ECRONICON OPEN ACCESS

EC MICROBIOLOGY
Research Article

Molecular Characterization of Efflux Pump Genes in Clinical Isolates of *E. coli* from Urinary Tract Infection UTI and Diarrheic Patients in Zaria, Nigeria

Igwe JC[1]*, Michael G[2], Bolaji RO[3], Durowaye MT[4], Olayinka BO[3], Ehnimidu JO[3] and Onaolapo JA[3]

[1]*Department of Pharmaceutical Microbiology and Biotechnology, Kaduna State University, Kaduna State, Nigeria*

[2]*Department of Medical Microbiology/Immunology, Federal Teaching Hospital Gombe, Gombe State, Nigeria*

[3]*Department of Pharmaceutics and Pharmaceutical Microbiology, Ahmadu Bello University, Zaria, Nigeria*

[4]*Department of Pharmaceutics and Pharmaceutical Microbiology, University of Ibadan, Nigeria*

***Corresponding Author:** Igwe JC, Department of Pharmaceutical Microbiology and Biotechnology, Kaduna State University, Kaduna State, Nigeria.

Received: December 06, 2018; **Published:** April 01, 2019

Abstract

Efflux pump mechanisms have been reported to contribute significantly to antimicrobial drug resistance; influencing treatment failure, high medical bills, and increased mortality/morbidity. This study evaluates the prevalence and antibiotic susceptibility profile of efflux pump encoding *E. coli* isolated from UTI and diarrheic patients in Zaria, Nigeria using standard microbiological, epidemiological and molecular techniques. The results showed that out of 65 presumptive *E. coli* collected from UTI (40) and diarrheic (25) patients for the period of 6 months (April - September, 2014) in 4 health care centers in Zaria, Nigeria; biochemical examination using Microgen kit showed that 73.8% (48) were *E. coli* [UTI (29) and diarrheic (19)]. The resistance profile of the isolates to 15 antibiotics showed that the isolates were highly resistant to Amoxicillin (93.6%), Cefpirome (89.6%), Cefpodoxime and Cefotaxime (77.1%), Tetracycline (68.8%) and Cotrimoxazole (60.4%); mildly resistant to Ofloxacin and Ciprofloxacin (45.8%), Gentamicin (37.4), Aztreonam (35.4), Ceftriaxone (33.3) and Nitrofurantoin (20.8), but highly susceptible to Imipenem and Amikacin (0%). High percentage (89.6%) of the isolates had MARI > 2 and 72.5% (29) were MDR. Out of the MDR isolates, those that were resistant to Betalactams, Cotrimoxazole and Tetracycline were evaluated for efflux pump genes. The result showed that all the isolates evaluated encode the 7 efflux pump genes (*mdfA, emrB, emrD, emrE, acrA, acrB, tolC*), which belongs to the 3 families of the efflux pump antibiotics resistance genes, except *emrE* gene of 156 base pair that amplified in 95.2% of the isolates. This study showed that *E. coli* isolates with efflux pump gene could express resistance to structurally unrelated antibiotic.

Keywords: *Efflux Pump Genes; E. coli; UTI; Diarrheic*

Introduction

Efflux pumps are transport proteins involved in the extrusion of toxic substrates (including virtually all classes of clinically relevant antibiotics) from within cells into the external environment. This mechanism primarily decrease lethal concentration effect of antibiotic or substrate within the bacteria cytoplasm. These proteins (*mdfA, emrB, emrD, emrE, acrA, acrB, tolC*) are found in both Gram positive and negative bacteria as well as in eukaryotic organisms [1]. Efflux pumps may be specific for one substrate or may transport a range of structurally dissimilar compounds (including antibiotics of multiple classes); such pumps can be associated with multiple drug resistance (MDR). In prokaryotic kingdom, there are five major families of efflux transporter, which include the MF (major facilitator), MATE (multidrug and toxic efflux), RND (resistance-nodulation-division), SMR (small multidrug resistance) and ABC (ATP binding cassette). All these systems utilize the proton motive force as an energy source, apart from the ABC family, which utilizes ATP hydrolysis to drive the export

of substrates [2]. Research have shown that a typical efflux pump consists of the following four components: (a) outer membrane proteins (e.g. TolC) (b) middle periplasmic protein (e.g. AcrA) (c) inner membrane protein (e.g. AcrB) and (d) transmembrane duct. The interplay between these components lays in the ability of the periplasmic membrane protein to interact with the outer and inner membranes, which helps to stabilize the duct (channel) in a closed state. Opening of the duct is triggered by binding of the substrate (drug) to the inner membrane protein, which is known to exchange the substrate (drug) with H^+, creating an energy-dependent protein-protein interaction between the outer membrane protein and periplasmic membrane protein [3]. According to Bohnert., *et al.* [4], efflux pump genes expression are controlled by a regulatory gene, which is contained in an operon, and increased expression is associated with resistance to the substrates, e.g. resistance to bile salts and some antibiotics in *Escherichia coli* is mediated by over-expression of *acrAB*. Although genes encoding efflux pumps can be found on plasmids, the carriage of efflux pump genes on the chromosome gives the bacterium an intrinsic mechanism that allows survival in a hostile environment (e.g. the presence of antibiotics), and so mutant bacteria that over-express efflux pump genes can be selected without the acquisition of new genetic material [5]. Among the efflux pump genes are *Emr* proteins that expels drugs from cells, contributing significantly to the continued rise in multidrug resistant bacteria, and thus re-emergence of drug-resistant strains in disease conditions [6]. *Emr* proteins belong to the major facilitator superfamily; a group of transporters among the most prevalent in microbial genomes [7]. These operons are distinctive in their ability to recognize and expel a highly diverse range of amphipathic compounds (hydrophobic and hydrophilic groups) [8]. Edgar and Bibi [9] had reported that *E. coli* with *MdfA* gene from a multicopy plasmid expressed more resistance to a diverse group of cationic or zwitterionic lipophilic compounds such as ethidium bromide, tetraphenylphosphonium, rhodamine, daunomycin, benzalkonium, rifampin, tetracycline, and puromycin. This *MdfA* also confers resistance to chemically unrelated, clinically important antibiotics such as chloramphenicol, erythromycin, and certain aminoglycosides and fluoroquinolones [10]. The wide spread of these resistance genes has been associated with mobile genetic elements including plasmids, transposons, and gene cassettes in integrons [10] and the alteration in *mar* locus regulation [11]**.** A deep study into this identified resistant determinants genes will add significantly to general understanding of the mechanism of drug transport through the cell membrane and provide the structural basis for how these proteins go about selecting specific drugs to expel.

