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Full Length Research Paper

Plasmid-mediated resistance of some gram-negative bacterial species to brands of cefuroxime and ceftriaxone

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Twenty two different strains of Gram-negative bacteria, namely *Pseudomonas aeruginosa, Proteus mirabilis, Klebsiella pneumoniae, Escherichia coli* and *Haemophilus influenzae* were tested for susceptibility to 10 different brands of ceftriaxone and cefuroxime. Some of the highly resistant strains were processed for antibiotic resistance curing and extraction of plasmid DNA. Of the 10 brands of ceftriaxone and cefuroxime, 2 brands of ceftriaxone were more effective on the Gram-negative bacteria than the remaining ceftriaxone and cefuroxime brands. The highly resistant strains were resistant not only to these 2 brands but also to tetracycline, amoxicillin, cotrimoxazole, nitrofurantoin, gentamicin, nalidixic acid and ofloxacin, used in antibiogram and as also evident in high minimum inhibitory concentrations recorded for the strains against the brands of ceftriaxone and cefuroxime. Some of the resistant strains were found to lose their resistance to curing following treatment with different concentrations of a mutagen, acridine orange. Of the 8 multiple drug resistant (MDR) strains of the bacteria selected for plasmid DNA, 5 strains and a sensitive strain of *H. influenzae* had single plasmid DNA copy ranging in size between 24 - 28 kb. The high level multiple drug resistance of the bacterial strains tested was due not only to variation in the brands of ceftriaxone and cefuroxime but more especially to R-plasmid mediation.

Key words: Plasmid mediated resistance, bacterial spp., cefuroxime and ceftriaxone.

INTRODUCTION

It has been estimated that of the instances of counterfeit pharmaceuticals reported to world health organization (WHO) from 28 countries in the past 15 years, 65% were produced in developing countries (McGreagor, 1997). Yet there are both the international and national drug regulating agencies providing standards aimed at regulating the manufacture, distribution and quality of drug products for ensuring products of proven quality, safety and efficacy (Phillips and williams, 1978; WHO, 2006). These regulations notwith-standing, reports abound in the literature on the existence of substandard pharmaceutical products associated with decrease in the safety, efficacy and reliability of such drugs thereby implicating life threatening infections in patients (WHO, 2006) resultant from therapeutic failure.

With respect to antibiotics, the undesirable therapeutic failure will encourage development of resistant mutants from the hitherto sensitive bacteria. Hence, the relevance of various suggestions on the need for regular monitoring of antibiotics and assessment of commonly used antibiotics in recognition of the role of R-plasmid in acquired resistance to antibiotics (Bauchmona and Chang, 1989; Quinn, 1994; Livermore, 1995; Lucet, 1999).

Cefuroxime and ceftriaxone are second and third generation cephalosporins, respectively. Their inactivation by β-lactamases is the primary mechanism of resistance to them by bacteria. The advent of extended spectrum β-lactamases (ESBL) producers is posing a great threat to the use of many classes of antibiotics, particularly the cephalosporins (Paterson et al., 2001; Adeniyi et al., 2006). ESBL are encoded by transferable conjugative plasmids which also quite often code resistant determinants to other antibiotics such as amino-

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Table 1. Clinical Sources of the bacterial isolates.

Clinical Source	Bacterial isolates
High Vaginal Swab	*E. coli* (1) *P. mirabilis* (1)
Wound swab	*E. coli* (2) *K. pneumoniae*, (2) *P. aeruginosa*, (2)
Wound biopsy	*K. pneumoniae* (1) *P. aeruginosa* (2) *P. mirabilis* (1)
Breast milk	*E. coli* (1)
Pleural aspirate	*K. pneumoniae* (1)
Sputum	*K. pneumoniae* (1) *P. mirabilis* (1)
Catheter tip	*P. aeruginosa* (1)
Cerebrospinal fluid	*H. influenzae*

N. B. Number of strains in parenthesis.

glycosides, tetracyclines and also among related and unrelated Gram-negative bacteria (Butaye et al., 2003; Adeniyi et al., 2006, Soge, 2007) thereby making infections caused by ESBL producing organisms difficult to control.

