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Research article

Detection, Inhibition and Molecular Analysis of Multidrug Resistant Aerobic Gram-negative Clinical Isolates from a Tertiary Hospital in Nigeria

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ABSTRACT

The challenge of combating the ever emerging multi-drug resistant (MDR) clinical isolates in the face of a slow rate of discovery of new classes of antibiotics is a problem in antibiotic chemotherapy. This study was aimed at (i) linking phenotypic antibiotic drug-resistance characteristics detected in randomly-sampled clinical isolates with detectable genetic markers. (ii) screening a suspected efflux pump inhibitor (EPI) [1-(3-(trifluoromethyl)benzyl]-piperazine (TFMBP)], which could be helpful in combating this challenge. Fifty-one isolates; 28 *Klebsiella pneumonia,* 3 *E. Coli,* 1 *Enterobacter cloacae,* 1 *E. aerogenes,* 5 *Proteus mirabilis,* 4 *Providencia rettgeri,* 1 *P. stuartii,* 1 *Serratia liquefaciens,* 6 *S. odorifera,* and 1 *Acinetobacter baumannii* obtained from infections of urinary tract, upper respiratory tract, gastrointestinal tract, ear swab, eye swab, and blood culture were screened for (i) antibiotic-susceptibility over a range of 11 classes of antibiotics, (ii) β-lactamase production, (iii) ESBL production and (iv) Efflux pump activity (EPA) in the presence and absence of 1-[3-(trifluoromethyl) benzyl]-piperazine (TFMBP). Molecular analysis was done using DNA extraction by boiling and the randomly-amplified polymorphic DNA (RAPD) polymerase chain reaction (PCR) procedure with 2% agarose gel electrophoresis stained with ethidium bromide at 10 µg/ml and visualized by UV trans-illumination. AmpC *β-lactamase* (4%) and K1 *β-lactamase* (5.8%) were detected with no carbapenemase producers. *AcrA and AcrB marker* genes were detected in 12% of the isolates while *bla*CTX-M (8%) and *bla*TEM (4%) were also detected. Antibiotic resistance due to EPA can be combated with a suitable EPI as demonstrated by TFMBP when combined with specific antibiotics.

Keywords: TFMBP, Efflux Pump Activity, ESBL, MDR, Carbapenemase

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INTRODUCTION

With the global emergence of various forms of ESBLs and MDR bacteria at a rate much faster than the rate at which new drug molecules are being developed, a molecular analysis of the drug resistance characteristics of randomly-sampled bacterial isolates, from patients attending Lagos University Teaching Hospital (LUTH) is imperative. The methods of PCR and DNA sequencing offer the promise of increased sensitivity, specificity and speed in the detection of specific known resistance genes (Perreten *et al.,* 2005.). The level and extent of involvement of EPA in the multidrug resistance patterns observed among the isolates was demonstrated by use of trifluoro-methyl benzyl piperazine TFMBP as the efflux pump inhibitor (EPI). Molecular analysis of ESBL and efflux pump types present in the isolates was carried out by determination of genetic markers. This study gives an insight into possible solution to the challenge of emergent EPA among MDR strains from LUTH through the incorporation of suitable EPIs into antibiotic formulations for treatment of infected patients.

MATERIALS AND METHODS

The 51 isolates used in this study were collected from the Medical microbiology laboratories of LUTH. These include

28 *Klebsiella pneumonia,* three *Escherichia Coli,* one *Enterobacter cloacae,* one *E. aerogenes,* five *Proteus mirabilis,* four *Providencia rettgeri,* one *P. stuartii,* one *Serratia liquefaciens,* six *S. odorifera,* and one *Acinetobacter baumannii* isolates.

The isolates were identified with the analytical profile index (API) and the analytical profile index web (APIWeb). *E. coli* (ATCC 25922) and *K. pneumoniae*- β-lactamase-producing (ATCC 700603) were included at every stage for quality control. Antibiotic susceptibility tests were carried out on each of the isolates as described by Bauer (Bauer *et al.*, 1966).

