sensitive to imipenem, ceftriaxone sulbactam, and levofloxacin with 2(50%) respectively. Specific resistance was also observed for *K. pneumoniae* and *P. mirabilis*. Levofloxacin, azithromycin, and ofloxacin were seen to be effective antibiotics against *S. aureus* with 2(100%) respectively. The bacterial isolates were mainly resistant to ciprofloxacin, imipenem, cefuroxime, cefixime, amoxicillin-clavulanate, cefotaxime, gentamycin, and ceftriaxone. *Citrobacter* spp, *Enterobacter* spp, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* were multidrug-resistant and identified as biofilm producers. Following curing, *Enterobacter* spp and *P. aeruginosa* which had previously showed high resistance to majority of the antibiotics became sensitive while *S. aureus* still remained resistant to all the antibiotics. *Enterobacter* spp. and *Pseudomonas aeruginosa* were sensitive to tetracycline and ciprofloxacin with a diameter zone of 19mm and 24mm respectively. Plasmid analysis indicated the presence of MDR plasmids in *Enterobacter* spp, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* thus, suggesting their role in drug resistance in UTIs. The study underscores the importance of promoting proper hygiene and monitoring antibiotic resistance, to address the growing issue of UTIs, especially among females.

Keywords: Uropathogens, biofilm formation, antibiotic susceptibility, resistance profiles, *P. aeruginosa, S. aureus*.

Introduction

Urine is the liquid waste excreted through the urinary tract for the needs of humans and vertebrates for metabolism. It is a body fluid normally metabolized by the human body, and contains essential information about the human health, dietary intake and exposure to certain environmental pollutants (Zhang and Liang, 2016). The urine contains rich physiological and pathological information of the human body (Bouatra *et al*., 2013). Microorganisms do not live as pure cultures of dispersed single cells but instead accumulate at interfaces to form poly-microbial aggregates such as films, mats, flocs, sludge, or biofilms (Flemming and Wingender, 2010). Biofilms are the structured microbial communities that occur as surface-attached cells. They consist mainly of microbial cells which includes bacteria and fungi that are embedded in a self-produced extracellular matrix that are composed of polysaccharides, extracellular DNA and other components.

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Biofilms are the result of complex intra- and intercellular signaling and communication processes, regulated by a complex quorum sensing regulation system, which are ubiquitous in the microbial world (Anghel *et al*., 2013). This social behavior is an evolutionary adaptation strategy for survival in hostile environments including the human host (Macia *et al*., 2014). Biofilms cause significant problems in many areas, both in the medical settings e.g. persistent and recurrent infections, device-related infections and in the non-medical industrial settings e.g. bio-fouling in the drinking water distribution systems and in the food processing environments (Niveditha *et al*., 2012). Biofilm is estimated to account for 80% of microbial infections (Javed *et al*., 2021). They confer numerous advantages to the biofilm forming bacteria. These include protection from antimicrobial agents, exchange of nutrients and metabolites and also genetic exchange between organisms. The incidence of limited penetration of antibiotics into the biofilm and slow rate of cell multiplication of organisms in the biofilm contribute to the development of chronic infections (Tayal *et al*., 2015). Biofilm can be found in the urothelium, renal stones and also in implanted foreign bodies (Soto, 2014). Its formation represents the cornerstone in the pathogenesis of urinary tract infections. This is because bacterial biofilms play a pivotal role in catheter-associated urinary tract infections which account for 40% of all nosocomial infections (Niveditha *et al*., 2012; Rishpana *et al*., 2015).

Bacterial pathogens which include both Gram-negative and Gram-positive are the most common suspects behind urinary tract infections (Delcaru *et al*., 2016; Medina and Castillo-Pino, 2019). *Escherichia coli*, *Proteus mirabilis*, *Klebsiella pneumoniae*, *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*, *Staphylococcus aureus* and *Enterococcus faecalis* are among the most frequent bacterial pathogens that are common in uncomplicated and complicated urinary tract infections (Oumer *et al*., 2021; Bono *et al*., 2022; Venkataraman and Yadav, 2022).

Microbial resistance among uropathogens has increased as a result of indiscriminate prescription and administration of antimicrobial drugs, posing a serious health threat globally (Sharif *et al*., 2012; Prestinaci *et al*., 2015) and has been acknowledged by the society as a critical health concern (Timothy *et al*., 2014).

Plasmids are extra genetic material found in many different types of cells, including bacteria, which are generally responsible for conferring a particular characteristic on the host cell (David and Nanette, 2013). Among these traits include toxin production and drug resistance. Because they enable pathogenic bacteria to acquire several resistance genes in a single transfer event, plasmids are critical in the establishment of antibiotic resistance, posing a threat to human health (Peterson and Kaur, 2018).

The determination of plasmid is very crucial, as it is considered DNA techniques to determine antimicrobial resistance patterns (Nworie *et al*., 2013). Hence, this study was aimed to determine biofilm formation and antibiotic resistance profiles in bacteria from female students with urinary tract infections at a tertiary institution in Nigeria.

