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# Plasmid Profile of Uropathogens among Children

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***Authors' contributions***

*This work was carried out in collaboration between all authors. Author JCE brought the concepts, designed and did the clinical studies. Authors KMEO, CNA and CCE defined all intellectual content, performed the literature search, experimental studies, data acquisition and data analysis. Authors CCE, NRA and CCE prepared, edited and reviewed the manuscript. All authors read and approved the final manuscript.*

***Original Research Article***

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## ABSTRACT

**Aims:** The study was carried out in order to determine the plasmid profile, antibiotic susceptibility pattern and the type of antimicrobial resistance (whether it is chromosomal or plasmid mediated) among producers of extended spectrum beta-lactamases of uropathogens in children.
**Study Design:** A cross-sectional study of three hundred children in a hospital.
**Place and Duration of Study:** Department of Pediatrics (Pediatrics Ward) and Department of Medical Microbiology and Parasitology, Nnamdi Azikiwe University Teaching Hospital, Nigeria between January 2009 to September 2010.
**Methodology:** Clean-catch urine samples were collected from 300 children aged 1 month to 16 years with suspected community acquired urinary tract infection. Isolated bacteria were identified using standard microbiological techniques. Antimicrobial susceptibility test was carried out by disc diffusion method. Extended Spectrum Beta-Lactamase (ESBL) was determined among the Gram-negative bacteria using double disc synergy test (DDST). The plasmid DNA of the bacterial isolates was extracted using

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alkalysis method and electrophoresed on 0.8% agarose gel stained with 2µl ethidium bromide (EtBr).
**Result:** The result of the study showed that *Staphylococcus aureus* had the highest prevalence among gram positive bacteria. *Escherichia coli* had the highest prevalence among gram negative bacteria. *Staphylococcus aureus* showed cross resistance towards some of the antimicrobial agents. *Escherichia coli and Pseudomonas* showed multiple drug resistance. All the uropathogens isolated were 100% susceptible to imipenem. The study highlights among the ESBL-producers, plasmids of higher molecular weight of 30Kb.
**Conclusion:** It is therefore suggested that appropriate antimicrobial agent be administered to reduce the risk of multi-drug resistance and avert the ineffectiveness of antimicrobial agents.

*Keywords: Uropathogens; children; plasmid profile; urinary tract infection.*

## 1. INTRODUCTION

Urinary tract infection (UTI) is one of the most common infectious diseases in children and is rated second after Upper respiratory tract infections [1-3]. The infections may be symptomatic or asymptomatic and either type of infection can result in serious sequelea if left untreated [4]. Urinary tract infections are associated with a lot of predisposing and risk factors ranging from bacterial virulence to host factors [5]. Different microorganisms can cause Urinary tract infections including fungi and viruses, but bacteria are the most causative agents and are responsible for 95% of cases worldwide. The predominant organisms are mostly the gram negative enteric bacilli [5]. Theoretically, UTI could be defined as colonization of a pathogen occurring anywhere along the Urinary tract: kidney, ureter, bladder, and urethra. Therefore in medical practice, a definition of urinary tract infection requires a combination of both clinical manifestations and laboratory findings [5]. Treatment of UTI is often started empirically [6] and these empirical treatments, most often have resulted to treatment failure due to antimicrobial resistance that could cause renal failure among children in their later years [5]. Bacterial resistance is becoming a serious threat to the medical world that has left Clinicians with few therapeutic options [6]. Bacterial resistance to different classes of antimicrobial agents may be encoded on the bacterial plasmids which are mostly transferable by conjugation, transformation and phage-mediated transduction [7]. Plasmids have been reported to confer resistance to their host bacteria by some studies [8,9] and this conferred resistance may lead to therapeutic failures. This study provides a first report on the plasmid profile of uropathogens among children in South Eastern Nigeria.

## 2. MATERIALS AND METHODS

### 2.1 Sample Collection and Processing

Clean catch urine samples were collected from the children (less than 16 years) aseptically using sterile dry wide mouth container. Absolute care was taken to ensure that contamination from either the children's anterior urethra or perineal skin did not occur. The samples were analyzed using standard microbiological techniques.

## 2.2 Antimicrobial Susceptibility Testing

### 2.2.1 Standardization of inoculums

Some colonies of the test organism was picked with a sterile wire loop from a pure culture plate of the organism and inoculated into a 4mls nutrient broth medium (Lab M) and standardized to 0.5 McFarland standard against a white sheet to match the turbidity of standard suspension. An aliquot of 0.5 McFarland equivalent standard of the test organisms were streaked on the surface of a sterile Mueller Hinton (FROM OXOID, UK.) plate using a sterile swab stick.