The antibiotic discs used included ampicillin-10 µg (Amp-10), penicillinG-10 µg (P-10), ceftriaxone-30 µg (CRO-30), ciprofloxacin-5 µg (CIP-5), tetracycline-30 µg (Te-30), cotrimoxazole-1.25 µg+23.75 µg(SXT-1.25/23.75), neomycin-30 µg (N-30), erythromycin-15 µg (E – 15), polymyxin-B-300 i.u. (PB-300), lincomycin-2 µg (L-2), chloramphenicol-30 µg (C-30) and bacitracin-10 µg (B-10). Zones of inhibition obtained were compared and interpreted with the zone diameter interpretative standards (CLSI document M100-S20-U, 2010). All strains found to be either resistant or intermediate to CRO-30 were regarded as potential ESBL producers, while all strains resistant to more than three classes of antibiotics were classified as MDR. These were picked for further study.

β-lactamase Assay: Fifty-one strains found showing some level of resistance to the β-lactam antibiotics were further tested for the production of β-lactamase enzyme by a nitrocefin (NF) assay (Livermore and Brown, 2001). The tests were performed as follows. In the blank assay, 0.25 ml sterile MHB and 0.25 ml of NF assay solution was added to the 0.5 ml of 0.01 M Na-HEPES, while in the reaction assay the 0.25 ml sterile MHB was replaced with 0.25 ml of broth culture. A colour change from yellow to red in the reaction solution within 10 min compared with the blank is indicative of the presence of β-lactamase (Livermore and Brown, 2001). Laboratory standard strains of the McNeil Science and Technology Centre (Usciences, Philadelphia), *E. coli* A (non β-lactamase producing) and *E. coli* B (β-lactamase producing), were included in the test as the control strains. Strains showing the colour change after 10 min were considered as inducible β-lactamase producers as suggested by Livermore and Brown (Livermore and Brown, 2001).

A Modified Double Disc Synergy Test Method: A ten-disk procedure was carried out on the suspected ESBL producers in sterile normal saline (equiv. to 0.5 McFarland standard) from overnight MHB cultures, as recommended by the Centre for Disease Control (CDC) and CLSI (CLSI) (CLSI document M100-S20-U, 2010). The disks used were CRO-30, cefotaxime-30 µg/clavulanate-10 µg (CTX-30/10), cefotaxime-30 µg (CTX-30), ceftazidime-30 µg/clavulanate-10 µg (CAZ/CLA-30/10), ceftazidime-30 µg (CAZ-30), cefepime-30 µg (FEP-30), cefoxitin-30 µg (FOX-30), aztreonam-30 µg (ATM-30), eterpenem-10 µg (ERT-10) and imipenem-10 µg (IMP-10).

The zones of inhibition obtained after incubation were interpreted as recommended (CLSI document M100-S20-U, 2010) for possible detection of ESBLs, AmpC β-lactamases, K1 β-lactamases and carbapenemases.

An expanded zone of inhibition with a confluent zone between disks such as CAZ/CLA-30/10 and CAZ-30 is as a result of the synergy between the Clavulanic acid of CAZ/CLA-30/10 and CAZ-30. This is referred to as the 'çlavulanic effect' or the 'Key hole' configuration which is exhibited by ESBL-producing bacteria (CLSI document M100-S20-U, 2010).

Efflux Pump Activity (Epa) Test: The MDR isolates were selected for the EPA Test. MICs of the test drugs were determined in the presence and in the absence of an EPI, TFMBP. A reduction in MIC in the presence of the EPI indicated resistance due to EPA (Bohnert and Kern, 2005) . Nine of the isolates tested from the MDDST were picked for this test. The four drugs used in this test were amoxicillin, cefuroxime, ciprofloxacin and tetracycline. These represent four classes of antibiotics to which the MDR organisms were commonly resistant. The distribution of the organisms and the drugs to which they were previously resistant were amoxicillin – 9 strains, cefuroxime - 7 strains, ciprofloxacin - 6 strains, and tetracycline - 9 strains, a total of 31 tests performed.