This study was carried out to investigate the impact of brand variation on the antibacterial activity of cefuroxime and ceftriaxone and verify the involvement of plasmid in the bacterial resistance to both antibiotics.

MATERIALS AND METHODS

Bacterial strains

Clinical isolates of bacteria comprising *Pseudomonas aeruginosa* (5), *Proteus mirabilis* (3), *Klebsiella pneumoniae* (5), *Escherichia coli* (4) and *Haemophilus influenzae* (1) were obtained from the routine section, Medical Microbiology laboratory, university college hospital, (UCH), Ibadan, Nigeria. In addition, 3 control strains were used: *P. aeruginosa* ATCC 27853, *K. pneumoniae* ATCC 13883 and *E. coli* ATCC 25922. All the strains were identified by some conventional characterization tests (Holt, 1984; Cheesbrough, 2000) (Table 1).

Antibiotics

Multodisk paper strips (Abtek) impregnated with these antibiotics, Augmentin^{R} (30 µg), tetracycline (30 µg), amoxicillin (25 µg), cotrimoxazole (25 µg), nitrofurantoin (30 µg), gentamicin (10 µg), nalidixic acid (30 µg), ofloxacin (30 µg), ceftriaxone (30 µg), cefuroxime (30 µg) and cefotaxime (30 µg).

7 brands of ceftriaxone and 3 brands of cefuroxime as injection powder and also pure sample of cefuroxime were used. All the antibiotics were obtained locally.

Antimicrobial susceptibility testing

Antibiogram was determined for each of the 2 bacterial strains using the multodisk antibiotic paper strips, by the disk-diffusion method as described by the National committee for clinical laboratory standards (NCCLS, 1997). The zones of growth inhibition observed after incubation of the plate cultures were measured in mm and interpreted as sensitive or moderately sensitive or resistant where there was no zone according to NCCLS (2000) criteria.

The minimum inhibitory concentration (MIC) of each of the 10 brands of cefuroxime and ceftriaxone and a pure sample of cefuroxime was determined by the tube broth-dilution method (Waterworth, 1978). 2-fold serial dilutions in sterile nutrient broth of each antibiotic were prepared in graduated decreasing concentrations and into each dilution was inoculated 0.1 ml of 10^{-2} diluted broth culture of the organism. A 5 ml nutrient broth similarly inoculated and an uninoculated serially diluted drug in nutrient broth served as controls. All the tubes were incubated at 37°C for 24 h.

Plasmid processing

The alkaline phosphate method described by Brinboim and Dolly (1979) was used to isolate plasmid DNA from 9 strains of the bacteria tested. 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, 400 mM 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, 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 3000 g 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 3000 g 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 mM Tris 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 ml in single-strength Tris-borate/EDTA electrophoresis buffer.

The gel was run at 75 V for approximate 1½ h. To visualize the DNA after electrophorsis, 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 transilluminator and photographed. The DNA bands were matched with those for Lambda (λ) DNA Hind III digest M. wt marker in the range 0.1 - 23.1 kb. The approximate m.wt of each plasmid was consequently obtained by extrapolation on graphical plots of m.wt of marker against the distance traveled by the respective band.

Curing of antibiotic resistance

The modified method of Rotimi, Duerden (1981) as described

Table 2a. Minimum inhibitory and bactericidal concentrations of the brands of ceftriaxone (Ctr) against the bacterial strains.