Materials and Methods

Specimen Collection

Ninety (90) early morning mid-stream urine specimens from female students of different faculties of Federal University Otuoke, Bayelsa State were collected into sterile universal containers. Respondents were female students from the school's hostels on campus and off campus.

Culturing of specimens and identification of bacterial isolates

The urine specimens obtained were properly mixed to ensure any sediment was evenly distributed, and a small quantity of the well mixed urine specimen was then streaked with a sterilized inoculating loop onto MacConkey agar (Chaitanya agro biotech, India), Cysteine Lactose Electrolyte Deficient (CLED) agar (Hi flown biotech, Berkshire, UK), and Cetrimide agar (Hi Media, India) and incubated for 24hrs at 37ºC. Colonies which developed after incubation were further subcultured on nutrient agar plates to obtain pure cultures. Characterization and identification of isolates were carried out using a combination of colonial morphology (Dilnessa and Bitew, 2016) with slight modification adopted for the Gram stain and biochemical tests (catalase, coagulase, citrate, motility, indole, urease, and triple sugar iron test according to the procedures of Cheesbrough (2006).

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Antibiotic susceptibility testing of isolates

Pure bacteria isolates were subjected to antibiotic susceptibility test by Kirby-Bauer's disc diffusion method on Mueller-Hinton (MH) agar and the zones/diameter of inhibition (mm) were interpreted as per CLSI guidelines. A bacterial suspension equivalent to 0.5 McFarland standard was prepared for inoculation. A sterile swab stick was dipped into the prepared bacterial suspension and inoculated into a Mueller-Hinton agar plate by swabbing the suspension evenly across the surface of the plate. Sterile forceps were then used to place antibiotic disks onto the inoculated agar plate.

The discs (Celtech Diagnostic Belgium) contained the following antibiotics for Gram-positive discs: Amoxicillin Clavulanate - AUG (30µg), Cefotaxime - CTX (25µg), Ceftriaxone Sulbactam - CRO (45µg), Cefexime - ZEM (5µg), Levofloxacin - LBC (5µg), Ciprofloxacin - CIP (5µg), Imipenem - IMP (10µg), Cefuroxime - CXM (30µg), Ofloxacin - OFX (5µg), Erythromycin - ERY (15µg), Gentamycin - GN (10µg), and Azithromycin - AZN (15µg). The Gram-negative discs (Celtech Diagnostics Belgium) contained the following: Amoxicillin Clavulanate - AUG (30µg), Cefotaxime - CTX (25µg), Imipenem – IMP (10µg), Ofloxacin - OFX (5µg), Gentamycin - GN (10µg), Nalidixic Acid - NA (30µg), Nitrofurantoin NF (300µg), Cefuroxime – CXM (30µg), Ceftriaxone Sulbactam – CRO (45µg), Ampiclox – ACX (10µg), Cefexime – ZEM (5µg), and Levofloxacin - LBC (5µg). The discs were gently pressed down to ensure contact with the agar. The plates were then incubated at 37°C for 18 hours to allow for the growth of the bacteria. After incubation, the plates were observed. The clear zones (zones of inhibition) around each antibiotic disc indicating the effectiveness of the antibiotics against the bacteria, were measured using a ruler, recorded in millimeters (mm), and interpreted as sensitive, intermediate, and resistant using CLSI guidelines (CLSI, 2015; CLSI, 2020).

Antibiotic susceptibility testing of isolates before curing

A 18-24 h multidrug resistant bacterial isolates from the previous susceptibility testing was used before the curing. The bacterial isolates were swabbed with a sterile swab stick on Mueller-Hinton agar plates.

The antibiotic susceptibility testing was performed by using Kirby-Bauer disc diffusion method according to the recommended standard of Clinical Laboratory Standard Institute (CLSI), the discs (Maxicare Medical Lab) contained the following antibiotics for positive: chloramphenicol C (30µg), cefotaxime CTX (30µg), amoxicillin AUG (30 µg), tetracycline TE (30µg), erythromycin E (15 µg), linezolid LNZ (30 µg), vancomycin VA (30 µg), azithromycin AZM (15 µg). The discs used for Gram negative (Rapids Labs International) contained the following: chloramphenicol C (30µg), cefotaxime CTX (30µg), amoxicillin AUG (30µg), tetracycline TE (30µg), cefepime/clavlanic acid FEP (40µg), ciprofloxacin CIP (5µg), gentamicin CN (10µg), azithromycin AZM (15 µg). The results were recorded after 24hours of incubation and measurement was taken using the diameter of the inhibition zone (mm) around each disc and interpreted as sensitive, intermediate and resistant using CLSI guideline (CLSI, 2020).

Detection of biofilm production via phenotypic methods (congo red agar method)

A simple qualitative method for detection of biofilm production as described by Freeman *et al*., (1989) was adopted. The medium used was Congo Red Agar (CRA) which composes of Brain heart infusion broth (37 gms/L), sucrose (50 gms/L), agar no.1 (10 gms/L) and congo red stain (0.8 gms/L, Himedia). Biofilm producers form black colonies with a dry crystalline consistency after 24h of incubation and non-biofilm producers form pink colored colonies.