Table 1:
Preparation of microtiter wells for mic determination in the presence of 200µg/ml of TFMBP

	1	2	3	4	5	6	7	8	9	10	11	12
MHB µl	111	111	111	111	111	111	111	111	111	111	111	111
Drug µl	50	50	50	50	50	50	50	50	50	50	0	0
TFMBP stock 1179 µg/ml	34	34	34	34	34	34	34	34	34	34	0	0
Make up water/buffer µl	0	0	0	0	0	0	0	0	0	0	84	89
Broth culture µl	5	5	5	5	5	5	5	5	5	5	5	0
Total Volume µl	200	200	200	200	200	200	200	200	200	200	200	200
Final drug concentrations µg/ml	125	62.5	31.25	15.625	7.813	3.9065	1.95325	0.97663	0.49	0.24	0	0
Drug Stock solution µg/ml	500	250	125	62.5	31.25	15.625	7.813	3.9065	1.95325	0.97663	**Growth control**	**Sterility control**

Table 2:
Nucleotide Sequence of Primers Used

SN	PRIMER	NUCLEOTIDE SEQUENCE	PHENOTYPE
1.	moxM-F	GCT GCT CAA GGA GCA CAG GAT	AmpC β-lactamase
2.	moxM-R	CAC ATT GAC ATA GGT GTG GTG C	AmpC β-lactamase
3.	citM-F	TGG CCA GAA CTG ACA GGC AAA	AmpC β-lactamase
4.	citM-R	TTT CTC CTG AAC GTG GCT GGC	AmpC β-lactamase
5.	dhaM-F	AAC TTT CAC AGG TGT GCT GGG T	AmpC β-lactamase
6.	dhaM-R	CCG TAC GCA TAC TGG CTT TGC	AmpC β-lactamase
7.	blaCTX-M1	AAA AAT CAC TGC GCC AGT TC	Extended spectrum β-lactamase
8.	blaCTX-M2	AGC TTA TTC ATC GCC ACG TT	Extended spectrum β-lactamase
9.	blaTEM-F	TCC GCT CAT GAG ACA ATA ACC	Extended spectrum β-lactamase
10.	blaTEM-R	TTG GTC TGA CAG TTA CCA ATG C	Extended spectrum β-lactamase
11.	blaSHV-F	TGG TTA TGC GTT ATA TTC GCC	Extended spectrum β-lactamase
12.	blaSHV-R	GGT TAG CGT TGC CAG TGC T	Extended spectrum β-lactamase
13.	blaSHV-F	TGG TTA TGC GTT ATA TTC GCC	Extended spectrum β-lactamase
14.	blaSHV-R	GGT TAG CGT TGC CAG TGC T	Extended spectrum β-lactamase
15.	gyrA-F	AAT CTG CCC GTG TCG TTG GT	Quinolone resistance
16.	gyrA-R	GCC ATA CCT ACG GCG ATA CC	Quinolone resistance
17.	tolC-F	AAG CCG AAA AAC GCA ACC T	Efflux pump resistance
18.	tolC-R	CAG AGT CGG TAA GTG ACC ATC	Efflux pump resistance
19.	acrA-F	CTC TCA GGC AGC TTA GCC CTA A	Efflux pump resistance
20.	acrA-R	TGC AGA GGT TCA GTT TTG ACT GTT	Efflux pump resistance
21.	acrB-F	GGT CGA TTC CGT TCT CCG TTA	Efflux pump resistance
22.	acrB-R	CTA CCT GGA AGT AAA CGT CAT TGG T	Efflux pump resistance

Broth Macro-Dilution: The broth macro-dilution method was used to determine the initial range of the MICs of these drugs against the isolates by the tube method.

Four mls of sterile Mueller Hinton broth (MHB) was placed in each of thirteen sterile test tubes. The stock solution of the tetracycline standard (4 ml of 2mg/ml), was placed in the first tube of MHB to effect a double dilution of the tetracycline to a concentration of 1 mg/ml (1000 µg/ml). From this point a double dilution was carried out serially up to the 11th tube to arrive at 0.9765 µg/ml of tetracycline.