Methodology

Study area

This study was carried out using four hospitals within Zaria metropolis. The following hospitals were selected for this study based on patients' population, distance apart and good representation of Zaria metropolis: Ahmadu Bello University Teaching Hospital Shika, St. Luke Anglican Hospital Wusasa, GamboSawama General Hospital Kofangaya, and Ahmadu Bello University Clinic (Sickbay) Main Campus Samaru.

Specimen collection and processing

Suspected *E. coli* isolates (from diarrhea; Children age 0 - 5 years and adult with recurrent watery/bloody stool and UTI patients) were sub-cultured onto an already prepared nutrient agar slants and transported in an ice pack to Pharmaceutical Microbiology Laboratory, A. B. U, Zaria for incubation for 24 hrs at 37°C. This sample collection was carried out from April 2014 to September, 2014.

Isolation, identification and biochemical test

The size, shape, and colour of the grown suspected *E. coli* colonies were observed under a microscope [12]. The isolates were further sub-cultured onto eosin methylene blue agar plates and incubated at 37°C for 18 hours for primary identification of *E. coli*. All colonies that showed characteristic green metallic sheen were further analyzed by Microgen biochemical tests kit using manufacturer procedure.

Antibiotic susceptibility testing

Antibiotic susceptibility pattern of *E. coli* isolates from UTI and diarrhea patients to 15 different antibiotics were determined using disc diffusion method according to Cheesbrough [13] and CLSI [14].

Molecular analysis

Bacteria cell preparation

The preparation of the bacteria cell were carried out using the method described by Dubey [15]: Chemical ingredients of Luria and Bertani broth media were prepared as follow; peptone (10g), NaCl (5g), 1N NaOH (10ml), yeast extract (5g), distilled water (1litre), pH 7.0 adjusted with NaOH solution and sterilized at 121°C for 15 minutes. Single colonies were picked from freshly streaked isolates on MacConkey plate and inoculated into 5 ml Luria and Bertani (LB) broth medium and incubated overnight at 37°C for 18 - 24 hrs. Bacteria cells were then harvested by centrifugation at 4°C, 8000 rpm (6800xg) in a refrigerated microcentrifuge for 30 seconds in an Eppendorff's tube. The supernatants were decanted and cells harvested.

Genomic DNA extraction

Genomic DNA extraction was carried out using the method described by DNeasyBlood and Tissue Handbook [16].

The harvested cell pellets were dislodged and 20 µl of proteinase K was added and mixed thoroughly by vortexing. The mixture was further incubated at 56°C for 3 hrs and vortexed occasionally using a thermo-mixer until the cells were completely lysed and properly mixed to prevent clogging of the DNeasy Mini spin column. 200 µl of buffer AL was added to the sample, and mixed thoroughly by vortexing. This was further be accompanied by the addition of 200µl of absolute ethanol (96 - 100%) and mixed again by vortexing to yield homogenous solution. This mixture were then be pipetted into DNeasy Mini spin column placed in a 2 ml collection tube and centrifuged at 8000 rpm for 1 minute. The flow-through and collection tube was then discarded.

The DNeasy Mini spin column was then transferred into a new 2 ml collection tube and 500 µl buffer AW1 was added and centrifuged again at 8000 rpm for 1 minute before the flow-through was also discarded. For the third time, DNeasy Mini spin column was placed in 2 ml collection tube and 500 µl buffer AW2 was added and centrifuged at 14000 rpm for 3 minutes to dry the DNeasy membrane in other to remove the residual ethanol that might interfere with subsequent reactions. The flow-through and collection tube was removed and discarded carefully so that the column does not come into contact with the flow-through, resulting in carryover of ethanol. The used DNeasy Mini spin column was now placed in a clean 2 ml micro-centrifuge tube and 200 µl buffer AE was introduced onto the DNeasy membrane to elute, and increase the final DNA concentration and yield in the eluate. The column was then incubated at room temperature for 1 minute before being centrifuged at 8000 rpm for 1 minute. The elution of the membrane was then repeated twice to ensure increased overall DNA yield in the same microcentrifuge tube used in step 7, but care was taken to prevent dilution of the first eluate.

Agarose gel electrophoresis

To ascertain that genomic DNA is actually extracted, the eluent were subjected to agarose gel electrophoresis, prepared as stated below.

1% agarose gel was used to resolve the genomic DNA. The agarose gel was prepared by combining l.0g of agarose in ten times concentration (10X) of Tris acetate ethylene diamine tetra acetate (2 ml 10X TAE) buffer and 98 ml distilled water in a 250 ml beaker flask and heating in a microwave for 2 minutes until the agarose is dissolved. 20 µl ethidium bromide (5.0 mg/ml) was added to the dissolved agarose solution as dye and mixed. The gel was then poured onto a mini horizontal gel electrophoresis tank and the casting combs inserted at the red bands to ensure easy view of the well, while filling the genomic DNA. It's then allowed to set for 30 minutes.