Plasmid Extraction

Extraction of plasmid from bacteria isolates was carried put using TENS-miniprep method (Zhou *et al*., 1990). An overnight bacterial culture on agar plates was harvested using a sterile inoculating loop and transferred into 100µl of nutrient broth medium. Each Eppendorf tube was vortexed using a vortex machine (Sci Finetech vortex mixer microfield) at high speed to resuspend the cells completely. 300µl of TENS buffer was added and the tubes were mixed by inverting them 3-5 times until the mixture became sticky ensuring to prevent chromosomal DNA degradation which may affect the plasmid DNA. 150µl of 3.0m sodium acetate (pH 5.2) was added into the tubes respectively that contains the isolates harvested cells, all the tubes were vortex to mix the cells and the reagents completely.

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After vortexing, the tubes were spun using microcentrifuge (Beckman Coulter Microfuge) for 5 minutes at the highest revolution per minute (14,000rpm) in order to pellet the cell debris and chromosomal DNA. After spinning, the supernatant was transferred into a fresh labeled sterile Eppendorf tube, where it was mixed with 900µl of 100% ethanol (absolute)which has been pre-cooled to -2^{0}C.Then spun for two (2) minutes at 1000rpm to precipitate the plasmid DNA (white pellet is observed) from the supernatant. The supernatant was discarded; the pellet was rinsed twice with 500µl of 70% ethanol, mixed by vortexing and spun for two (2) minutes at 14,000rpm. The supernatant was discarded by decanting, blotted and dried for 3hours at a safe and sterile atmosphere. The plasmids extracted were kept inside the freezer for further use.

Agarose Gel Electrophoresis

The plasmid and DNA were analyzed by gel electrophoresis. Agarose gel (1.5%) was prepared by dissolving 1.5g agarose in 100ml of 1 X TBE (Tris borate EDTA) buffer. The slurry was heated on an electric cooker to dissolve the agarose and the solution was allowed to cool to about 50^{0}C. Two (2) drops of ethidium bromide (EtBr) as an intercalating agent was added to the solution and was gently swirl for even mixture. The solution was carefully poured into the horizontal gel casting tray of which two (2) combs has been inserted prior to the pouring and the gel was left to set at room temperature. The combs were carefully removed and 4µl each of the plasmid samples was mixed with 1µl of the loading dye giving 5µl each for nineteen (19) samples. The DNA ladder was loaded in the first well and the samples were loaded using a micropipette into each well starting from the second well. The gel was submerged in the electrophoresis tank and 0.5 X TBE buffer was poured into the tank the buffer covers the surface of the gel. The electrodes were connected to the power source and run at 80volts for 45 minutes. Plasmids were visualized on ultraviolet (UV-Transilluminator). The bands were photographed using gel documentation.

Plasmid Curing with Acridine Orange

After gel documentation, the two (2) bacterial isolates that produced bands were subjected to curing by treatment with acridine orange. The preserved bacterial isolates were subcultured by streaking on nutrient agar plates and incubated at 37^{0}C for 24 hours. The overnight bacteria culture were harvested in 1ml of lysogeny broth, each labeled and incubated at 37^{0}C for 24 hours. 85ml of nutrient agar was prepared into a conical flask and it was supplemented with 0.043g of acridine orange. The solution was carefully mixed by swirling and reaction (change in colour) was observed. The overnight broth culture was vortexed for 1 minute to mix completely and the micro-centrifuge was used for spinning at 10,000rpm for 5 minutes to pellet cell debris. After spinning, the supernatant was discarded by decanting leaving the cell debris. 1ml of the acridine orange broth was suspended in each of the Eppendorf tubes, mixed by vortexing and each tube was wrapped with aluminum foil because acridine orange is light sensitive. The tubes were incubated at 37^{0}C for 24 hours in a rotary incubator.

Antibiotic susceptibility testing of isolates after curing

Following curing, the three (3) bacterial isolates in acridine orange broth incubated in a shaking/rotary incubator was brought out and each was swabbed with a sterile swab stick on Mueller-Hinton agar plates. Antibiotic susceptibility testing was performed using Kirby-Bauer disc diffusion method according to the recommended standard of Clinical Laboratory Standard Institute (CLSI, 2020). The discs (Maxicare Medical Lab) contained the following antibiotics for positive: chloramphenicol C (30µg), cefotaxime CTX (30µg), amoxicillin AUG (30 µg), tetracycline TE (30µg), erythromycin E (15µg), linezolid LNZ (30µg), vancomycin VA (30µg), azithromycin AZM (15µg).

The discs used for Gram negative (Rapids Labs International) contained the following: chloramphenicol C (30µg), cefotaxime CTX (30µg), amoxicillin AUG (30µg), tetracycline TE (30µg), cefepime/clavlanic acid FEP (40µg), ciprofloxacin CIP (5µg), gentamicin CN (10µg), azithromycin AZM (15 µg). The results were recorded after 24hours of incubation and measurement was taken using the diameter of the inhibition zone (mm) around each disc and interpreted as sensitive, intermediate and resistant using CLSI guideline (CLSI, 2020).