An 18 hr fresh MHB culture of each strain was diluted to give a 1:103 dilution in normal saline (equivalent to 0.5 McFarland standard), and 0.1 ml of this was introduced into each tube from the second tube to the 12th tube. The twelfth tube, which contained no antibiotic was the growth control, while the 13th tube with only 4 ml of sterile MHB served as the sterility control. After 18 hrs.of incubation at 35oC, the MICs were determined by measuring optical density (OD) at 550 nm on a Spectronic Thermo 20 Genesys spectrophotometer against un-inoculated MHB as the reference blank. The tube with the lowest concentration that completely inhibited growth was taken as the MIC.

Broth Micro-Dilution; The MDR strains were subjected to another round of MIC tests with the micro-dilution assay method. The 96-well micro-titre plates were used to perform the MIC tests at different concentrations of the antibiotics against the bacterial strains based on the initial results of the macro-dilution assay.

Before the MIC tests in micro-titre plates, serial double-dilutions of each antibiotic in either sterile phosphate buffer, pH 6.0, for amoxicillin and cefuroxime or sterile water for tetracycline and ciprofloxacin were carried out in a twelve-channel basin to get the desired working concentrations. The serial dilution was performed in a twelve channel basin as discussed below.

The diluent (either sterile phosphate buffer, pH 6.0 for amoxicillin and cefuroxime or sterile water for tetracycline and ciprofloxacin), 1.5ml, was placed in channels 2 to 12. With Tetracycline as an example, 3 mls of the stock drug solution (2000 µg/ml) was placed in channel no. 1. From this, 1.5 ml was moved into channel 2, mixed and the same volume was moved from Channel 2 to 3 up to channel 12 to give a final concentration of 0.97663 µg/ml. This procedure was done for all the other antibiotics used in this study. One micro-titre plate was used to test two, different drugs at a time, at ten different concentration levels. The eleventh column of wells (A11-H11) was used as a growth control of the test strain, while the twelfth column of wells (A12-H12) was for sterility control of the process (Table 1).

Preparation of Micro-dilution in Micro-titre Plates: The EPA test for each drug was done in duplicate on the micro-titre plate. For example, rows A and B were for amoxicillin with TFMBP, C and D for amoxicillin without TFMBP, E and F for cefuroxime with TFMBP, and G and H for cefuroxime without TFMBP. This was done for all of the other drugs used. With the aid of the 12-channel multipipettor fitted with appropriate sterile tips, test materials for each of the 96 wells in a plate including the growth controls and the sterility controls were drawn into the corresponding row of wells (Table 1).

For determination of MICs in the absence of the EPI, 34 µl of make-up volume of sterile phosphate buffer or sterile water (for water soluble antibiotics) was then added to each of the 96 wells including growth and sterile controls, while 34 µl of the EPI solution was added to all the wells excluding the growth and sterile control wells in tests in the presence of TFMBP.

More of the buffer or water was then added to the growth control wells (34+50 µl) and sterile control wells (34+55 µl) to compensate for the drug and the bacterial culture not added respectively. Finally, 5 µl of the 1:103 dilution in normal saline solution of the overnight MHB culture of the test strain was now added with the 12-channel multipipettor fitted with 11 sterile tips for all the wells excluding those for sterility

control (Table 2). EPA tests carried out were 37. The plates were then covered and incubated at 35oC for 18 hr. The optical density (OD) of each well in the plates, were read at 650 nm in a micro-plate reader, Spectramax 384-plus.

Molecular analysis: The molecular analysis of the MDR organisms by randomly-amplified polymorphic DNA (RAPD)-PCR and agarose gel electrophoresis was done on DNA-extracted by boiling as described in literature (Pagani et al., 2003; Villegas et al., 2004.). DNA quantification and check for purity was also done as described in literature (Pagani et al., 2003; Villegas et al., 2004.). For each chromosomal DNA sample, a PCR mix of 25 µl containing magnesium chloride (6%; 1.5 µl), forward and reverse primers (0.8%; 0.2 µl each) was used. Nucleotide sequence of primers used is on Table 2.