### 2.2.2 Antimicrobial agents

The antimicrobial agents that were used include: Ceftazidime (30µg), Cefotaxime (30µg), Ceftriaxone (30µg), Augmentin -Amoxicillin plus Clavulanic acid (30µg), Ciprofloxacin (CIP) 5µg, Imipenem (10µg) (from Oxoid Laboratories, UK), then, Ampicillin plus Cloxacillin (30µg), Erythromycin (5µg), Cotrimoxazole (25µg), Nalidixic Acid (30µg), Colistin (5µg), Tetracycline (25µg) (from Abtek Biologicals Ltd USA). All plates were incubated for 18-24hrs at 37ºC aerobically. Interpretation was carried out according to the CLSI criteria. Controls were used as recommended by Clinical Laboratory Institute Standard [10].

## 2.3 Detection of Extended-spectrum Beta-lactamase

The presence of Extended-Spectrum Beta-Lactamase (ESBL) was detected by the Double Disk Synergy Test (DDST). A suspension of the test organism was inoculated evenly on Mueller-Hinton agar. A disk containing 30µg Amoxicillin plus Clavulanic acid was placed centrally on the plate. Ceftazidime and Cefotaxime disks were placed at a distance of 20mm (center to center) from the Amoxicillin + Clavulanic acid disk. The plates were incubated over night at 35ºC and the zones of inhibition patterns were noted. Isolates that exhibited a distinct shape/ size with potentiation towards Amoxicillin + Clavulanate disk were considered as ESBL producers. Controls were used as recommended by Clinical Laboratory Institute Standard [10].

## 2.4 Plasmid Processing

The alkaline phosphate method was used to isolate plasmid DNA from the bacteria. An overnight broth culture of each strain was obtained in 1.5 ml. Nutrient broth into Eppendorf tube capped and centrifuged at 8,000g for 2 mins. The supernatant was removed leaving the cell pellet, then suspended in 200 µl of ice-cold buffer solution, 400mM Tris (pH 8.0) to wash and suspend the cells into the liquid phase. A lysis solution, 4% sodium dodecyl sulphate was then added in 400 µl to the tubes which were then inverted x 20 at 28°C. Ice-cold-buffered solution, 3.0M sodium acetate pH 5.5, in 300 µl, was then added to stop the cell lysis and the tubes were centrifuged at 3000 g for 15 mins and the supernatant was transferred into fresh Eppendorf tubes. Chloroform 700 µl, was added to each tube and mixed gently by vortexing, followed by centrifugation at 3000g for 10 mins. To 500 µl of supernatant obtained in fresh tube, 1 ml of absolute alcohol was added to precipitate the plasmid DNA. The tubes were then held on ice for 1 h after which the tubes were centrifuged at 3000g for 30 mins. Then supernatant was removed and 70% ethanol added to wash the pellets in the tubes. The mixture was again centrifuged for 5 mins and the supernatant was removed. The tubes were then inverted on a paper towel to drain

the remaining traces of liquid. The pellets were resuspended on 100 µl of 10 mMTris buffer solution, pH 8.0. Agarose gel electrophoresis was carried out on the isolate using 0.8% w/v agarose gel measuring 20 x 10 cm in length and 3 mm deep, to determine the M. wt. of the plasmid isolate. The volume of agarose was 100 m in single-strength Tris-borate/EDTA electrophoresis buffer. The gel was run at 75 V for approximate 1½ h. To visualize the DNA after electrophoresis, the gel was transferred to a 0.5 µg/ml solution of ethidium bromide in de-ionized water and allowed to strain for 10 - 15 mins at room temperature, 28°C.The stained gel was visualized with short-wave UV light Trans illuminator and photographed. The DNA bands were matched with those for Lambda DNA Hind III digest molecular weight marker in the range 2,322Bp - 23,130Bp.

## 2.5 Curing Experiments

The ESBL producing isolates were grown in 10mL of nutrient broth and incubated at 37ºC for 24hours to get an overnight suspension of their culture respectively. 1ml of the Overnight cultures of the test organism was inoculated in 15mls double strength of nutrient broth supplemented with 1ml of different percentages concentration (5%, 9%, 9.5%, 10%, 11%, 12%, 13%, 15%, 20%) of Sodium dodecyl sulphate (SDS) (SIGMA,INDIA) solution, and incubated at 37ºC for 24hours. The progeny of each isolates were subcultured on a Mueller Hinton agar (From Oxoid Laboratory, UK) severally to get a homogenous colonies. To determine the most efficacious concentration, the plasmid DNA of the progenies were extracted using alkalysis method and separated by electrophoresis on 0.8% agarose gel at 90V. The DNA Samples were loaded into the gel wells along with Hind III digest of lambda DNA (Sigma chemicals),used as molecular weight standard. After viewing in the transilluminator the progeny of the concentration that successively cured the organisms of the plasmid they contained were re-identified to know whether they still posses the characteristics of the parent organisms and finally re-screened for ESBL using of the Double Disk Synergy Test (DDST). The positive control was the ESBL- producing isolates not subjected to SDS (Sodium dodecyl sulphate).