Statistical Analysis

The data obtained were subjected to Analysis of Variance (ANOVA) test. This is to determine the significant difference at 95% confidence interval.

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Results

A total of 90 urine specimens studied, 25 isolates were obtained. The bacterial isolates identified and their percentage occurrence in parenthesis were *Citrobacter* spp 8(32%), *Enterobacter* spp 4(16%), *P. aeruginosa* 5(20%), *K. pneumoniae* 4(16%), *P. mirabilis* 2(8%), and *S. aureus* 2(8%). The gram negative bacilli were the most prevalent bacteria isolated from the urine specimens.

The Age Distribution of the female students from Federal University Otuoke is as shown in Table 1. The highest bacterial isolate was seen within age brackets of 18-20 years and 21-23years. There was no significant difference between the age distributions of the female students at Federal University Otuoke (P> 0.05).

Table 1: Age Distribution of the female students from Federal University Otuoke

Age range (years)	Total number of specimen	Total number of bacteria isolated (%)
18-20	18	10 (40)
21-23	24	10 (40)
24-26	24	5 (20)
27-29	24	0 (0)
Total	90	25 (100)

The distribution of bacteria isolated from female students in Federal University, Otuoke is as shown in Table 2. *Citrobacter* spp has the highest percentage distribution with 8(32%) while *Pseudomonas aeruginosa* and *Staphylococcus aureus* both have the least percentage distribution with 2(8%) respectively is as shown in Table 2 below. Statistically, no significant difference was observed between the percentage distribution of bacterial isolate (P > 0.05).

Table 2: Distribution (%) of bacteria isolated from female students in Federal University Otuoke

Organisms	Number of isolates (%)
Citrobacter spp	8(32.0)
Enterobacter spp	4(16.0)
P. aeruginosa	5(20.0)
K. pneumoniae	4(16.0)
P. mirabilis	2(8.0)
S. aureus	2(8.0)
Total	**25(100)**

The antibiotic susceptibility pattern of the Gram negative bacterial isolates is as indicated in Table 3. Ofloxacin and nitrofurantoin was effective against *Citrobacter* spp 5(62.5%), and resistant to augumentin, cefotaxime, imipenem, nalixidic acid, cefuroxime and ampiclox. *Enterobacter* spp 2(50%) was susceptible to levofloxacin while resistance to most of the antibiotics. *P. aeruginosa* exhibited resistant to all antibiotics. Oflaxacin and cefixime were highly sensitive to *K. pneumoniae* with 2(50%) respectively, while imipenem, ceftriaxone sulbactam and levofloxacin were sensitive to *P. mirabilis* with 2(50%) respectively. However, resistance was also observed for *K. pneumoniae* and *P. mirabilis*. Significant difference was observed among the susceptibility pattern of the gram negative bacterial isolates (P < 0.05).

Table 4 shows the antibiotic susceptibility pattern of gram positive bacterial isolates. Levofloxacin, azithrromycin, and ofloxacin were seen to be effective antibiotics against the *S. aureus* with 2(100%) respectively. While resistant was observed with ciprofloxacin, imipenem, cefuroxime, cefixime, amoxicillin clavulanate, cefotaxime, gentamycin and ceftriaxone.

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Table 3: Antibiotic susceptibility of the Gram negative bacterial isolates from urine specimens of female students