The PCR was conducted by an initial denaturation at 95ºC for 5 min. followed by 30 cycles of denaturation at 95ºC for 30 sec. The annealing step was done for 60 sec. at various temperatures to suit each primer pair followed by elongation at 72ºC for 60 sec. The final elongation step was done at 72ºC for 10 min.

RESULTS

Antimicrobial susceptibility test result : The clinical isolates tested were mostly resistant to ampicillin but mostly susceptible to neomycin and ciprofloxacin (Table 3).

MDR isolates found, β-lactamase test and Modified Double Disc Synergy Test (MDDST) results: From the isolates tested, nine were found to be MDR strains, 20 (39.2%) isolates were found to be inducible β-lactamase producers, while seven (13.8%) turned out to be intrinsic β-lactamase producers. (Fig. 1).

The MDDST revealed the presence of ESBL producers, AmpC β-lactamase and K1 β-lactamase producers (Fig. 1).

Table 3:
Antimicrobial Susceptibility Test Results of the Isolates Tested

Antimicrobial agent used		Sensitive		Intermediate		Resistant	
	No of isolates tested	Number	%	Number	%	Number	%
Ampicillin 30 µg	51	7	13.73	0	0	44	86.27
Ceftriaxone 30 µg	51	42	82.35	2	3.92	7	13.73
Ciprofloxacin 5 µg	51	45	88.24	1	1.96	5	9.80
Tetracycline 30 µg	51	15	29.41	8	15.69	28	54.90
SXT-1.25/23.75	51	26	50.98	0	0	25	49.02
Neomycin 30 µg	49	47	95.92	0	0	2	4.08
PolymyxinB 300iu	49	40	81.63	0	0	9	18.37
Chloramphenicol 30 µg	49	31	63.27	3	6.12	15	30.61

KEY: **SXT-1.25/23.75 -** cotrimoxazole-1.25 µg+23.75 µg

Table 4:
Summary of EPA Findings on MDR Isolates

Organism code	Isolate	Source	Efflux Pump Activity (Fold Reduction in MIC)				Number of antibiotics
			TTC	CPF	AMC	CFX	
OG 1(4)	*K. pneumonae*	Semen	4	NS	NS	NS	1
N 19	*K. pneumonae*	Skin	NS	NS	NS	NS	0
N 24	*K. pneumonae*	Skin	NS	NS	NS	NS	0
Med. 5(1)	*K. pneumonae*	HVS	NS	NS	NS	NS	0
Med. 1(2)	*K. pneumonae*	HVS	NS	32	NS	NS	1
N 3	*K. pneumonae*	Skin	4	16	NS	2	3
OG 1(3)	*K. pneumonae*	Semen	4	2	NS	NS	2
N 14	*Ent. aerogenes*	Blood	4	16	NS	NS	2
N 21(2)	*E. coli*	Semen	8	16	NS	NS	2
N			5	5	0	1	

KEY: NS - Not significant **HVS –** High vaginal swab
TTC- Tetracycline; **CPF-** Ciprofloxacine; **AMC-** Amoxycillin; **CFX** – Cefuroxime

