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Antibiogram and Plasmid Profile of Some Multi-Antibiotics Resistant Urinopathogens Obtained from Local Communities of Southeastern Nigeria

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Abstract

Multi-antibiotic resistant strains of bacteria represent a global medical challenge, having reduced or threatened to completely eliminate the list of reserved, last-resort agents usually deployed in serious and/or life-threatening infections. Even newer and very potent antibiotics are not spared from the emergence of resistant strains of organisms. In this study, the antibiotic resistance profile and the plasmid profile of some multi-antibiotic resistant bacteria strains isolated from the urine samples of fifty volunteers (ages 15-30) from a community in south eastern Nigeria were analyzed. Eight multidrug-resistant bacteria were isolated from the 50 urine samples, of which approximately 60% showed resistance to nitrofurantion, amoxicillin, ciprofloxacin, ampicillin, gentamicin, ampiclox and erythromycin. The MIC of the isolated *S. aureus*, *E. coli*, and *Klebsiella* species to ofloxacin, ciprofloxacin, pefloxacin, and co-trimoxazole were greater than 500 µg/ml. Plasmid profile studies revealed the presence of

R-plasmids of size range 11-18 kb. The cultures of resistant isolates irreversibly lost their antibiotic resistance with acridine orange and SDS treatment, which suggests that the resistant genes could be harboured in the plasmids.

Key words: Antibiogram, antibiotic-resistance, plasmid profile, urinopathogens

Introduction

Multi-antibiotic resistant strains of bacteria pose greater challenges to infectious disease specialists since they have either reduced, or threatened to completely eliminate, the available reserved and last-resort agents usually deployed as final options in very serious and life-threatening infections. Even newer and very potent antibiotics are not spared from the emergence of resistant strains of organisms. These underscore the importance of research efforts aimed at understanding the spread of antibiotic resistance as a step to devising a means of curtailing, or possibly reversing, the

spread of resistant genetic material.

Transferable antibiotic resistance is due to extra-chromosomal genetic elements called plasmids. A plasmid is an extra-chromosomal DNA molecule, which is capable of replicating independently (1). In many cases, it is circular and double-stranded. Plasmids are widely spread in bacteria and they provide an important extra-dimension to the adaptation of the organisms to hostile changes in its environment, like the presence of antibiotics. Plasmid size varies from 1 to >200 kilobase pairs (kbp) (2). The plasmids hold genetic information that determines antibiotic-resistance, replication, and transmissibility (1). Bacterial plasmids (with few exceptions) exist within the cell as circular DNA molecules with a very compact conformation due to super-coiling.

Plasmid-encoded antibiotic resistance encompasses most, if not all, classes of antibiotics currently in clinical use, and includes resistance to many that are at the forefront of antibiotic therapy. Notable among these are commonly used cephalosporins, fluoroquinolones, and aminoglycosides. Many resistant plasmids are conjugative, and encode the functions necessary to promote cell-to-cell DNA transfer, particularly their own transfer. Others are mobilizable when helped by a conjugative plasmid co-resident in the cell. Conjugation is a replicative process that leaves both donor and recipient cells with a copy of the plasmid (3).

In this study, the antibiotic resistance profile and the plasmid profile of some multi-antibiotic resistant strains isolated from the urine samples of fifty volunteers (ages 15-30) in a local community in southeastern Nigeria were analyzed. This trial in this region is very important since earlier studies have shown a high rate of antibiotic self-medication, abuse, and under-dosing which could also encourage the spread of resistance (4). We set out to determine not only the resistant pattern, but also the plasmid profile, and the possible role of plasmids in transmission of the resistant gene in these bacteria.

Methodology

Culture Media

Mueller Hinton Agar, Mueller Hinton Broth, McConkey Agar, Mannitol Salt Agar, Nutrient Broth and Nutrient Agar (Oxoid, UK) were all prepared according to the manufacturers' recommendations.