## 3. RESULTS AND DISCUSSION

The plasmid profile of uropathogens among 300 children was studied. The prevalence of uropathogens (Gram-positive and Gram-negative organisms) were: *E. coli* (35%), *Pseudomonas* (8.17%), *Klebsiella pneumoniae* (25.83%), *Klebsiella oxytoca* (8.17%), *Staphylococcus aureus*(39.17%), *Staphylococcus xylosus* (16.5%), and *Staphylococcus chromgenes* (8.27%). *Pseudomonas* showed 100% resistance to all the beta-lactam antibiotics except Imipenem (Table 1). (Table 2) showed 100% resistance of *E.coli* to Cotrimoxazole. All the gram positive bacteria were susceptible to Imipenem (Table 3). Among the gram positive bacteria, *Staphylococcus chromgenes* showed decreased susceptibility across various antibiotics, (Table 4). The Plasmid profile of Gram-negative and positive organisms are shown in (Table 5) with ESBL-producing gram-negative bacteria having plasmid of higher molecular weight of 30 Kb. The Plasmid profile of Gram-positive organisms had plasmid of molecular weight 11Kb, 20Kb and 30Kb respectively.

**Table 1. Cumulative susceptibility of gram negative bacteria to beta-lactam antibiotics**

| Organism | AS | CFX | CTZ | CEF | APX | AUG | IMP |
|---|---|---|---|---|---|---|---|
| *E. coli* | S (%) | 16.67 | 16.67 | 16.67 | 0 | 50.00 | 100.00 |
| | I (%) | 16.67 | 33.33 | 16.67 | 16.67 | 16.67 | 0 |
| | R (%) | 66.67 | 50.00 | 67.67 | 83.33 | 33.33 | 0 |
| *K. oxytoca* | S (%) | 0 | 0 | 0 | 0 | 0 | 100.00 |
| | I (%) | 0 | 100.00 | 0 | 0 | 100.00 | 0 |
| | R (%) | 100.00 | 0 | 100.00 | 100.00 | 0 | 0 |
| *K. pneumoniae* | S (%) | 40.00 | 20.00 | 40.00 | 60.00 | 20.00 | 100.00 |
| | I (%) | 0 | 40.00 | 0 | 20.00 | 20.00 | 0 |
| | R (%) | 60.00 | 40.00 | 60.00 | 20.00 | 60.00 | 0 |
| *Pseudomonas* | S (%) | 0 | 0 | 0 | 0 | 0 | 100.00 |
| | I (%) | 0 | 0 | 0 | 0 | 0 | 0 |
| | R (%) | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 0 |

*Key: AS - Antibiotic Susceptibility, Resistant (R), Intermediate (I), Sensitivity (S), CFX - Cefotaxime (30µg), CTZ- Ceftazidime (30µg), CEF - Ceftriaxone (30µg), APX - Ampicillin plus Cloxacillin (30µg) AUG - Augmentin - Amoxicillin plus Clavulanic acid (30µg), IMP- Imipenem (10µg)*

**Table 2. Cumulative susceptibility of gram negative bacteria to other antibiotics**

| Organism | AS | TET | ERY | CIP | NAX | COL | COT |
|---|---|---|---|---|---|---|---|
| *E. coli* | S (%) | 50.00 | 0 | 33.33 | 0 | 0 | 0 |
| | I (%) | 16.67 | 16.67 | 33.33 | 16.67 | 16.67 | 0 |
| | R (%) | 33.33 | 83.33 | 33.33 | 83.33 | 83.33 | 100.00 |
| *K. oxytoca* | S (%) | 0 | 0 | 0 | 0 | 0 | 0 |
| | I (%) | 100.00 | 0 | 100.00 | 0 | 0 | 0 |
| | R (%) | 0 | 100.00 | 0 | 100.00 | 100.00 | 100.00 |
| *K. pneumoniae* | S (%) | 20.00 | 0 | 40.00 | 40.00 | 0 | 40.00 |
| | I (%) | 40.00 | 20.00 | 40.00 | 0 | 20.00 | 20.00 |
| | R (%) | 40.00 | 80.00 | 20.00 | 60.00 | 80.00 | 40.00 |
| *Pseudomonas* | S (%) | 0 | 0 | 0 | 0 | 0 | 0 |
| | I (%) | 0 | 0 | 100.00 | 0 | 0 | 0 |
| | R (%) | 100.00 | 100.00 | 0 | 100.00 | 100.00 | 100.00 |