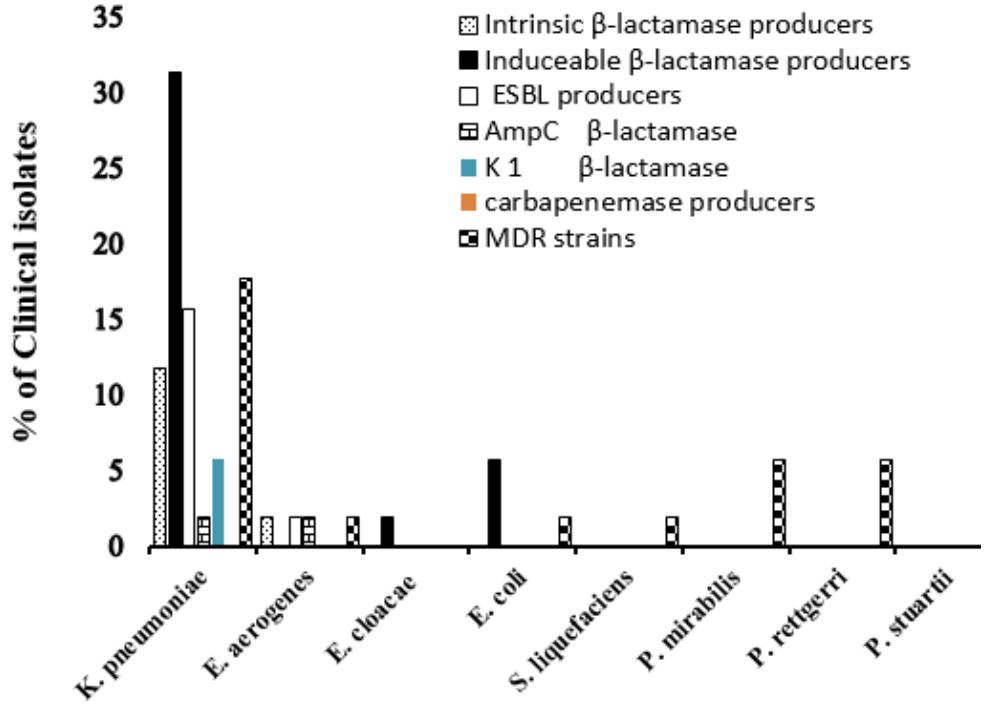

Figure 1:
Types of resistance traits exhibited by isolates found.

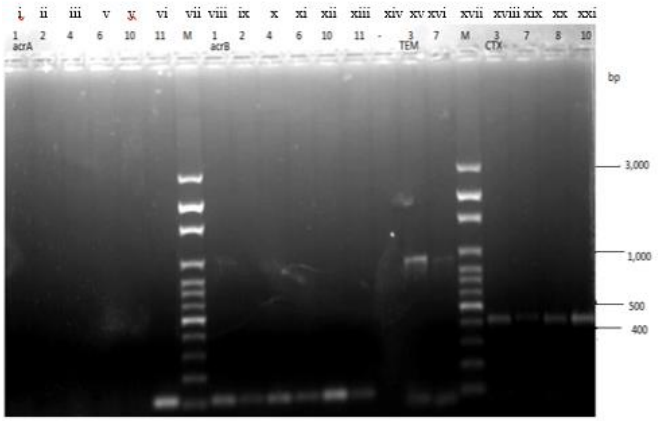

Figure 2:
Agarose gel electrophoresis of AcrA, AcrB, TEM and CTX amplicons. AcrA amplicons on lanes i to vi (with samples 1, 2, 4, 6, 10 & 11 respectively). Only sample 11 gave the band at 107 bp for acrA. AcrB amplicons on lanes viii to xiii (with samples 1, 2, 4, 6, 10 & 11 respectively). Samples 1, 2, 4, 6 and 10 gave the bands at 107 bp for acrB. TEM amplicons on lanes xv & xvi (with samples 3 & 7) and CTX on lanes xviii to xxi (with samples 3, 7, 8 & 10) gave the appropriate bands for TEM at 931 bp for samples 3 and 7 while samples 3, 7, 8 and 10 gave the bands for CTX at 415 bp. M on lanes vii and xvii is molecular size marker (100 bp DNA ladder).

Efflux Pump Activity (EPA) of MDR Isolates: Out of the nine MDR isolates tested, six (31.6%) isolates gave evidence of EPA. One *K. pneumoniae* strain showed efflux pump activity to up to three drugs tested while one other gave as much as a 32-fold reduction in MIC for ciprofloxacin in the presence of the EPI (Table 4).