Antibiotic Disks

The antibiotic disk used in this study was Optudisc® (Optun Laboratory Nigeria Ltd). The following antibiotic-containing discs were used: Perfloracin (PEF, 10 µg),

Gentamicin (GN, 10 µg), Ampiclox (APX, 30 µg), Cefuroxime (Z, 12 µg), Amoxicillin (AMX, 30 µg), Ceftriaxone (R, 125 µg), Ciprofloxacin (CPX, 10 µg), Streptomycin (S, 10 µg), Sulphamethoxazole/Trimethoprim (SXT, 30 µg), Erythromycin (E, 10 µg). These disks were selected on the basis of antibiotics commonly available and easily abused in the local communities.

Reagents

Tris-EDTA/SDS (TENS) buffers: (10 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0; 0.1 N NaOH; 0.5% SDS); Tris-EDTA (TE) (10 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0); 95% EtOH precooled to -20 ° C; 70% EtOH at room temperature; 3 M Na Acetate, pH 5.2; Tris-acetate/EDTA (TAE) Running Buffer (0.04 M Tris-acetate, 0.001M EDTA).

Collection and Purification of Bacteria Isolates

The organisms studied were isolated from urine samples collected under aseptic conditions from healthy subjects in some local communities in southeastern Nigeria. A loop-full of the urine samples were collected with a sterile wire loop and streaked on a solid nutrient agar plates under aseptic conditions. The plates so inoculated were incubated at 37 °C for 24 h. Purification of the culture was achieved by successive picking and sub-culturing of the identified colonies from each plate on different selective media (McConkey and Mannitol salt agar). Thereafter, a further identification and confirmation was achieved by specific biochemical tests and by Gram staining. The isolated colonies were identified to be common urinopathogens (*Staphylococcus aureus*, *Escherichia coli*, and *klebsiella species*)

Test for Antibiotic Sensitivity

The strains were tested for antibiotic resistance by the disk diffusion method, performed on Mueller Hinton agar plates. A suspension of each isolate was made by transferring loop-full of the colony in a 3 ml sterile nutrient broth and adjusting to 10³ cfu/ml using McFarland standards. About 0.1 ml of each of the isolates was inoculated to solid nutrient agar plates and multi-antibiotics disc placed gently on the surface of each plate using sterile forceps. All the plates were incubated at 37°C for 24 h and the inhibition zone diameter (IZD) produced by each antibiotic on the various isolates was measured.

The antibiotics sensitivity/resistance profiles (antibiogram) of the isolates were determined from the inhibition zone

diameters (IZD) produced on the inoculated Mueller-Hinton agar plates by the antibiotics multi-disc. The MIC of ofloxacin, ciprofloxacin, perfloxacin, and co-trimoxazole were also determined using the agar dilution technique.

Plasmid Isolation

A colony of test organism, cultured on fresh agar plates, was picked with the aid of a sterile wire loop and inoculated into sterile test tubes containing 8 ml of fresh nutrient broth and then incubated at 37 °C for 72 h. The antibiotic resistance plasmids in the cultures were isolated using the alkaline lyses protocol (5,6).

The broth culture was centrifuged in a microfuge at 6000 rpm for 10 minutes to pellet the cells, and the supernatant gently decanted leaving 50–100 µl together with cell pellet. The cells were completely pelleted by further vortexing at high speed.

Tris-EDTA/SDS (300 µL, TENS buffer) was added and vortexed for 2-5 seconds to resuspend the pellets. Thereafter, 150 µl 3M Sodium acetate, pH 5.2 was added and vortexed 2-5 s to mix completely. The mixture was spun again at 6000 rpm for 10 min to pellet cell debris and chromosomal DNA. The supernatant was transferred into a fresh micro-test tube and mixed with 0.9 ml of 95% EtOH which has been pre-cooled to –20 °C and further spun for 2 minutes to pellet plasmid DNA and RNA. The supernatant was also discarded, and the pellet rinsed twice with 1 ml of 70% EtOH and dried in vacuum. For the subsequent steps, the isolated plasmid DNA was re-suspended in 200 µl of TE buffer; pH 8 and 200 ng/µl RNase were also added to aid dissolution of the pellet and the digestion of RNA.

Plasmid Separation and Profiling

Agarose gel (1%) was prepared by weighing out 1 g of agarose and dissolving in 100 ml of 1x TBE (Tris-Boric Acid-EDTA buffer) with the aid of heat. The hot gel solution was cooled until the glass conical flask could be touched without discomfort and then ethidium bromide (5 µl of 10 mg/ml stock) was added.