The casting combs will then be carefully removed after the gel had completely solidified, one time concentration (1X) TAE electrophoresis buffer was then added to the reservoir until the buffer covers the agarose gel. 5 µl of gel tracking dye (bromophenol blue) was added to 15 µl of each sample with gentle mixing. 20 µl of the sample was then loaded onto the wells of the gel, the mini horizontal electrophoresis gel set up was covered and the electrodes connected running from cathode (-) to anode (+). Electrophoresis was carried out at 100 mV for 45 minutes to allow easy separation of sample based on molecular weight. At the completion of the electrophoresis, the gel was removed from the buffer (Tris) and the gel viewed under a trans-illuminator UV light of wavelength 302 nm. The band pattern of the DNA fragments was then photographed with a Polaroid camera and documented using an electrophoresis gel documentation system. Electrophoresis is employed to identify the number of plasmid copies present in different isolates. However, standard DNA molecular weight marker (1kb) was used to estimate the genomic DNA size.

Detection of efflux pump genes using their respective primer (Polymerase Chain Reaction)

Amplification of efflux pump genes were carried out using Dream Taq™ DNA polymerase, which is an enhanced multiplex PCR Taq DNA polymerase, optimized for all standard PCR applications as described by DNeasy Blood and Tissue Handbook (2006).

It ensures higher sensitivity, longer PCR products and higher yield compared to conventional Taq DNA. Dream Taq™ DNA polymerase uses the same reaction set-up and recycling conditions as conventional Taq™ DNA polymerase. Extensive optimization of reaction is not required. The enzyme is supplied with optimized Dream Taq™ buffer, which includes 20 mM $MgCl_2$. Dream Taq™ DNA polymerase generates PCR products with 3'-dA overhangs. The enzyme is inhibited by dUTP but can incorporate modified nucleotides: Dream Taq™ PCR master mix (2X) was vortexed and centrifuged for 30 seconds at 8000 rpm after thawing. The thin - walled PCR tube was then placed on an ice pack and the following components was added for each isolate for single reaction: (a) Dream Taq™ PCR master mix. (b) Forward primers (c) reverse primers (d) template DNA, Taq buffer, dNTP (e) the nuclease - free water. The samples were vortexed gently and spin down. The primers used for PCR are as contained in the table 1.

S/N	Primer name	Sequence (5′→3′)	PCR product size (bp)	Annealing temp (°C)	References	Antibiotics Resistance To
1	acrA-F	CTCTCAGGCAGCTTAGCCCTAA	107	53	Viveros., *et al.* (2007)	Resistance nodulation cell division family
	acrA-R	TGCAGAGGTTCAGTTTTGACTGTT				
2	acrB-F	GGTCGATTCCGTTCTCCGTTA	107	53	Viveros., *et al.* (2007)	
	acrB-R	CTACCTGGAAGTAAACGTCATTGGT				
3	*MdfA*-F	CATTGGCAGCGATCTCCTT	103	52	Michelle., *et al.* (2011)	Major facilitator supper family
	MdfA-R	TTATAGTCACGACCGACTTCTTTCA				
4	tolC-F	AAGCCGAAAAACGCAACCT	100	51	Michelle., *et al.* (2011)	
	tolC-R	CAGAGTCGGTAAGTGACCATC				
5	*Emr*B-F	ATTATGTATGCCGTCTGCTT	196	52	Viveros., *et al.* (2007)	
	*Emr*B-R	TTCGCGTAAAGTTAGAGAGG				
6	*Emr*D-F	TGTTAAACATTGGGGATTCTC	243	52		
	*Emr*D-R	TCAGCATCAGCAAATAACAG				
7	*Emr*E-F	GGATTGCTTATGCTATCTGG	156	52	Viveros., *et al.* (2007)	Small multidrug resistance family
	*Emr*E-R	GTGTGCTTCGTGACAATAAA				

Table 1: *Multidrug resistance efflux pumping genes.*

Results

Isolation, identification and biochemical test

The result showed that 73.8% of the presumed *E. coli* isolates from both disease conditions are *E. coli.*

S/N	Hospitals Sampled (n = 4)	Diarrhea (S)		UTI (U)		Total Isolates Collected
		Isolates collected	*E. coli*	Isolates collected	*E. coli*	
1	ABUTH	7	5	16	11	23
2	ABUSB	4	4	11	8	15
3	SLAH	8	6	6	5	14
4	HGSGH	6	4	7	5	13
	Total (%)	25	19 (76)	40	29 (72.5)	65 (100)
	% (Occurrence of *E. coli*)			73.8 (48)		

Table 2: *Distribution of E. coli isolates among UTI and diarrheic patients.*

Antibiotic susceptibility profile

The isolates were highly resistant to Amoxicillin (93.6%), Cefpirome (89.6%), Cefpodoxime and Cefotaxime (77.1%), Tetracycline (68.8%) and Cotrimoxazole (60.4%); mildly resistant to Ofloxacin and Ciprofloxacin (45.8%), Gentamicin (37.4), Aztreonam (35.4), Ceftriaxone (33.3) and Nitrofurantoin (20.8), but highly susceptible to Imipenem and Amikacin (0%).