Molecular analysis on MDR Isolates: *AcrA* and *AcrB* genes were detected in six (12%) of the 51 isolates tested. This confirms the EPA detected in the four *K. pneumoniae* strains, one *Enterobacter aerogenes* and one *E. coli* (Figs. 2, 3). *bla*CTX-M was detected as the gene conferring ESBL characteristics in four isolates (8%) including three *K. pneumoniae* strains and one *E. aerogenes*, (Figs. 2,3). *bla*TEM was detected as the gene conferring ESBL characteristics in two (4%) *K. pneumoniae* isolates (Figs. 2,3).

The agarose gel electrophoresis of acrA and acrB (for EPA) for some of the other isolates, blaSHV, blaOxa (for ESBLs), Mox-M, Cit-M and Dham (for AmpC) and gyrA (for Quinolone), dham, mec, gyr Primers, aacA-aphD and TolC Primers did not produce any amplicons from the isolates tested.

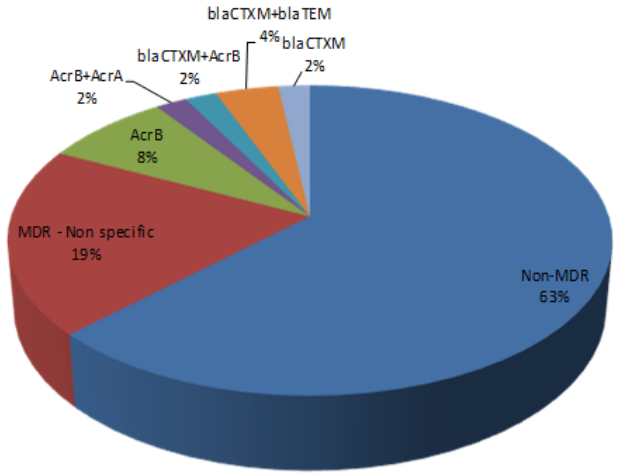

Figure 3:
Distribution of types of resistance genetic markers on MDR isolates tested

DISCUSSION

All of the CRO-30-resistant strains studied were also found to be MDR, a known characteristic of ESBL producers. About half of these were also either resistant or intermediate to ciprofloxacin, thus corroborating the statement on increasing correlation between ESBL-producing and fluoroquinolone-resistant strains (Dalhoff, 2012).

Some isolates were found to produce β-lactamase enzyme intrinsically. However, there were others that produced the NF colour change much later than 10 minutes. These were considered as either inducible β-lactamase or secondary β-lactamase activity of penicillin binding proteins forming unstable acyl complexes (Livermore and Brown, 2001).
Those isolates that did not give any NF colour reaction even beyond 10 minutes, recorded 100% sensitivity to ceftriaxone. Among them were *K. pneumoniae* strains sensitive to ampicillin, thus showing no β-lactamase production. The seven isolates that gave immediate NF colour change were among the eight strains initially suspected to be ESBL producers from their resistance to CRO-30. The prevalence level obtained for the ESBLs among these isolates, is higher than that found in Hong Kong, Korea, Taiwan, Japan, Australia and Netherlands but less than that found in places like the USA, France, Singapore, Asia Pacific and Latin America (Ghafourian *et al.,* 2014). These results are within the range reported in Lagos (Aibinu *et al.,* 2003).

One *K. pneumoniae* isolate was found to be sensitive to ceftazidime but resistant to aztreonam and was thus diagnosed to produce the chromosomally-borne K1-β-lactamase (CLSI document M100-S20-U, 2010)**.** However cephalosporinase overproduction may be the reason for the detection of ESBL in this strain (Gottlieb and Wolfson, 2000).

Typical of plasmid-borne AmpC β-lactamase producer, a *Klebsiella* isolate was shown to be resistant to cefoxitin and sensitive to cefepime. It did not give the clavulanic effect typical of ESBL-producers in the MDDST, and was sensitive to ceftazidime and the monobactam aztreonam. This isolate proved to be an inducible ESBL-producer. Induced and stable over-production of AmpC cephalosporinase can coexist with acquired ESBL production as was found in one *E. aerogenes* isolate that was resistant to cefoxitin, intermediate to aztreonam and cefepime but gave the clavulanic effect typical of ESBLs with cefotaxime and ceftazidime (Miro *et al.,* 2013). The level of occurrence of AmpC in this study is similar to levels reported for *E. coli* and *Klebsiella* respectively by other researchers (Ding *et al.,* 2008).