The gel solution (100 ml) was poured into electrophoresis tray to which a comb was fixed to create holes on the solidified agarose gel. The combs were removed after gelling and, using the micropipette, the plasmid preparations (to which bromophenol blue/glycerol loading dye, 3 µl was added) were loaded into the holes. A standard DNA ladder was also loaded in a hole between the sample plasmid preparations. The tray with the gel was buffered with TBA buffer and the plasmid preparations from the different bacterial isolates, added to the different holes, was set up to migrate towards the positive charged electrode. The gel was allowed to run

for 3 h at 63 V. The gel containing the separated plasmids was removed and visualized under UV light with the aid of UV goggle. A picture of the DNA was taken showing size and mobility on the agarose gel. The mobility (mm) and the size (kb) were determined relative to the standard DNA ladder loaded in between the sample plasmid preparations.

Plasmid Curing

Acridine Orange Treatment

The resistant isolates were cured with acridine orange to ascertain the involvement of plasmids in antibiotics-resistance determination. A small inoculum of 100 to 300 cfu/ml was added to acridine orange nutrient broth (pH 7.6) in varying concentration of acridine orange up to 0.25 mg/ml and incubated at 37 °C for 24 h (7,8). Cultures containing the highest concentration of acridine orange in which growth was clearly visible were diluted and spread on nutrient agar plates with appropriate antibiotics for susceptibility testing.

Sodium Dodecyl Sulphate (SDS) Treatment

The isolates that showed resistance to some standard antibiotics due to presence of plasmids were selected and subjected to SDS plasmid curing treatment as earlier described (9-11). Briefly, 1g of sodium dodecyl sulphate (SDS) was added to 100 ml of nutrient broth. The solution was autoclaved at 121 °C for 15 minutes, the pH adjusted to 7.6, and thereafter steamed for 1 hr. Fresh 24 h old cultures of the samples were standardized and 0.5 ml of 10³ cfu/ml was pipetted into the 100 ml broth. Control broth containing no SDS was also subjected to similar treatment. Cultures were incubated with aeration at 37 °C for 24 h. The cells including those from the control broth were then tested for antibiotic susceptibility.

Results and Discussion

It is well established that antibiotic therapy can select for antibiotic-resistant strains and R-plasmid-mediated antibiotic resistance can spread in a population subjected to heavy antibiotic therapy (12). Earlier survey studies showed that antibiotic self-medication and under-dosage is common and almost the norm in communities in south eastern Nigeria (4), but the incidence of resistant strains, the efficiency and mode of antibiotic resistance transfer are not well known in these communities. Most cases of multiple-antibiotics resistant strains have been demonstrated to be due to transferable, extrachromosomal circular DNA, plasmids (13,14). Therefore, an understanding of how

antibiotic resistance develops and is spread in these communities is a desirable pre-requisite to the design and development of interventional strategies intended to minimize the threat of bacterial infections.

In our present investigation, we isolated multi-drug resistant uropathogens from urine samples of healthy volunteers in local communities in southeast Nigeria. We then characterized the plasmid profile of the isolates with respect to presence, number, and size. Three different multidrug-resistant bacteria isolates were cultured from eight of fifty urine samples collected, and these included *Escherichia coli*, *Staphylococcus aureus*, and *Klebsiella*

a plasmid (Table 3) with the exception of *Staphylococcus aureus* isolates (S1 and S5) (Figure 1). The plasmid profile studies showed plasmid sizes ranging from 11-18 kb in the isolated resistant strains (Table 2 and 3). In earlier studies, some antibiotic-resistant plasmids were shown to be of similar size ranges (15). Generally, the resistant pattern correlates well with the list of antibiotics, which are available and easily obtained from medicine shops in the area. These include ciprofloxacin, perfloxacin, gentamicin, ampicillin, ampicillin/cloxacillin (Ampiclox®), cefuroxime, sulphamethoxazole/trimethoprim, erythromycin, amoxicillin, ceftriaxone, streptomycin.