Antibiotics	*Citrobacter* spp n=8(%)			*Enterobacter* spp n=4(%)			*Pseudomonas aeruginosa* n=5(%)			*Klebsiella pneumoniae* n=4(%)			*Proteus mirabilis* n=2(%)		
	NS	**NI**	**NR**	**NS**	**NI**	**NR**	**NS**	**NI**	**NR**	**NS**	**NI**	**NR**	**NS**	**NI**	**NR**
AUG	0(0.0)	0(0.0)	8(100)	0(0.0)	0(0.0)	4(100)	0(0.0)	0(0.0)	5(100)	0(0.0)	0(0.0)	4(100)	0(0.0)	0(0.0)	2(100)
CTX	0(0.0)	0(0.0)	8(100)	0(0.0)	0(0.0)	4(100)	0(0.0)	0(0.0)	5(100)	0(0.0)	1(25)	3(75)	0(0.0)	0(0.0)	2(100)
IMP	0(0.0)	0(0.0)	8(100)	0(0.0)	0(0.0)	4(100)	0(0.0)	0(0.0)	5(100)	0(0.0)	0(0.0)	4(100)	2(100)	0(0.0)	0(0.0)
OFX	5(62.5)	1(12.5)	2(25)	0(0.0)	0(0.0)	4(100)	0(0.0)	0(0.0)	5(100)	2(50)	2(50)	0(0.0)	1(50)	0(0.0)	1(50)
GN	1(12.5)	0(0.0)	7(87.5)	0(0.0)	0(0.0)	4(100)	0(0.0)	0(0.0)	5(100)	0(0.0)	0(0.0)	4(100)	0(0.0)	0(0.0)	2(100)
NA	0(0.0)	0(0.0)	8(100)	0(0.0)	0(0.0)	4(100)	0(0.0)	0(0.0)	5(100)	0(0.0)	0(0.0)	4(100)	0(0.0)	0(0.0)	2(100)
NF	3(37.5)	3(37.5)	2(25)	0(0.0)	0(0.0)	4(100)	0(0.0)	0(0.0)	5(100)	1(25)	1(25)	2(50)	1(50)	0(0.0)	1(50)
CXM	0(0.0)	0(0.0)	8(100)	0(0.0)	0(0.0)	4(100)	0(0.0)	0(0.0)	5(100)	0(0.0)	0(0.0)	4(100)	0(0.0)	0(0.0)	2(100)
CRO	2(25)	3(37.5)	3(37.5)	0(0.0)	0(0.0)	4(100)	0(0.0)	1(20)	4(80)	1(25)	0(0.0)	3(75)	2(100)	0(0.0)	0(0.0)
ACX	0(0.0)	0(0.0)	8(100)	0(0.0)	0(0.0)	4(100)	0(0.0)	0(0.0)	5(100)	0(0.0)	0(0.0)	4(100)	0(0.0)	0(0.0)	2(100)
ZEM	1(12.5)	2(025)	5(62.5)	1(25)	0(0.0)	3(70)	0(0.0)	0(0.0)	5(100)	2(50)	0(0.0)	2(50)	1(50)	0(0.0)	1(50)
LBC	0(0.0)	4(50)	4(50)	2(50)	1(25)	1(25)	0(0.0)	2(40)	3(60)	0(0.0)	1(25)	3(75)	2(100)	0(0.0)	0(0.0)

*NS-number of sensitive, NI-number of intermediate, NR-number of resistant, %-percentage.

Key: AUG; amoxicillin clavulanate-30µg, CTX; cefotaxime-25µg, IMP; imipenem-10µg, NF; nitrofurantoin-300µg, CXM; cefuroxime-30µg, CRO; ceftriaxone sulbactam-45µg, OFX; ofloxacin- 5µg, GN; gentamycin-10µg, NA; nalidixic acid-30µg, ACX; ampiclox-10µg, ZEM; cefixime-5µg, LBC; levofloxacin-5µg.

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Table 4: Antibiotic susceptibility of Gram positive bacterial isolates from urine specimens of female students

Antibiotics	*Staphylococcus aureus* n=2(%)		
	NS	NI	NR
LBC - Levofloxacin- (5µg)	2(100)	0(0.0)	0(0.0)
CIP - Ciprofloxacin- (5µg)	0(0.0)	1(50)	1(50)
AZN - Azithromycin-15µg	2(100)	0(0.0)	0(0.0)
IMP - Imipenem-10µg	0(0.0)	0(0.0)	2(100)
CXM - Cefuroxime-30µg	0(0.0)	0(0.0)	2(100)
ZEM - Cefixime-5µg	0(0.0)	0(0.0)	2(100)
AUG - Amoxicillin clavulanate-30µg	0(0.0)	0(0.0)	2(100)
CTX - Cefotaxime-25µg	0(0.0)	0(0.0)	2(100)
GN - Gentamycin-10µg	0(0.0)	1(50)	1(50)
ERY - Erythromycin-15µg	1(50)	1(50)	0(0.0)
CRO - Ceftriaxone sulbactam-45µg	0(0.0)	0(0.0)	2(100)
OFX - Ofloxacin- 5µg	2(100)	0(0.0)	0(0.0)

Key: -NS-number of sensitive, NI-number of intermediate, NR-number of resistant, %-percentage.

Figure 1 shows the susceptibility pattern of the multidrug resistant *Enterobacter* spp, *P. aeruginosa* and *S. aureus* before curing. Prior to curing, the three bacterial isolates exhibited resistance to all the antibiotics. Following curing, *Enterobacter* spp and *P. aeruginosa* which had previously showed high resistance to majority of the antibiotics became sensitive while *S. aureus* still remained resistant to all the antibiotics. *Enterobacter* spp was susceptible to tetracycline with inhibtion zone of 19mm, chloramphenicol and gentamicin with 18mm respectively while *P. aeruginosa* was susceptible to ciprofloxacin with inhibition zone of 24mm, chloramphenicol and gentamicin with 20mm respectively as shown in Figure 2.

The result of the biofilm formation by the bacterial isolates isolated from urine of female students using phenotypic method is as shown in Table 5. *Citrobacter* spp, *Enterobacter* spp, *P. aeruginosa* and S. *aureus* showed biofilm formation.

The detection of plasmid DNA through agarose gel electrophoresis is shown in Plate 1. The plasmid analysis revealed that multiple drug resistant *Enterobacter* spp, *S. aureus* and *P. aeruginosa* isolates harbor plasmid with molecular weight of at molecular weight above 23130bp respectively. Lane 4; no plasmid was detected for *Citrobacter* spp.

