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Impact of Outer Membrane Protein OmpC and OmpF on Antibiotics Resistance of *E. coli* Isolated from UTI and Diarrhoeic Patients in Zaria, Nigeria

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Abstract

Multidrug resistant (MDR) *E. coli* associated infections remains one of the most bacterial infections that have contributed significantly to increased morbidity and mortality in clinical settings. One of the known resistant mechanisms of MDR bacteria is reduced cell wall permeability, which is controlled by outer membrane protein OmpF and OmpC. This study evaluates the difference in molecular weight of outer membrane protein of MDR *E. coli* isolated from UTI and diarrhoeic patients in Zaria, Nigeria and antibiotic susceptible ATCC29522 strain using standard microbiological and molecular techniques. Eighty seven (87) confirmed *E. coli* isolates from UTI and diarrhoeic patients in selected hospitals in Zaria, Nigeria were evaluated for MDR using 15 antibiotics commonly prescribed for *E. coli* associated infections. The results showed that the 21 suspected multidrug isolates were 100% susceptible to Imipenem and Amikacin, and 71.4 % susceptible to Nitrofurantoin but highly (100%) resistant to Amoxicillin, Ofloxacin, Ciprofloxacin, Cefpodoxime and Ceftaxime, 95.2% resistant to Cefpirome, 85.7% to Tetracycline and Sulphamethonidazole-Trimethroprim, 76.2% to Gentamicine, 66.7% to Chloramphenicol, 61.9% to Aztreonam and 57.1% to Ceftriaxone. Cell wall protein evaluation using SDS-PAGE showed that both the MDR isolates and susceptible strain had equal OmpC bands at 38kDa while the OmpF varied from one MDR isolate to another compared with the ATCC29522 used as control. This study contributes to other findings that a decrease in cell wall outer protein OmpF could contribute to high resistance to antibiotics.

Keywords: Antibiotic resistance, Outer membrain protein, OmpF, OmpC, SDS-PAGE

Introduction

Antibiotic translocation across membranes of Gram-negative bacteria is a key step for its activity on specific intracellular targets [1]. This translocation across membranes is controlled by an outer membrane (OM), which consists of the carbohydrate (lipopolysaccharide) in its outer leaflet, phospholipids in its inner leaflet and proteins (lipoproteins also called porins or outer membrane proteins (OMPs)) moiety [2]. The OM serves as first line protective structure against osmotic pressure, external stress, environmentally induced toxins and antibiotic resistance [3]. The OMPs are characterized by β-barrel structure and they form water-filled channels for the passage of a large variety of hydrophilic molecules [4]. A modification of membrane permeability could induce resistance to small hydrophilic antibiotics such as β-lactams and fluoroquinolones resulting in the closure of the general outer membrane porins and limited intracellular accesses of antibiotic [1].

OMs are mainly prominent in Gram negative peptidoglycan like *E. coli* compared to Gram positive bacteria [5]. The pathogenicity of the OM is linked to its lipid A and immunogenic polysaccharide that aggravate immunoresponse as they form endotoxins if found in blood vessels, in which patient infected with such, develop high temperature, increased respiration rate and a low blood pressure [6]. This OM has been highlighted as one of the major mechanism of multiple antibiotics resistance as bacteria intrinsically possess it [7]. Antibiotics resistance associated with OM have been shown to develop due to pre-treatment of the organism with low doses of antibiotics, and subsequent development of cross resistance to a broad range of unrelated antibiotic [8]. Some other studies also showed that growth of bacteria in the presence of sub-lethal doses of some antibiotics could protect the bacterial cells against a broad range of other unrelated antibiotics (tetracycline, ampicillin, streptomycin and kanamycin), organic solvents and biocides [9-11]. However, this mechanism develops at random in strains that are multidrug resistant [12] and mutation or loss of the genes (*OprD2, nalB, nfxB, and nfxC*) encoding an outer membrane protein could be the causative agent in carbapenem-resistance *P. aeruginosa* [13]. In *E. coli*, two known porins (OmpC and OmpF) of about 10^5 copies per cell which are mainly regulated by OmpR (cytosolic response regulator) and EnvZ (membrane-bound sensor kinase) regulatory system are the most predominant OMPs, and they constitute the major channel of entry of small molecules [14].