*Key: AS - Antibiotic Susceptibility, Resistant (R), Intermediate (I), Sensitivity (S), TET - Tetracycline (25µg), ERY- Erythromycin (5µg), CIP - Ciprofloxacin (5µg), NAX - Nalidixic Acid (30µg), COL - Colistin (5µg), COT- Cotrimoxazole (25µg)*

**Table 3. Cumulative susceptibility of gram positive bacteria to beta-lactam antibiotics**

| Organism | AS | CFX | CTZ | CEF | APX | AUG | IMP |
|---|---|---|---|---|---|---|---|
| *S. aureus* | S (%) | 28.57 | 28.57 | 42.86 | 28.57 | 14.28 | 100.00 |
| | I (%) | 42.86 | 28.57 | 42.86 | 28.57 | 28.57 | 0 |
| | R (%) | 42.86 | 42.86 | 14.28 | 42.86 | 57.14 | 0 |
| *S. chromogens* | S (%) | 0 | 100.00 | 0 | 0 | 0 | 100.00 |
| | I (%) | 100.00 | 0 | 100.00 | 0 | 0 | 0 |
| | R (%) | 0 | 0 | 0 | 100.00 | 100.00 | 0 |
| *S. xylosus* | S (%) | 66.67 | 0 | 33.33 | 33.33 | 33.33 | 100.00 |
| | I (%) | 33.33 | 66.67 | 33.33 | 0 | 33.33 | 0 |
| | R (%) | 0 | 33.33 | 33.33 | 66.67 | 33.33 | 0 |

*Key: AS - Antibiotic Susceptibility, Resistant (R), Intermediate (I), Sensitivity (S), CFX - Cefotaxime (30µg), CTZ- Ceftazidime (30µg), CEF - Ceftriaxone (30µg), APX - Ampicillin plus Cloxacillin (30µg) AUG - Augmentin - Amoxicillin plus Clavulanic acid (30µg), IMP- Imipenem (10µg)*

**Table 4. Cumulative susceptibility of gram positive bacteria to other antibiotics**

| Organism | AS | TET | ERY | CIP | COL | COT |
|---|---|---|---|---|---|---|
| *S. aureus* | S (%) | 42.86 | 42.86 | 28.57 | 42.86 | 0 |
| | I (%) | 42.86 | 42.86 | 28.57 | 28.57 | 14.28 |
| | R (%) | 14.28 | 14.28 | 42.86 | 28.57 | 85.71 |
| *S. chromogens* | S (%) | 100.00 | 0 | 0 | 0 | 0 |
| | I (%) | 0 | 100.00 | 0 | 0 | 0 |
| | R (%) | 0 | 0 | 100.00 | 100.00 | 100.00 |
| *S. xylosus* | S (%) | 33.33 | 0 | 33.33 | 33.33 | 33.33 |
| | I (%) | 33.33 | 66.67 | 66.67 | 33.33 | 0 |
| | R (%) | 33.33 | 33.33 | 0 | 33.33 | 66.67 |

*Key: AS - Antibiotic Susceptibility, Resistant (R), Intermediate (I), Sensitivity (S), TET - Tetracycline (25µg), ERY- Erythromycin (5µg), CIP - Ciprofloxacin (5µg), COL - Colistin (5µg), COT- Cotrimoxazole (25µg)*

**Table 5. Plasmid profile of the uropathogens**

| Uropathogens | ESBL-producing | Plasmid profile (size) |
|---|---|---|
| **Gram-positive** | | |
| S. aureus | ND | 30kb(23130bp) |
| S. chromogens | No | 20kb(6557bp) |
| S. xylosus | No | 11kb(2027bp) |
| **Gram-negative** | | |
| E. coli | No | 11kb(2027bp) |
| E. coli | Yes | 30kb(23130bp) |
| K. oxytoca | No | 11kb(2027bp) |
| K. oxytoca | Yes | 30kb(23130bp) |
| K. pneumoniae | Yes | 30kb(23130bp) |
| Pseudomonas | No | 11kb(2027bp) |