Strain	Brands of Ctr (µg/ml)						
	Ctr 01	**Ctr 02**	**Ctr 03**	**Ctr 04**	**Ctr 05**	**Ctr 06**	**Ctr 07**
P. aeruginosa UCH 197	>250	125	250	>250	>250	>250	>250 *
	>250	>250	250	>250	>250	>250	>250 **
P. aeruginosa UCH 095	>250	62.5	250	62.5	250	250	250
	>250	>250	>250	>250	>250	>250	>250
E. coli UCH 950	>250	>250	>250	>250	>250	>250	>250
	>250	>250	>250	>250	>250	>250	>250
E. coli UCH 848	6.25	6.25	12.5	50	12.5	12.5	12.5
	>50	50	50	>50	>50	>50	>50
K. pneumoniae UCH 166	>250	>250	>250	>250	>250	>250	>250
	>250	>250	>250	>250	>250	>250	>250
K. pneumoniae UCH 898	15.63	62.5	15.63	15.63	125	62.5	31.25
	31.25	125	31.25	31.25	250	125	62.5
P. mirabilis UCH 179	>50	>50	>50	>50	>50	>50	>50
	>50	>50	>50	>50	>50	>50	>50
H. influenzae UCH 1245	1.56	1.56	6.25	12.5	25	3.13	12.5
	6.25	6.25	12.5	25	50	12.5	25
P. aeruginosa ATCC 27853	>250	>250	>250	>250	>250	>250	>250
	>250	>250	>250	>250	>250	>250	>250
E. coli ATCC 25922	>50	>50	>50	>50	>50	>50	>50
	>50	>50	>50	>50	>50	>50	>50
*K. pneumoniae*ATCC 13883	15.63	62.5	31.25	31.25	31.5	62.5	125
	31.25	125	62.5	62.5	62.5	125	12.5
P. mirabilis UCH 214	6.25	1.56	3.13	12.5	25	6.25	6.25
	6.25	6.25	6.25	>50	25	12.5	12.5

Ctr 01–07: Brands of Ceftriaxone, coded 01-07
*: Minimum inhibitory concentrations (MIC)
* *: Minimum bacterial concentrations (MBC)
UCH: University College Hospital, Ibadan, Nigeria.

by Obaseki -Ebor (1988) was used with some modifications (Adeleke and Odetola, 1997). Overnight cultures of the resistant strains found with plasmid were obtained into 250, 200, 150, 125 µg/ml of acridine orange (mutagen) in nutrient broth. Each mutagen exposed culture was then plated on drug-free nutrient agar and incubated overnight at 35 - 37°C.

Five colonies were randomly selected from each of the plates and tested for loss of antibiotic resistance by exposure to 1 and 5 µg/ml of each of ceftriaxone and cefuroxime in tube broth-dilution. The tests and controls (as stated earlier) were incubated at 37°C for 24 h.

RESULTS

The antibiogram produced varied results with the antibiotics tested showing the following percentage resistance against them by the bacteria Aug (68.18%), Tet (68.18%), Amx (82.36%) Cot (100%), Nit (50%), Gen (31.8%), Nal (31.8%), Ofl (4.5%), Ctr (54.5%), Cef (81.8%) and Cft (54.5%).

Notably, ofloxacin had the strongest activity against *P.*

Table 2b. MIC and MBC of the brands of cefuroxime (Cfr) against the bacterial strains.

Strain	Brands of Cfr (µg/ml)			
	Cfr 01	Cfr 02	Cfr 03	Cfr (PC)
P. aeruginosa UCH 197	>500 >500	>500 >500	>500 >500	>500 >500
P. aeruginosa UCH 095	>500 >500	>500 >500	>500 >500	>500 >500
E. coli UCH 848	100 >100	100 >100	>100 >100	100 >100
E. coli UCH 950	>500 >500	>500 >500	>500 >500	>500 >500
K. pneumoniae UCH 166	>500 >500	>500 >500	>500 >500	>500 >500
K. pneumoniae UCH 898	500 >500	500 >500	500 >500	500 >500
P. mirabilis UCH 179	>100 >100	>100 >100	>100 >100	>100 >100
P. mirabilis UCH 214	>100 >100	12.5 25	50 >100	12.5 25
H.influenzae UCH 1245	12.5 50	6.25 12.5	12.5 50	3.13 12.5
P. aeruginosa ATCC 27853	>500 >500	>500 >500	>500 >500	>500 >500
E. coli ATCC 25922	>100 >100	>100 >100	>100 >100	>100 >100
K. pneumoniae ATCC 13883	62.5 125	62.5 125	125 250	62.5 125