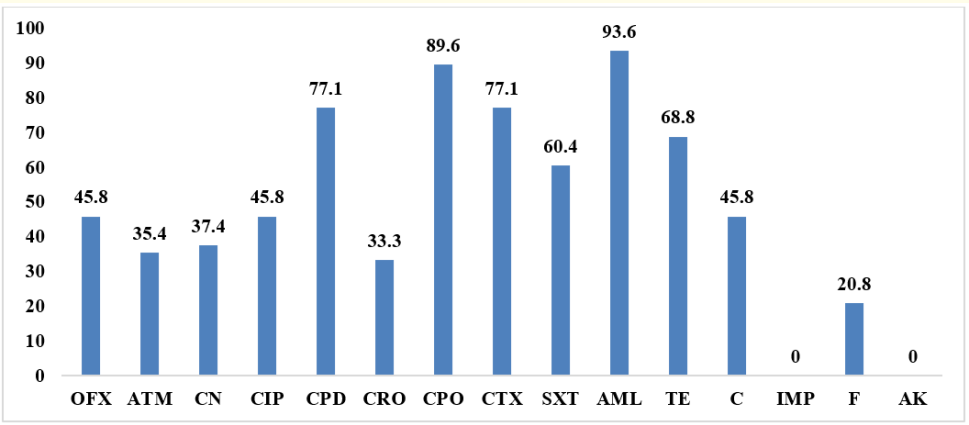

Figure 1: *Antibiotics susceptibility of E. coli isolated from UTI and diarrheic patients.*
OFX: Ofloxacin; F: Nitrofurantoin; ATM: Aztreonam; CN: Gentamicin; CIP: Ciprofloxacin; CPD: Cefpodoxime; CRO: Ceftriaxone; CPO: Cefpirome; CTX: Cefotaxime; SXT: Cotrimoxazole; TE: Tetracycline C: Chloramphenicol; IPM: Imipenem; AML: Amoxicillin; AK: Amikacin.

S/N	Isolates	Antibiotic Resistance Pattern	NART	GART	NGART	CR
1	THU1	OFX, ATM, CN, CIP, CPD, CRO, CPO, CTX, SXT, C, AML, TE	12	FLU, AMIN, MISC, TE, BET	5	MDR
2	SLS6	OFX, CIP, CPD, CPO, CTX, SXT, C, AML	8	FLU, MISC, BET	3	MDR
3	THU10	OFX, ATM, CN, CIP, CRO, CPD, CPO, CTX, SXT, F, AML, TE	12	FLU, AMIN, MISC, TE, BET	5	MDR
4	THU13	OFX, ATM, CIP, CRO, CPD, CPO, CTX, C, AML, TE	10	FLU, MISC, TE, BET	5	MDR
5	THU19	OFX, ATM, CIP, CRO, CPD, CPO, CTX, SXT, AML, F, TE	11	FLU, AMIN, TE, BET, MISC	5	MDR
6	THU25	OFX, ATM, CN, CIP, CRO, CPD, CPO, CTX, SXT, C, AML, F, TE	13	FLU, AMIN, MISC, TE, BET	5	MDR
7	THU27	OFX, CN, CIP, CRO, CPD, CPO, CTX, SXT, AML, F, TE	11	FLU, AMIN, MISC, TE, BET	5	MDR
8	THS2	OFX, ATM, CIP, CRO, CPD, CPO, CTX, C, AML	9	FLU, MISC, BET	3	MDR
9	THS8	CN, OFX, ATM, CIP, CRO, CPD, CPO, CTX, SXT, C, AML, TE	12	FLU, AMIN, MISC, TE, BET	5	MDR
10	THS12	CN, OFX, ATM, CIP, CRO, CPD, CPO, CTX, SXT, C, AML, TE	12	FLU, AMIN, MISC, TE, BET	5	MDR
11	THS15	CN, OFX, CIP, CPD, CPO, CTX, SXT, AML, TE	9	FLU, AMIN, MISC, TE, BET	5	MDR
12	SBS1	CN, OFX, CIP, CPD, CPO, CTX, SXT, C, AML, TE	10	FLU, AMIN, MISC, TE, BET	5	MDR
13	SBU2	CN, OFX, CIP, CPD, CPO, CTX, SXT, AML, TE	9	FLU, AMIN, MISC, TE, BET	5	MDR
14	SBU12	CN, ATM, OFX, CIP, CRO, CPD, CPO, CTX, SXT, C, AML, F, TE	13	FLU, AMIN, MISC, TE, BET	5	MDR
15	SBU13	CN, ATM, OFX, CIP, CRO, CPD, CPO, CTX, SXT, C, AML, TE	12	FLU, AMIN, MISC, TE, BET	5	MDR
16	SBU15	CN, OFX, CIP, CPD, CPO, CTX, SXT, C, AML, TE	10	FLU, AMIN, MISC, TE, BET	5	MDR
17	SBU16	CN, ATM, OFX, CIP, CPD, CPO, CTX, SXT, C, AML, TE	11	FLU, AMIN, MISC, TE, BET	5	MDR
18	SLU10	OFX, CIP, CPD, CPO, CTX, SXT, C, AML	8	FLU, MISC, BET	3	MDR
19	HGS5	CN, ATM, OFX, CIP, F, CPD, CPO, CTX, SXT, AML, TE	11	FLU, AMIN, MISC, TE, BET	5	MDR
20	HGU1	CN, OFX, CIP, CPD, CPO, CTX, SXT, AML, TE	9	FLU, AMIN, MISC, TE, BET	5	MDR
21	HGS9	CPO, CPD, AML	3	BET	1	NMR
22	HGU16	CN, ATM, OFX, CIP, CRO, CPD, CTX, C, AML, TE	10	FLU, AMIN, MISC, TE, BET	5	MDR
23	THU9	CPD, CPO, CTX, AML, TE	5	TE, BET	2	NMR
24	THU12	CPO, CTX, AML	3	BET	1	NMR
25	THU17	CPO, CTX, F, AML, TE	5	MISC, TE, BET	3	MDR
26	SBU9	CPD, CPO, SXT, C, AML	5	MISC, BET	2	NMR