*Key: ND – 'Not detected'*

Urinary tract infections are mainly due to the invasion of the urethra, bladder or kidneys by pathogens belonging to the family *Enterobacteriacae*. From the study, *E. coli, Klebsiella oxytoca* and *Pseudomonas* showed 100% resistance to Cotrimoxazole. The reason for such resistance exhibited towards Cotrimoxazole could be attributed to widespread and indiscriminate use of the drug. The enzymes responsible for cotrimoxazole resistance are plasmid encoded and the gram-positive cocci could have acquired the property innately or artificially through either mutation or other processes. There was high degree of plasmids relatedness among the bacteria isolates from the various patients because of the presence of the similar size plasmids of molecular weight approximating 30Kb, 20Kb, and 11Kb. The results showed that to a great extent, there was homogeneity between the isolates but some isolates (*E. coli* and *Klebsiella oxytoca)* also had two plasmids. Our findings partially agrees with reports by Wax, et al. [11] and Ezeonu and Ayogu [12] that showed some level of homogeneity among the *Staphylococcus aureus*s trains.

Antibiotic resistant *enterobacteriaceae* can cause major clinical problems in human healthcare. This resistance is related to increasing mis-use of antimicrobial drugs [13]. Beta-lactam agents such as penicillins and cephalosporins are the most widely used antibiotics and β-lactamase production is the most common type of resistance mechanism exhibited by these bacteria. *Pseudomonas* species isolated showed 100% resistance to all the third

generation cephalosporins in the study. Multidrug resistance was observed among gram-negative bacteria. Two (02) *E. coli* strains, one (01) *Klebsiella pneumoniae*and one (1) *Pseudomonas* species were ESBL producers. Other Gram-negative isolates were non-ESBL producers but showed resistance to at least one beta-lactam antibiotic. However, the non-ESBL producers were suspected to be ESBL producers by CLSI criteria. Akujobi and Ewuru [14] isolated 144 bacteria resistant to at least one beta-lactam antibiotic of which only 40 were ESBL producers, confirming our results. They postulated that this could have resulted from other resistant enzymes other than ESBL; such as inhibitor resistant beta-lactamases (IRT). Furthermore, some strains harbour both ESBL and inhibitor resistant beta-lactamase Amp-C which prevent the recognition of the ESBL phenotypically [14,15]. Plasmid profile showed homogeneity among the isolated gram-negative bacilli except for ESBL producers that had extra plasmid of molecular weight 30kb.In Nepal, among 29 multi drug resistant *E.coli* isolates plasmid of size ranging 2-51kb were obtained with the most common plasmid size of 32kb [16]. This study is in close similarity with our study that showed *E. coli* having a plasmid size of 30kb. Study in Iran, showed an average of 5.5kb plasmid size among 76 *E. coli* isolates [17]. This differs from result from this study. Ayten, et al. studied 118 uropathogen *E. coli* strain with some isolates having plasmids ranging from 1 to 24kb in size. The most common plasmid size of 19kb was detected in almost all strains isolated [18] which agrees with this study. Plasmid curing on ESBL producing isolates with sodium dedocylsulphate (SDS) eliminated plasmid resistance markers of ESBL producing organisms, corroborating the findings of Iroha, et al. [19]. The fluoroquinolones have been reported as first line drugs in treatment of urinary tract infections caused by gram-negative bacteria that are resistant to most beta-lactam antibiotics [20]. Gram-negative bacteria exhibited increased resistance to fluoroquinolones (Ciprofloxacin and Nalixidic Acid) as earlier by Akujobi and Ewuru [14]. *Pseudomonas* species showed intermediate sensitivity to ciprofloxacin. The clinicians' only alternative to this challenge is using imipenem, a carbepenem that is administered with an enzyme inhibitor cilastatin which prevents its rapid degradation in the kidneys and it is concentrated in the urine [21]. This antibiotic was seen to be 100% susceptible to all the isolates including the ESBL producers, in agreement with the report of Aiyegoro et al. [22].

## 4. CONCLUSION

The relatedness of the plasmid profiles of the uropathogens implies the presence of common causative agents responsible for urinary tract infection in children. Urinary tract infections are predominantly caused by gram-negative bacilli and infections caused by this group of bacteria are becoming a serious global health threat due to resistance exhibited by these bacilli. The prevalence of community-acquired urinary tract infections among children has been on the increase. The findings from the study revealed high prevalence. This certainly calls for immediate attention of relevant health authorities as well as hospital administrators. Thus, this study recommends proper antibiotic susceptibility testing before drug administration for confirmed cases of urinary tract infections in children in study area.

## CONSENT

All authors declare that ‘written informed consent was obtained from the patient and other approved parties for publication of this research study.

## ETHICAL APPROVAL

The study was done according to the existing ethical guidelines on human subjects. An ethical approval was issued by the Ethical Committee of the hospital.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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