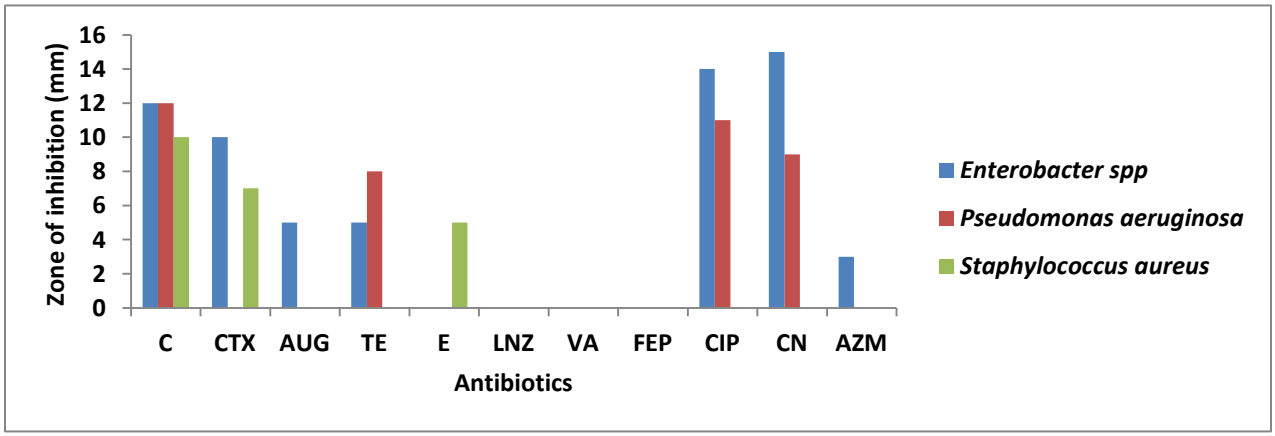

Fig. 1: Antibiotic susceptibility of bacterial isolates from urine of female students before plasmid curing

Key: C=Chloramphenicol, CTX=Cefotaxime, AUG=Amoxicillin, TE=Tetracycline, E=Erythromycin, LNZ=Linezolid, VA=Vancomycin, FEP=Cefepime clavlanic acid, CIP= Ciprofloxacin, CN=Gentamicin, AZM=Azithromycin,

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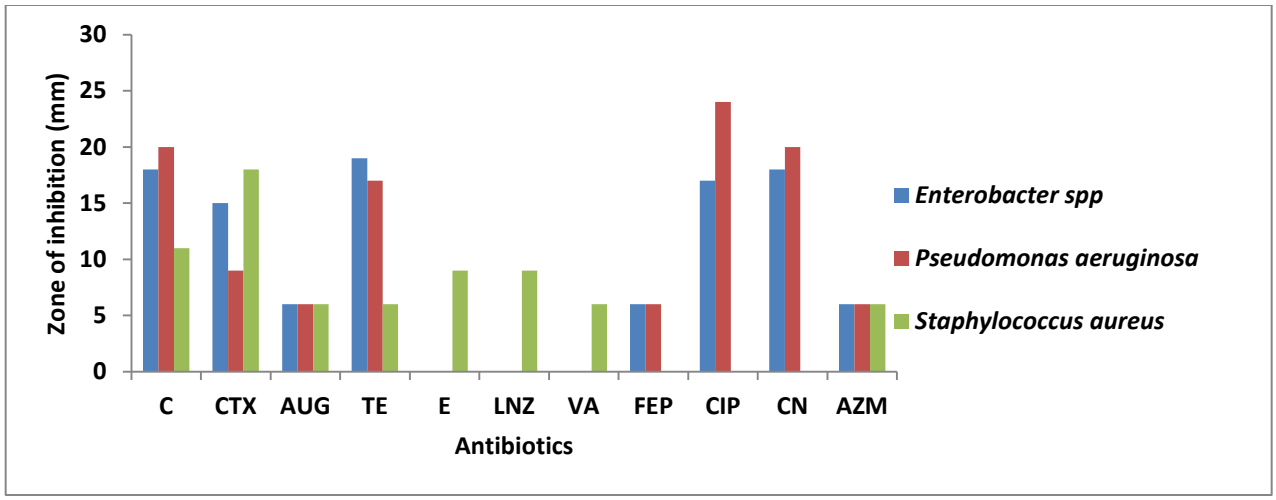

Fig. 2: Antibiotic susceptibility of bacterial isolates after plasmid curing

Key: C=Chloramphenicol, CTX=Cefotaxime, AUG=Amoxicillin, TE=Tetracycline, E=Erythromycin, LNZ=Linezolid, VA=Vancomycin, FEP=Cefepime clavlanic acid, CIP= Ciprofloxacin, CN=Gentamicin, AZM=Azithromycin,