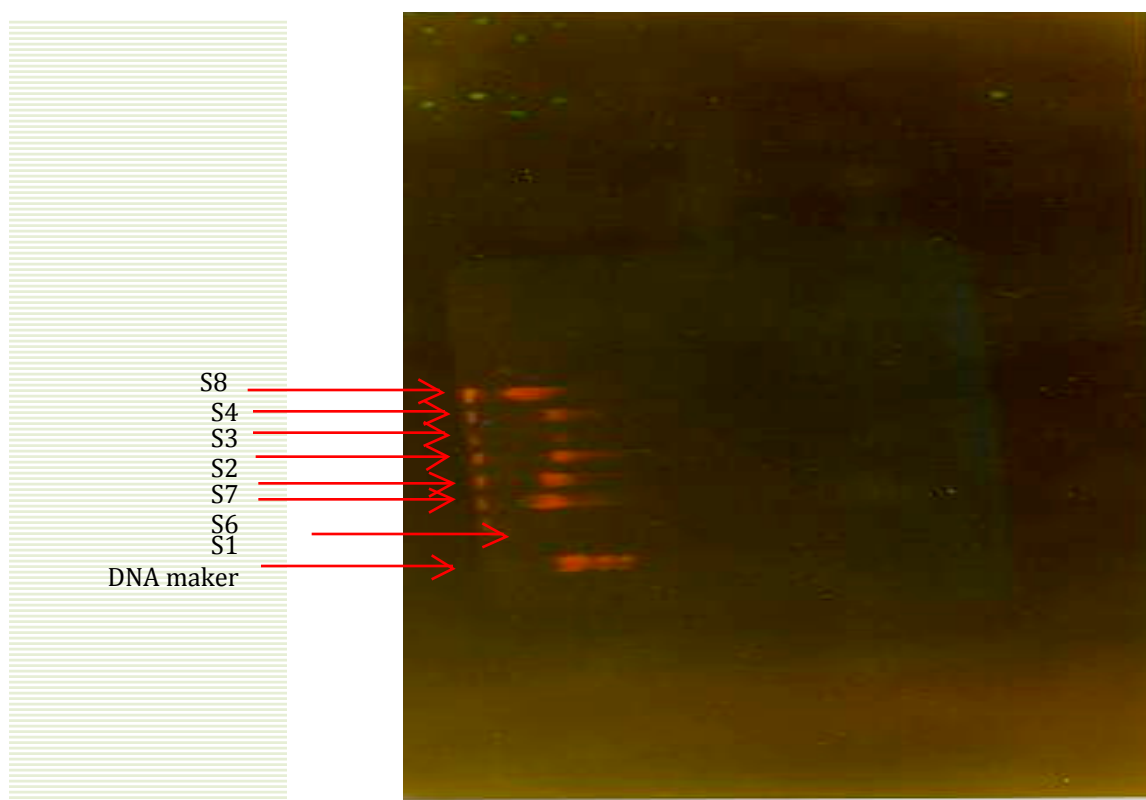


Fig 1: Picture of the plasmid bands of isolates and the DNA ladder

S1 (*S. aureus*) -No plasmids; S2 (*S. aureus*); S3 (*S. aureus*); S4 (*S. aureus*); S5 (*S. aureus*) - No Plasmids, not shown; S6 (*E. coli*); S7 (*E. coli*); S8 (*K. pneumoniae*)

pneumoniae.

These isolates were resistant to ampicillin/cloxacillin, amoxicillin, cefuroxime, nitrofurantoin, erythromycin, norfloxacin and streptomycin (Table 1), but Sample 8 (*K. pneumoniae*) was sensitive to norfloxacin and streptomycin with an IZD of 25mm and 13mm, respectively. All the isolated antibiotic-resistant bacteria were shown to possess

Resistance plasmids could either be conjugative or mobilizable. In general, mobilizable plasmids lack the genes that encode functions that enable cells to couple prior to DNA transfer (which are provided by the conjugative plasmid) but do encode functions needed specifically for transfer of their own DNA. Accordingly, mobilizable