Cfr 01-03: Brands of Cefuroxime, coded 01-03
Cfr (pc): Cefuroxime, pure compound
UCH: University College Hospital, Ibadan, Nigeria.

aeruginosa while cotrimoxazole was the weakest of all the antibiotics. The brands of cefuroxime and ceftriaxone also varied in their minimum inhibitory and minimum bactericidal concentrations (MIC and MBC) against the bacterial specie tested. *H. influenzae* was found exceptionally relatively sensitive, with the brands of ceftriaxone recording MIC's 1.56 - 12.5 µg/ml and cefuroxime, 3.13 - 12.5 µg/ml against the organism, followed by 4 of the 5 strains of *E. coli* and 2 of the 3 strains of *P. mirablis*. All the strains of *P. aeruginosa* and *K. pneumoniae* showed multiple resistance to all the brands of ceftriaxone and cefuroxime with the MIC ranging from 15.63 - > 250 µg/ml (Ceftriaxone), > 500 µg/ml. (Cefuroxime) against *K. pneumoniae*, and 62.5 - > 250 µg/ml (ceftriaxone), >500 µg/ml (cefuroxime) against *P. aeruginosa*. Remarkably, the control strains used showed multiple drug resistance against all the brands of ceftrioxone and cefuroxime as well as pure cefuroxime sample (Tables 2a and b).

Figure 1 shows the plasmid DNA (as bands) isolated

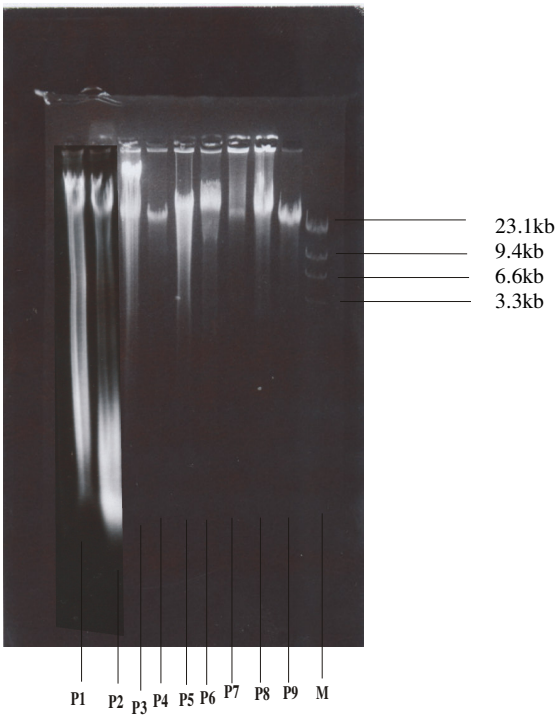

Figure 1. Plasmid profile of isolates. Lane M:MWM Lambda DNA Hind 111-Digest. Lane P1 and P9: *P. aeruginosa*, Lane P2 and P8: *E. coli*, LaneP4 and P5: *P. mirabilis*, Lane P6 and P7: *K. pneumoniae*, Lane P3: *Haemophilus influenzae*

from the highly resistant bacterial strains and also the sensitive *H. influenzae.* 5 of the strains had one plasmid DNA copy of size 24 - 28 Kb as obtained from Figure 2. Most of the strains lost their plasmids due to cure by the mutagen (acridine orange) particularly at 250 and 200 µg/ml. Exposure of the resistant strains to these 2 concentrations made the resistant strains become sensitive to 1 and 5 µg/ml of both ceftriaxone and cefuroxime.

DISCUSSION

The general antibiotic susceptibility profile produced by the bacterial strains whereby all but one (ofloxacin) of the 11 antibiotics employed in the antibiogram recorded 30 - 100% resistance against the bacterial strains corrobates the generally antibiotic resistant nature of Gram-negarive bacteria and in particular *P. aeruginosa* (Brown, 1975; Odelola et al., 1985).