Table 5: Detection of biofilm production by phenotypic method via Congo red agar

Organism	Description	Remark
Citrobacter spp	Colour of medium turn from red to black after 24 h incubation	Positive
Enterobacter spp	Colour of medium turn from red to black after 24 h incubation	Positive
P. aeruginosa	Colour of medium turn from red to black after 24 h incubation	Positive
S. aureus	Colour of medium turn from red to black after 24 h incubation	Positive

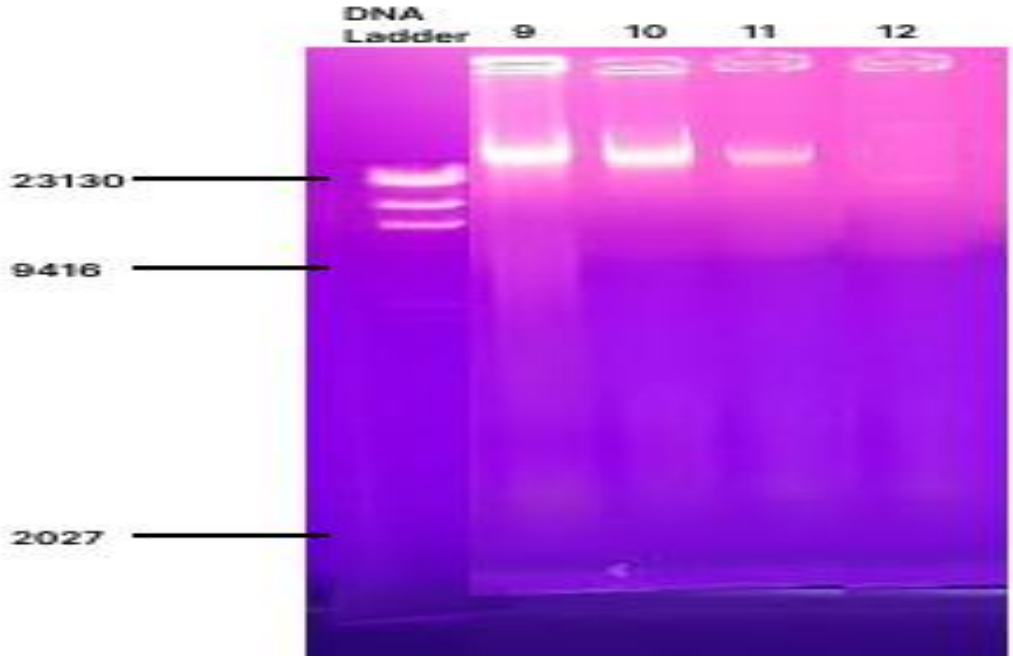

Plate 1: Molecular weight of plasmid DNA on an agarose gel stained with ethidium bromide

Lane M marker DNA - 23130bp DNA ladder, lanes 9, 10, and 11=plasmid DNA bands of multidrug drug-resistant *P. aeruginosa*, *Enterobacter* spp, and *S. aureus* respectively at molecular weight above 23130bp. Lane 12; no plasmid of *Citrobacter* spp.

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Discussion

Urinary tract infections are most common in women due to their anatomic and physical factors. This cuurent study showed the presence of *Citrobacter* spp, *Enterobacter* spp, *P. mirabilis, K. pneumoniae, P. aeruginosa* and *S. aureus* in the urine of the female students. *Citrobacter* spp was seen to be predominant bacteria with 8(32%) followed by *P. aeruginosa* 5(20%). *Enterobacter* spp and *K. pneumonae* both showed a prevalence of 4(16%). The least prevalence was exhibited by *P. mirabilis* and *Staphylococcus aureus* at 2(8%). The only gram positive isolate identified was *S. aureus*. This presence of these bacterial isolates can be due to improper toilet habits and poor hygiene. Our result of the isolated organisms revealed the prevalence of the most identified uropathogen was Gram negative organism; *Citrobacter* spp with 8/25 (32%). This fnding is in contrast with the researches by Majumder *et al*. (2014) and Haritha *et al*. (2023) who identified *Escherichia coli* as the most prevalent Gram negative organism isolated from urine specimens and the most common cause of UTI in both males and females.

The most susceptible age group with the bacterial isolates was 18-23years, followed by 24-27years and 27-30years. However, the highest number of *Citrobacter* spp was found in the age group of 18-23 years which is similar to the findings of the study by Sood and Gupta, (2012) as their anatomy makes them more vulnerable and prone to this organism. Gram negative organisms were the most isolated pathogens in this study. This finding can be compared with report by Flores-Mireles *et al*. (2015) and Stefaniuk *et al*. (2016).