27	SBU11	CPD, CPO, AML	3	BET	1	NMR
28	THU16	CRO, CPO, CTX, AML, F	5	MISC, BET	2	NMR
29	SLU2	CPO, CTX, SXT, TE	4	MISC, BET	2	NMR
30	SLU18	CPD, AML	2	BET	1	NMR
31	SLU4	CPD, CPO, C, AML, TE	5	MISC, TE, BET	3	MDR
32	SLU7	CPD, CPO, ATM, TE	5	TE, BET	2	NMR
33	HGU4	F, CPO, SXT, AML, TE	5	MISC, TE, BET	3	MDR
34	HGU6	C, CTX, AML, TE	4	AMIN, TE, BET	3	MDR
35	SBU22	CPD, CPO, AML, TE	4	TE, BET	1	NMR
36	HGU15	CN, CPD, CPO, CTX, SXT, C, AML	7	AMIN, MISC, BET	3	MDR
37	THS10	CPD, CPO, SXT, AML	4	MISC, BET	2	NMR
38	SBS4	CPD, CPO, CTX, SXT, C, AML	6	MISC	2	NMR
39	SBS9	CPD, CPO, CTX, SXT, C, AML	6	MISC, BET	2	NMR
40	SBS10	CPD, CPO, CTX, SXT, C, AML	4	MISC, BET	2	NMR
41	SLS1	CPO, CTX, SXT, AML, TE	5	MISC, TE, BET	3	MDR
42	SLS2	AML, TE	2	TE, BET	2	NMR
43	SLS3	CPD, CPO, CTX, SXT, AML, TE	6	TE, BET	2	NMR
44	THU2	OFX, CIP, CN, CRO, CPO, CTX, AML, TE	8	FLU, AMIN, TE, BET	4	MDR
45	SLS7	F, CRO, CPO, SXT, C, AML	6	MISC, BET	2	NMR
46	SLS8	AK, CRO, ATM, CPO, CTX, TE	6	AMIN, TE, BET	3	MDR
47	HGS3	ATM, CPD, CTX, AML, TE	5	TE, BET	2	NMR
48	HGS4	ATM, CPD, CPO, CTX, AML, TE	4	TE, BET	2	NMR

Table 3: *Antibiotic Resistance Profile of E. coli from Diarrhea and UTI.*

Keys: FLU: Fluoroquinolone; MON: Monobactam; AMIN: Aminoglycoside; CEPH: Cephalosporin; MISC: Miscellaneous antibiotics; CAB: Carbapenems; PEN: Penicillin; AK: Amikacin; OFX: Ofloxacin; F: Nitrofurantoin; ATM: Aztreonam; CN: Gentamicin; CIP: Ciprofloxacin; CPD: Cefpodoxime; CRO: Ceftriaxone; CPO: Cefpirome; CTX: Cefotaxime; SXT: Cotrimoxazole; C: Chloramphenicol; IPM: Imipenem; AML: Amoxicillin; MDR: Multidrug-resistant; NMDR: Not Multidrug-Resistant; NAR: Number of Antibiotics Resistance; CART: Class of Antibiotics Each Isolate of E. coli is Resistant to; MDR: Non-Susceptible to ≥ 1 agent in ≥ 3 Antimicrobial Categories.

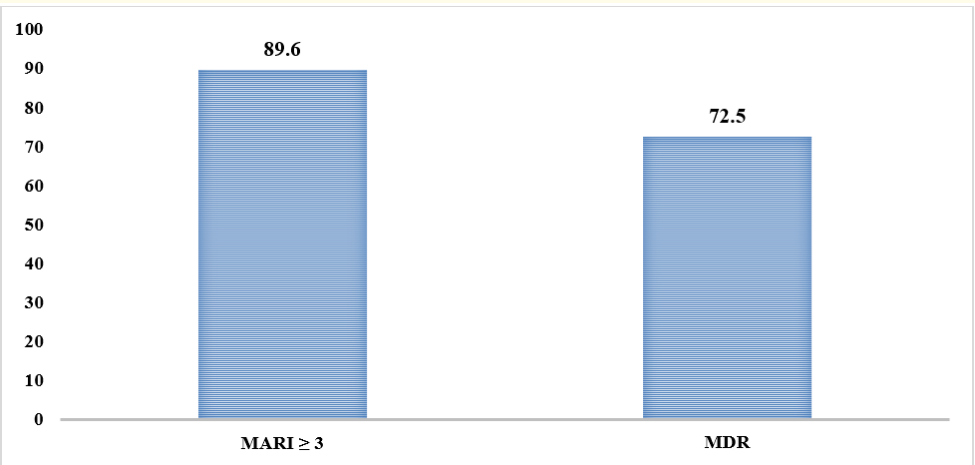

Figure 2: *Classification of multiple antibiotic resistant index and percentage multidrug resistant.*

Molecular analysis

Unique multidrug resistant pattern to Betalactams, Cotrimoxazole and Tetracycline simultaneously was observed in most MDR isolates and they were selected for efflux pump evaluation. All the isolates (Lane 2 to Lane 22) have harbored *mdfA*, *emrB, tolc, emrE* and *emrD* that are responsible for efflux pump, except lane 21 (HGU1) which do not have *emrE* gene that amplified at 156 base pair.