The contrasting antibacterial activity of ceftriaxone (54.5% resistance) and cefuroxime (81.8% resistance) in the antibiogram, which also manifested in the brands of these 2 antibiotics agrees with the previous reports of a better activity for ceftriaxone than Cefuroxime (Foye et al., 1995; Williams, 1995; Chamber et al., 1998). Nonetheless, the equally contrasting multiple drug resistance against the brands of ceftriaxone and cefuroxime corroborates the alarm raised by Paul et al. (1997) on the alarming rate of resistance to cephalosporins by the hospital strains of Gram-negative bacteria and more so with the advent of the extended spectrum β- lactamases (ESBL) producers (Butaye et al., 2003; Soge, 2007). This confirms a report that in Nigeria, β-lactams are the most frequently prescribed antibiotics in Gram-negative bacilli infections and selective pressure exerted by the use of these β-lactam drugs have resulted in the strains producing the extended spectrum beta-lactamase enzyme (Aibinu et al., 2003).

The production of ESBL can be chromosomal or plasmid in origin (Iroha et al., 2008). The isolation of plasmid DNA in some of the highly resistant strains lends credence to the relevance of plasmid genes encoding ESBL to the acquisition of antibiotic resistance in gram-negative bacteria (Shukla, 2004; Soge, 2007). These transferable plasmids also code for resistant determinants to other antimicrobial agents, hence, multidrug resistance is expected to be more common in ESBL producing organisms. ESBLs that are chromosomal in origin cannot easily be transferred from organism to organism (Aibinu et al., 2003).

The reversion obtained in the resistance of some of the multiple drug resistant strains used following exposure to acridine orange agrees with the reports of linkage between curing of antibiotic resistance and plasmid borne

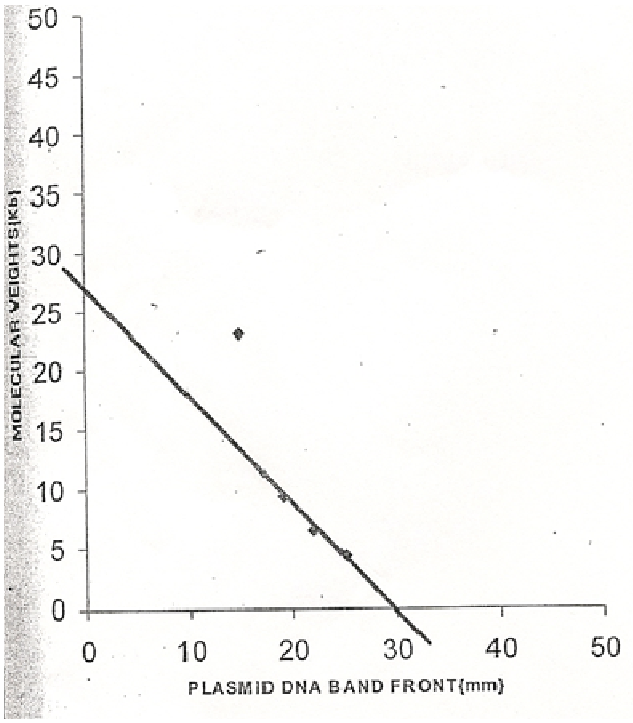

Figure 2. Determination of molecular weights of plasmids DNA isolates.

resistance (Naomi, 1978; Darini, 1996; Adeleke and Odelola, 1997). The sensitivity of *H. influenzae* to the brands of ceftriaxone and cefuroxime would not support a suggestion of brand variation as being responsible for the overall resistance observed for other bacterial spp.

However, the sensitivity of *H. influenzae* despite the isolation of plasmid DNA from this organism supports the recognition of other functions for plasmid DNA beyond antibiotic resistance (Smith et al., 1967; Adeleke, 1998). Marketing of different brands of antibiotics is a reality but regular monitoring of the brands through susceptibility testing is an important measure to check on their authenticity. Previous studies conducted in Nigeria have demonstrated that resistant genes reservoirs are increasing in healthy persons (Okeke et al., 2000). Hence, a suggestion that surveillance of emerging resistant bacteria should not only be carried out in clinical settings but in healthy individuals and environments that act as reservoirs of resistant genes.

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