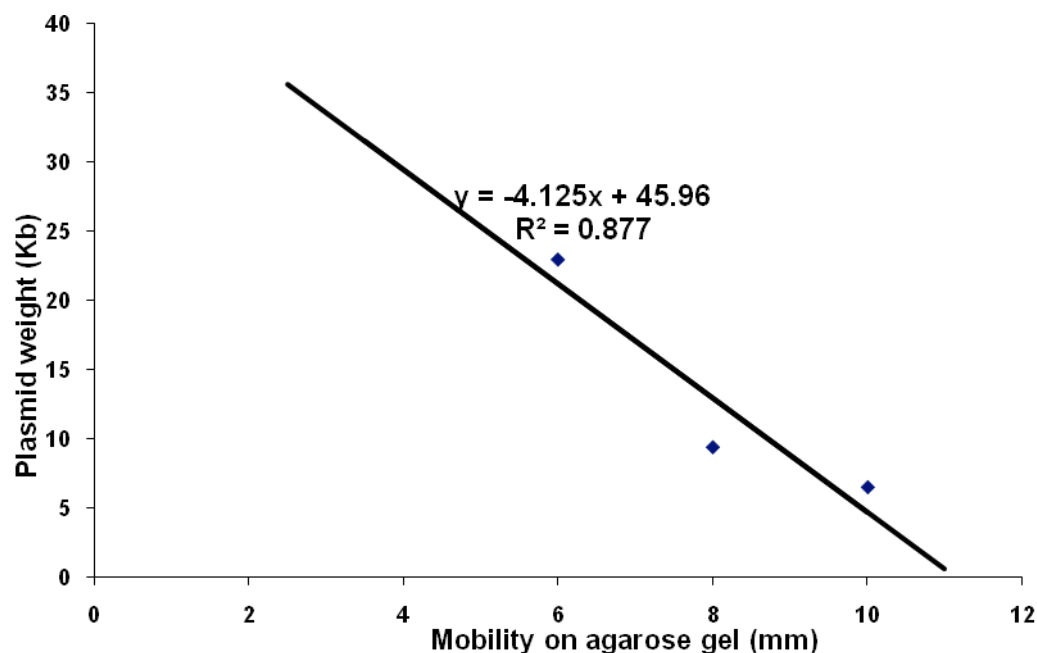


Fig 2: Mobility-weight calibration plot of DNA marker

Table 1: The antibiogram of the isolated samples on a Gram positive multidisc											
Inhibitory Zone Diameter (IZD in mm)											
Sample	Sample identity	PEF (10µg)	GN (10µg)	APX (30µg)	Z (12µg)	AMX (30µg)	R (125µg)	CPX (10µg)	S (10µg)	SXT (30µg)	E (10µg)
S1	S. aureus	12	-	-	-	-	16	17	-	12	-
S2	S. aureus	11	12	-	-	-	13	14	-	6	-
S3	S. aureus	15	-	-	-	-	12	17	-	15	-
S4	S. aureus	15	-	-	-	-	16	13	-	17	-
S5	S. aureus	16	-	-	-	-	18	14	-	16	-
S6	E. coli	12	-	-	-	-	25	12	-	11	-
S7	E. coli	16	-	-	-	-	28	24	-	17	-
S8	K. pneumonia	11	-	-	-	-	2	-	13	-	-
Perfloxacin (PEF), Gentamicin (GN), Ampiclox (APX), Cefuroxime (Z), Amoxicillin (AMX), Ceftriaxone (R), Ciprofloxacin (CPX), Streptomycin (S), Sulphamethoxazole/Trimethoprin (SXT), Erythromycin (E)											

resistant plasmids tend to be relatively small, often less than 10 kb in size, encoding only a handful of genes including the resistance gene(s). On the other hand, conjugative plasmids tend to be somewhat > 30 kb (resistant plasmids of ≥100 kb are not unusual), reflecting the sizable amount of DNA

(20–30 kb) needed to encode the conjugation functions that permit cell-to-cell coupling, particularly between Gram-negative bacteria. Conjugative plasmids in Gram-positive bacteria tend to be smaller than those in Gram-negative bacteria, reflecting a somewhat different mechanism of

Table 2: Mobility and molecular weight of plasmids isolated from the test cultures