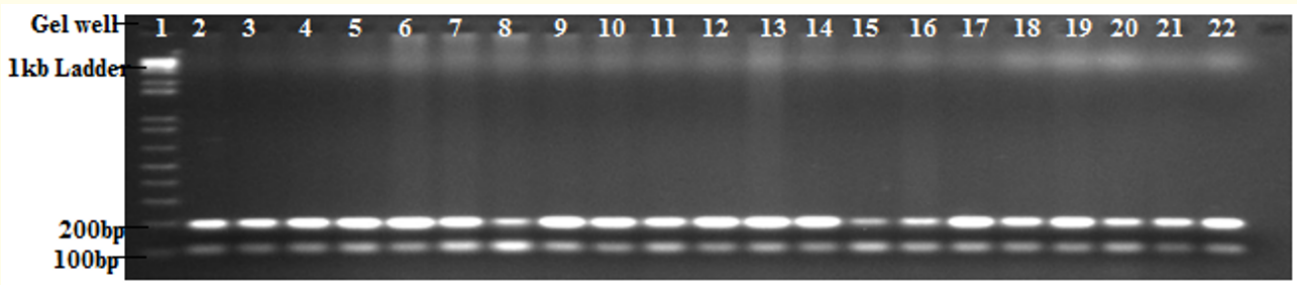

Figure 3: *Multiplex amplification of mdfA (103), emrB (196bp) genes on electrophoretic gel.*

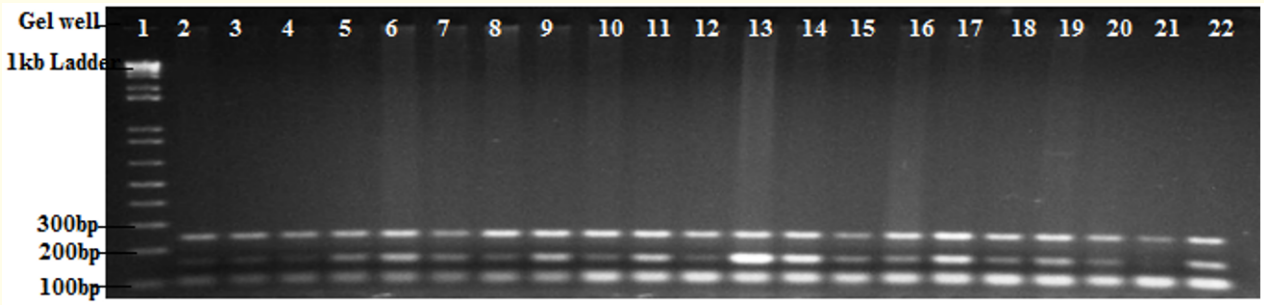

Figure 4: *Multiplex amplification of tolc (100bp), emrE (156), emrD (243bp) genes on electrophoretic gel.*
Keys: Lane 1= 1 kb DNA Ladder; Lane 2 (THU1); Lane 3 (THU2); Lane 4 (THU10); Lane 5 (THU13); Lane 6 (THU19); Lane 7 (THU25); Lane 8 (THU27); Lane 9 (THS2); Lane 10 (THS8); Lane 11 (THS12); Lane 12 (THS15); Lane 13 (SBS1); Lane 14 (SBU2); Lane 15 (SBU12); Lane 16 (SBU13); Lane 17 (SBU15); Lane 18 (SBU16); Lane 19 (SLU10); Lane 20 (HGS5); Lane 21 (HGU1); Lane 22 (HGU16).

Discussion

Efflux pumps are transport proteins involved in the extrusion of toxic substrates (including virtually all classes of clinically relevant antibiotics) from within cells into the external environment. This resistant model is enhanced in the presence of cell wall protective enzymes, plasmid encoded resistant genes and efflux transporters. In *E. coli*, seven different proton-dependent MDR pump systems have been identified in biological studies (*AcrAB-TolC, EmrAB, MdfA, TehA, EmrE, AcrEF,* and*EmrD*) and others have been identified by comparative amino acid sequence analysis [17]. Efflux pump mechanisms have been reported to contribute significantly to antimicrobial drug resistance; influencing treatment failure, high medical bills, and increased mortality/morbidity [18]. Biochemical confirmation test showed that out of the 65 presumed *E. coli* isolates [UTI (40) and diarrheic (25) patients] for the period of 6 months (April - September, 2014) in 4 health care centers in Zaria, Nigeria; 73.8% (48) were confirmed as *E. coli* [UTI 60.4% (29) and diarrheic 39.6% (19)]. The percentage of *E. coli* isolated in this study concur with the study conducted by Cunha., *et al.* [19] in Brazil while that of diarrheic sample is in line with the study of Kilic., *et al* [20]. According to Caliendo., *et al.* [21], the use of presumptions in the identification of clinical pathogens contributes significantly to antibiotics misuse as it influences the use of unempiric antimicrobial therapy rather than appropriate therapy. Hence, rapid biochemical kits or techniques are needed in the hospitals for specific pathogen diagnosis. These techniques should be easy to use and provide a rapid result (ideally within an hour) to have a positive impact on care. The resistance profile of the isolates to 15 antibiotics showed that the isolates were highly resistant to Amoxicillin (93.6%), Cefpirome (89.6%), Cefpodoxime and Cefotaxime (77.1%),

Tetracycline (68.8%) and Cotrimoxazole (60.4%); mildly resistant to Ofloxacin and Ciprofloxacin (45.8%), Gentamicin (37.4), Aztreonam (35.4), Ceftriaxone (33.3) and Nitrofurantoin (20.8), but highly susceptible to Imipenem and Amikacin (0%). These findings concur with the findings of Olorunmola., *et al.* [22] and Aboderin., *et al.* [23] in Ile-Ife, and Chiyangi., *et al.* [24] in Zambia, whose studies observed widespread and increasing resistance to amoxicillin, gentamicin, cotrimoxazole, ciprofloxacin, ofloxacin, and tetracycline and also variations in percentage antibiotic resistance among *E. coli* isolates to most commonly prescribed antibiotics in the hospitals. High percentage (89.6%) of the isolates had MARI > 2 and 72.5% (29) were MDR. These high resistant profile and characteristics emphasize the need for continuous antimicrobial surveillance as well as the implementation of prevention programmes for *E. coli* associated infections.