The EPI used in this study, TFMBP, with an intrinsic MIC >400 µg/ml, and used at 200 µg/ml, is unlikely to have any appreciable antibacterial activity of its own. This is in line with other reports (Stermitz *et al.,* 2002) that compounds exhibiting MICs of more than 200 µg/ml are generally considered as weak antibacterial agents and potential EPA inhibitors. The MICs given by these organisms in the absence of TFMBP are obviously much higher than clinically-useful *in vivo* concentrations. With active efflux pumps, even normal clinical doses of an antibiotic will be present as a sub-clinical dose *in vivo* which will encourage emergence of resistant strains (Xian-Zhi and Hiroshi; 2009).

The TFMBP used in this work was found to appreciably lower MICs of up to two or three drugs in some of the MDR isolates tested. This agrees with reports (Sun *et al.,* 2014) that a single pump is able to confer resistance to multiple compounds. Results showing efflux pump inhibition on organisms from clinical sources such as semen, high vaginal swab (HVS), catheter, skin, blood, eye and ear swabs among others, further underscores the suggestion that the use of such inhibitors, in association with substrate antibiotics, may be useful in increasing both the activity and the range of species for which the drugs may be effective (Webber and Piddock 2003).

The threat of resistance mechanisms due to EPA in these MDR strains further underscores the need for more research into EPIs. This is in consonance with the assertion (Fiamegos *et al.,* 2011) that one plausible antimicrobial alternative to MDR could be the combination of conventional antibiotics with efflux pump inhibitors. Appreciable lowering of MICs obtained from this study reveals that design of newer drugs or modification of existing drug molecules with the inclusion of EPIs may lead to the development of more potent compounds for tackling resistant organisms.

The use of EPIs could facilitate the re-introduction of therapeutically-ineffective antibiotics back into clinical use and even suppress the emergence of MDR strains (Kourtesi *et al.,* 2013). The most prevalent ESBL gene in this study was found to be *bla*CTX-M. From studies on enterobacteriaceae, *bla*CTX-M have been found as highly prevalent including very high prevalence rates in *E. coli* and *K. Pneumoniae* from hospital- acquired infections in various parts of Europe and Asia. A preference for the hydrolysis of cefotaxime over ceftazidime was found in this study; differences in zone diameters of about 3 mm to 7 mm were noticed for *Klebsiella* samples and one *E. aerogenes* that showed *bla*CTX-M genes as detected from other works (Choi *et al.,* 2015).

Half of the strains bearing the *bla*CTX-M gene tested also carried the *bla*TEM gene. The *bla*TEM-24 was reported (Mammeri *et al.,* 2001) as an epidemic clone established in the hospital ecology of Amiens Teaching Hospital in France in 1996. In this same period another study (De Gheldre *et al.,* 2001) in Belgium reported that half of their *E. aerogenes* were carrying a similar *bla*TEM-24, thus supporting the theory of international dissemination of resistance genes.
Cefepime, which was thought to have excellent *in vitro* activity against *Enterobacter* spp. (Magiorakos *et al.,* 2012), gave an intermediate result for the *E. aerogenes* strain and four of the *K. pneumonae* strains. This adds to worldwide concerns about strains of Enterobacteriaceae, especially with cases of *K. pneumoniae* and *E. coli* harbouring the variety of ESBL-producing *bla*TEM and *bla*SHV; the expression of these genes give high MICs for cefepime a fourth generation cephalosporin. The detection of *acrA* and *acrB* efflux pump genes in some isolates confirmed EPA as the origin of the MDR.

In conclusion, the result of this work buttresses the theory of international dissemination of various forms of MDR pathogenic bacteria found in this study and as found elsewhere in the world. It also gives hope that the emergent EPA among MDR strains can be tackled with discovery of useful EPIs which can be incorporated into antibiotic formulations to

bring about the lowering of MICs of antibiotics to clinically useful levels.

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