Sample	Sample identity	Mobility of plasmid (mm)	Estimated molecular weight of plasmid (kb)
S1	S. aureus	-	-
S2	S. aureus	4	15.5
S3	S. aureus	5	14.0
S4	S. aureus	6	12.5
S5	S. aureus	-	-
S6	E. coli	6.5	11.75
S7	E. coli	6	12.50
S8	K. pneumonia	3	17.1

Table 3: Identification of sample cultures and their plasmid profiles

Sample	Lane	Sample identity	No of plasmid isolated	Plasmid size (kb)
DNA maker	1	Standard		6.5, 9.4, 23.0
S1	2	S. aureus	0	No plasmid
S2	5	S. aureus	1	15.5
S3	6	S. aureus	1	14.0
S4	7	S. aureus	1	12.5
S5		S. aureus	0	No plasmid
S6	3	E. coli	1	11.75
S7	4	E. coli	1	12.5
S8	8	K. pneumonia	1	17.1

Table 4: Results of curing of plasmid DNA with acridine orange and SDS

Samples	Identity of Culture	Exposure to acridine orange	Exposure to SDS
S1	S. aureus	-	-
S2	S. aureus	-	+
S3	S. aureus	-	-
S4	S. aureus	+	-
S5	S. aureus	+	-
S6	E. coli	+	+
S7	E. coli	+	+
S8	K. pneumonia	+	+
- = No growth (Cured), + = Growth (Uncured)			

Table 5: Sensitivity pattern of antibiotic resistant isolates, before and after curing to some antibiotics

		Inhibitory Zone Diameter (IZD in mm)					
		Pefloxacin, 10 µg		Gentamycin, 10 µg		Ampiclox®, 30 µg	
Sample	Organism	Before	After	Before	After	Before	After
S1	<i>S. aureus</i>	12	-	-	-	-	-
S2	<i>S. aureus</i>	11	8	12	-	-	-
S3	<i>S. aureus</i>	15	-	-	-	-	-
S4	<i>S. aureus</i>	15	-	-	-	-	-
S5	<i>S. aureus</i>	16	12	-	10	-	-
S6	<i>E. coli</i>	12	10	-	13	-	12
S7	<i>E. coli</i>	16	13	-	-	-	9
S8	<i>K. pneumonia</i>	11	9	-	12	-	14

cell-to-cell coupling (16,17), which requires less genetic information.

It is acknowledged that limited size accuracy is obtained by this method of plasmid size estimation, which involves comparison with linear DNA standards (18). It is also usually difficult to establish the size limits of plasmids or the real size distribution in any organisms because the method of isolation of plasmid is more effective with the smaller plasmids than with the larger ones, which are more difficult to isolate (19).

In other to further prove the involvement of plasmids R-factor in these multi-antibiotic resistant isolates, we treated the cultures with acridine orange and SDS. The culture samples S1, S2, and S3 were cured by exposure to acridine orange while the exposure to SDS cured samples S1, S3, S4, and S5. The cured samples irreversibly lost their antibiotic resistance and became susceptible to the inhibition by similar concentrations of the different antibiotics to which they were previously resistant. This further suggests that the antimicrobial resistance demonstrated by most of these isolates may be mediated by R-plasmids (Tables 4 and 5).

The result of this study shows that the plasmid-encoded antibiotic resistance encompasses most, if not all, classes of antibiotics commonly employed at the forefront of clinical antibiotic therapy including the cephalosporins, fluoroquinolones, and aminoglycosides. Development of resistance to antibiotics by bacteria constitutes a very serious challenge in medical practice. Without effective procedures to limit bacterial infection, many modern medical procedures would be more risky, if not a complete waste of time and resources, and rates of morbidity and mortality from bacterial infection would be considerably

high (17). The successes and investments in antibiotic drug development would also be lost if appropriate measures are not put in place to curtail the emergence of resistant strains of bacteria. The situation is more worrisome in rural communities in less developed countries where inappropriate use of antibiotics has encouraged the spread of resistance genes in the bacteria. It is important to observe that these drug resistant pathogens were isolated from asymptomatic subjects, which suggests that the organisms may have sacrificed their pathogenic potentials in order to acquire resistance to these antibiotics which are often abused during symptomatic acute infections. The understanding how these genes are acquired and spread is a step at devising strategies and implementing policies that could reverse the trend and ultimately save many lives.

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