In this study, 3 efflux pump families such as the major facilitator super family (MFS) pumps (*mdfA* (responsible for multidrug transporter) *emrB, emrD);* the small multidrug resistance (SMR) family (*emrE*) and resistance nodulation division (RND) family [*acrA* (that is responsible for protein fusion)*, acrB* (a cytoplasmic membrane transporter protein) *and tolC* (an outer membrane channel protein)] were investigated. The results showed that the 7 efflux pump genes belonging to the 3 families were present in all the 21 MDR ESBL producing *E. coli* from UTI and diarrhea patients evaluated in Zaria, Nigeria, except *emrE* gene of 156 base pair that could not be amplified in HGU1 *E. coli* isolate (Lane 21 of figure 4). This result showed an over-expression of efflux pump genes in MDR ESBL producing *E. coli* isolates, which might have contributed to the MDR observed in the antibiotic susceptibility study of the *E. coli* isolates in this study. Our findings also purported a correlation between ESBL production in *E. coli* and efflux pump genes. This result is in-line with the study of Morita., *et al.* [25] who reported that several efflux pumps could be expressed in a single bacterial species, thus conferring on it resistance to many antimicrobials. Oethinger., *et al.* [26] and Tomihiko., *et al.* [27] also had demonstrated the contribution of these gene to be responsible for Fluoroquinolones resistance in *E. coli* and a deletion of these genes (*acrAB* and *mdfA*) from the genome of the organism restored the sensitivity of the isolates to Fluoroquinolones. The overproduction of *acrAB* and *mdfA* was also reported to lead to an 8-fold-increased resistance to Ciprofloxacin [5]. Strains that over-expressed *emrE* and *mdfA* have been associated with Ethidium bromide, Tetraphenylphosphonium, Rhodamine, Daunomycin, Benzalkonium, Rifampin, Tetracycline, Puromycin, Chloramphenicol, Erythromycin, some Aminoglycosides, and Fluoroquinolones resistance [17]. The presence of these genes in clinical isolates should indeed be of concern, as often over-expression of a pump will result in resistance to antibiotics of more than one class (Fluoroquinolones, β-lactams, Chloramphenicol and Trimethoprim) as well as some dyes, detergents and disinfectants (including some commonly used biocides) [28]. The problem of cross-resistance could also arise as exposure to any one agent that belongs to the substrate profile of a pump would favour over-expression of that pump and consequent cross-resistance to all other substrates of the pump [29].

This significant resistant mechanism could be overcome by developing structural analogs of an antimicrobial agent, as newer generation agents are less susceptible to efflux pumps than the older generation agents of the same class. For example, the Glycyclines are less susceptible than Tetracycline's, and Ketolides are less susceptible than Macrolides [30]. Drugs that are competitive and non-competitive inhibitors of efflux pumps could be used as adjuncts to reverse or prevent the development of efflux-mediated drug resistance [18]. Although efflux pump inhibitors that prevent the energy-dependent efflux of drugs and some endogenous metabolites from the microbial cells are available, and thus reduce MDR to existing antimicrobial agents, majority of the efflux pump inhibitors (some Lipophilic alkaloids, Terpenoids, Flavonoids, Verapamil, Reserpine, Quinazolinones, Chlorpromazine, Omeprazole and Pantoprazole) are not used as pump inhibitors in routine clinical practice because of high plasma concentrations that is required to achieve efflux inhibition *in vitro* are rarely achieved *in vivo* without serious toxicities [31]. Chlorpromazine enhanced the antimicrobial activity of Aminoglycosides and Macrolides, and also had a synergistic effect in combination with penicillin G against *E. coli* by inhibition of bacterial efflux pumps [32]. Flavonoids (herbal constituents) also exhibited P-gp inhibitory and direct antitumor activity, thereby acting synergistically with Taxanes, Vinca alkaloids, and Camptothecins, in cancer chemotherapy [33]. It must be noted that efflux pumps represent a greater threat with regard to antibiotic resistance in those microorganisms that couple efflux with a low-permeability cell envelope, as is the case for Gram-negative bacteria and mycobacteria, due to the existence of synergy between these two resistance strategies [34].

Conclusion

This study observed that *E. coli* isolates with efflux pump genes develop resistance to structurally unrelated antibiotics and are majorly multidrug resistant. The antibiotics of choice for the treatment of *E. coli* associated infections with efflux pump genes were imipenem, amikacin and nitrofurantoin.