The antibiotic susceptibility pattern of the gram negative bacterial isolates; resistance was observed with *Citrobacter* spp while high resistance was seen with *Enterobacter* spp, *P. aeruginosa* and *S. aureus. Enterobacter* spp exhibited resistance to amoxicillin clavulanate, cefotaxime, imipenem, ofloxacin, gentamycin, nalidixic acid, nitrofurantoin, cefuroxime, ceftriaxone sulbactam and ampiclox, while showing susceptibility to ofloxacin 5(62.5%), and levofloxacin 2(50%) respectively. *Klebsiella pneumoniae* and *P. mirabilis* also exhibited resistance to amoxicillin clavulanate, cefotaxime, nalidixic acid, cefuroxime and ampiclox while *K. pneumoniae* showed susceptibility to ofloxacin and cefixime; and *Proteus mirabilis* to imipenem, ceftriaxone sulbactam and levofloxacin with 2(50%) respectively. High resistance to all the antibiotics was observed with *P. aeruginosa*. *S. aureus* exhibited resistance to ciprofloxacin, imipenem, cefuroxime, cefexime, amoxicillin clavulanate, cefotaxime, gentamycin and ceftriaxone sulbactam. while susceptible to levofloxacin and ofloxacin with 2(100%) respectively. Significant difference was observed among the susceptibility pattern of the gram negative bacterial isolates ($P< 0.05$). The resistance exhibited by these bacteria to more than two antibiotics, classifies them as mulitidrug-resistant (MDR).

This report is in accordance with the studies by Alabi and Lawal, (2018) and Wolters *et al*. (2020). The high resistance observed may be due to poor hygiene practices, abuse or excessive intake of antibiotics, Furthermore, the female students could be harbouring the resistance strains of the bacteria acquired from the environment since plasmids conferring antibiotic resistance between strains and species of organisms could be readily transferred.

The biofilm assessment was carried out only on *Citrobacter* spp, *Enterobacter* spp, *P. aeruginosa* and S. *aureus.* This was due to high multidrug resistance shown by these bacterial isolates. The analysis revealed these bacteria to biofilms producers. The production of biofilms by these uropathogens, mostly Gram positive bacteria have been connected with their continuous persistence in urinary tract infection and could be due to their virulence mechanism. This report can be compared with the sudy by Harika *et al*. (2020) who reported *S. aureus, K. pneumoniae P. aeruginosa* and *A. baumanii* as major organism that produce biofilms. Shrestha *et al*. (2019) also reported that 80% of uropathogenic Gram-positive cocci are biofilm producers.

The plasmid analysis revealed that multiple drug resistant *Enterobacter* spp, *S. aureus* and *P. aeruginosa* isolates harbor plasmids with molecular weight appearing higher than the DNA molecular weight of 23130bp respectively while *Citrobacter* spp lack plasmids. This could be due to the supercoiled nature of the plasmid or secondary structures (such as hairpin loops). These two factors may have influenced the mobility of the plasmid DNA during gel eletrophoresis, causing them to migrate differently than linear DNA molecules of the same size.

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The supercoiling occurs when the plasmid twist on itself, thereby affecting its migration rate on the gel electrophoresis, while the slowing down of the plasmid bands movement by secondary structures, makes them appear higher than the DNA ladder (Gibson *et al*., 2020). Before plasmid curing, all the antibiotics exhibited resistance to the three bacterial isolates. While after curing, multiple antibiotics resistant *P. aeruginosa* and *Enterobacter* spp became sensitive to chloramphenical and gentamicin with diameter zone of 18mm respectively while *P. aeruginosa* was sensitive to ciprofloxacin with 24mm after curing with ethidium bromide. *S. aureus* were resistant prior to plasmid curing still retain their resistant to prescribed antibiotics after curing. This result is similar to the report by Yah *et al*. (2007) who stated that resistance of some bacteria might be chromosomal mediated since they remained resistant after curing. Another study by Nmesirionye *et al.* (2022) demonstrated that majority resistant bacteria carrying plasmids continued to be resistant to antibiotics even after curing. Furthermore, our finding on resistance is comparable to the other work carried out by other researchers who reported that plasmid have encoded gene that confer resistance to naturally occurring antibiotics in modest environmental niche (Kroll *et al*., 2010). The absence of plasmid in *Citrobacter* spp may not demonstrate that the characteristic is plasmid-encoded because many plasmids can incorporate into the DNA of the bacterial host as reported by Patwardhan *et al*. (2018).

In addition, the resistance may not be chromosomal borne, it may be due to MDR plasmids that might have be acquired by susceptible bacteria through treatment with antibiotics that can be induced and handpicked for horizontal gene transfer, as similarly reported by Ebele *et al*. (2022). However, other scientist assumed that the acquisition of resistance may be due to chromosomal mutation or plasmids that are capable of transferring from one strain of organism to another even across the species in addition to environmental influence. Therefore, the gene coding for antibiotics resistance may perhaps either encode on plasmid or chromosomal DNA (Haavisto, 2023).

In this present study, molecular characterization and genotyping of the bacterial isolates was not carried, due to financial constraints. This we acknowledge as our limitation.

In conclusion, with the multidrug resistance pattern presented, this report should be useful to clinicians in the selection of appropriate antimicrobial treatment options. The findings from this study have revealed and offer practical insights for healthcare providers, potentially enhancing the effectiveness of urinary tract infection prevention and treatment strategies in this specific demographic. Educational programs aimed at female students to increase awareness of urinary tract infections (UTIs), their causes, and prevention measures should be implemented.

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