Bibliography

1. Bambeke VF., *et al*. "Antibiotic efflux pumps". *Biochemical Pharmacology* 60.4 (2000): 457-470.
2. Lomovskaya O., *et al*. "Identification and characterization of inhibitors of multidrug resistance efflux pumps in Pseudomonas aeruginosa: novel agents for combination therapy". *Antimicrobial Agents and Chemotherapy* 45.1 (2001): 105-116.
3. Symmons MF., *et al*. "The assembled structure of a complete tripartite bacterial multidrug efflux pump". *Proceedings of the National Academy of Sciences of the United States of America* 106.17 (2009): 7173-7178.
4. Bohnert JA., *et al*. "Altered spectrum of multidrug resistance associated with a single point mutation in the Escherichia coli RND-type MDR efflux pump YhiV (MdtF)". *Journal of Antimicrobial Chemotherapy* 59.6 (2007): 1216-1222.
5. Yang S., *et al*. "Relative contributions of the AcrAB, MdfA and NorE efflux pumps to quinolone resistance in Escherichia coli". *Journal of Antimicrobial Chemotherapy* 51.3 (2003): 545-556.
6. Lomovskaya O., *et al*. "EmrR is a negative regulator of the Escherichia coli multidrug resistance pump EmrAB". *Journal of Bacteriology* 177.9 (1995): 2328-2334.
7. Pao SS., *et al*. "Major facilitator super-family". *Microbiology and Molecular Biology Reviews* 62.1 (1998): 1-34.
8. Geoffrey A Chang., *et al*. "New Study Reveals Structure of E. coli Multidrug Transporter Protein Could Aid". In: Fight Against Drug-resistant Bacteria. Scripps Research Institute (2006).
9. Edgar R and Bibi E. "MdfA, an Escherichia coli multidrug resistance protein with an extraordinarily broad spectrum of drug recognition". *Journal of Bacteriology* 179.7 (1997): 2274-2280.
10. Carattoli A. "Importance of integrons in the diffusion of resistance". *Veterinary Research* 32.3-4 (2001): 243-259.
11. Ethinger M., *et al*. "Over expression of the marA or soxS regulatory gene in clinical topoisomerase mutants of Escherichia coli". *Antimicrobial Agents and Chemotherapy* 42.8 (1998): 2089-2094.
12. Chakraborty P and Nishith KP. "Manual of practical microbiology and parasitology". New central book agency limited, West Bengal, India (2008).
13. Cheesbrough M. "District laboratory practice in tropical countries (Part 11)". Cambridge, University Press UK. (2000): 134-143.
14. Clinical Laboratory Standard Institute (CLSI). Performance standards of antimicrobial disc and dilution susceptibility tests for bacteria isolated from animal, approved standard. 3rd edition. 28.8 (2008).
15. Dubey RC. "Multicolour Textbook of Biotechnology. 4th Edition". S. Chand and Co. India. (2009): 71-80.
16. DNeasy Blood and Tissue Handbook. Animal Tissues (spin-column protocol): purification of Total DNA from animal tissue (07/2006): 28-31.
17. Mark C Sulavik., *et al*. "Antibiotic Susceptibility Profiles of Escherichia coli Strains Lacking Multidrug Efflux Pump Genes". *Antimicrobial Agents and Chemotherapy* 45.4 (2001): 1126-1136.
18. Ughachukwu PO and Unekwe PC "Efflux Pump-Mediated Resistance in Chemotherapy". *Annals of Medical and Health Science Research* 2.2 (2012): 191-198.
19. Cunha MA., *et al*. "Antibiotic resistance patterns of urinary tract infections in a northeastern Brazilian capital". *Revista do Instituto de Medicina Tropical de São Paulo* 58 (2016): 2.
20. Kilic IH., *et al*. "Investigation of Diarrhea Agents Less Than 5 Years of Age in Summer in Gaziantep/Turkey". *Pakistan Journal of Biological Sciences* 10.17 (2007): 2915-2919.

21. Caliendo MA., *et al*. "Better Tests, Better Care: Improved Diagnostics for Infectious Diseases". *Clinical Infectious Diseases* 57.3 (2013): S139-S170.

22. Olorunmola FO., *et al*. "Antibiotic Resistance and Virulence Properties in Escherichia Coli Strains from Cases of Urinary Tract Infections". *African Journal of Infectious Diseases* 7.1 (2013): 1-7.

23. Chiyangi H., *et al*. "Identification and antimicrobial resistance patterns of bacterial enteropathogens from children aged 0-59 months at the University Teaching Hospital, Lusaka, Zambia: a prospective cross sectional study". *BMC Infectious Diseases* 17.1 (2017): 117.

24. Aboderin OA., *et al*. "Antimicrobial resistance in Escherichia coli strains from urinary tract infections". *Journal of the National Medical Association* 101.12 (2009): 1268-1273.

25. Morita Y., *et al*. "Antibiotic inducibility of the MexXY multidrug efflux system of Pseudomonas aeruginosa: involvement of the antibiotic-inducible PA5471 gene product". *Journal of Bacteriology* 188.5 (2006): 1847-1855.

26. Oethinger M., *et al*. "Ineffectiveness of topoisomerase mutations in mediating clinically significant fluoroquinolone resistance in Escherichia coli in the absence of the AcrAB efflux pump". *Antimicrobial Agents and Chemotherapy* 44.1 (2000): 10-13.

27. Tomihiko Yasufuku., *et al*. "Correlation of Overexpression of Efflux Pump Genes with Antibiotic Resistance in Escherichia coli Strains Clinically Isolated from Urinary Tract Infection Patients". *Journal of Clinical Microbiology* 49.1 (2011): 189-194.

28. Webber MA and LJV Piddock. "The importance of efflux pumps in bacterial antibiotic resistance". *Journal of Antimicrobial Chemotherapy* 51.1 (2003): 9-11.

29. Chuanchuen R., *et al*. "Cross resistance between triclosan and antibiotics in Pseudomonas aeruginosa is mediated by multidrug efflux pumps: exposure of a susceptible mutant strain to triclosan selects nfxB mutants overexpressing MexCD-OprJ". *Antimicrobial Agents and Chemotherapy* 45 (2001): 428-432.

30. Li XZ and Nikaido H. "Efflux-mediated drug resistance in bacteria". *Drugs* 64.2 (2004): 159-204.

31. Van Bambeke F., *et al*. "Inhibitors of bacterial efflux pumps as adjuvants in antibiotic treatments and diagnostic tools for detection of resistance by efflux". *Recent Patents on Anti-Infective Drug Discovery* 1.2 (2006): 157-175.

32. Chan YY., *et al*. "Synergistic interaction between phenothiazines and antimicrobial agents against Burkholderia pseudomallei". *Antimicrobial Agents and Chemotherapy* 51.2 (2007): 623-630.

33. Bansal T., *et al*. "Emerging significance of flavonoids as P-glycoprotein inhibitors in cancer chemotherapy". *Journal of Pharmacy and Pharmaceutical Sciences* 12.1 (2009): 46-78.

34. Lucía Fernández and Robert EW Hancock. "Adaptive and Mutational Resistance: Role of Porins and Efflux Pumps in Drug Resistance". *Clinical Microbiology Reviews* 25.4 (2012): 661-681.

Volume 15 Issue 4 April 2019