OmpC and OmpF consist of three 16-stranded β-barrels, each of which forms a channel that is restricted in the middle due to the inward folding of a loop (loop L3) [15]. It has been reported that high nutrition presence (e.g. as found in mammal intestinal tracts) encourages the expression of OmpC, which has a smaller channel than OmpF, thus limiting the influx of large and charged molecules such as bile salts and antibiotics, while low osmotic pressure encourages the activity of OmpF [1]. However, this OMPs have been reported to work in collaboration with *tolC* OMP which is a multifunctional protein involved in efflux of wide range of xenobiotics including antibiotics, biocides, toxins, bile salts and organic solvents [16], this makes the emergence of MDR phenotypes of clinical isolates and increases the virulence of pathogenic species [1]. This study therefore compares the

molecular weight of OmpC and OmpF in MDR *E. coli* from UTI and diarrhoeic patients in Zaria with that of ATCC29522 which is sensitive to the antibiotics tested and also detect the presence of *tolC* Omp, since these outer membranes are involved in non-specific solute transport and antibiotics resistance.

Primer name	Sequence (5′→3′)	PCR product size (bp)	Annealing temp (°C)	References
tolC-F	AAGCCGAAAAACGCAACCT	100	51	Michelle et al., (2011)
tolC-R	CAGAGTCGGTAAGTGACCATC			

Table 1: PCR amplification of *tolC* gene.

Hospitals	Incidence			
	Diarrhoeic Confirmed *E. coli*	Hyper MDR *E. coli* (%)	UTI Confirmed *E. coli*	Hyper MDR *E. coli* (%)
ABUTH	11	4 (36.4)	21	7(33.3)
ABUSB	7	1(14.3)	15	5(33.3)
SLAH	8	0 (0)	9	1 (11.1)
HGSGH	6	1 (16.7)	9	2 (22.2)
Total	32	6 (18.8)	54	15(27.8)

Keys: ABUTH: Ahmadu Bello University Teaching Hospital Shika; ABUSB: Ahmadu Bello University Sick Bay; SLAH: St. Luke Anglican Hospital Wusasa; HGSGH: Hajiya Gambo Sawaba General Hospital, Kofan-Gayan

Table 2: Occurrence of presumed hyper multidrug resistant *E. coli* isolates among UTI and diarrheic patients in Zaria, Nigeria.

Methodology

Ethical approval, study area, isolates collection and processing

Ethical clearance with the number ABUTH/HRECG04/2013 was obtained from the ethical committee of Ahmadu Bello University Teaching Hospital Shika (ABUTH) for sample collection. This study was carried out using four hospitals within Zaria metropolis, which Ahmadu Bello University Teaching Hospital Shika, St. Luke Anglican Hospital Wusasa, Gambo Sawaba General Hospital Kofan-Gaya, and Ahmadu Bello University Clinic (Sickbay), Main Campus Samaru. Presumptive non-duplicated hyper multidrug resistant *E. coli* isolates (resistant to 8 and above antibiotics) from urine and stool samples submitted to the Medical Microbiology Laboratory units of the selected health facilities/hospitals were collected over a period of 6 months (April-September, 2014).

Purification, confirmation and antibiotics susceptibility test

The isolates were sub-cultured onto Eosin methylene blue agar and incubated at 37°C for 24 h. Colonies that showed characteristic green metallic sheen were further analysed by Microgene GNA test kit. Antibiotic susceptibility pattern of the isolates were determined using disc diffusion method according to Cheesbrough [17] and CLSI [18].

Molecular analysis

Bacteria cell preparation: The preparation of the bacteria cells were carried out using the method described by Dubey [19]. Chemical ingredients of Luria and Bertani broth media were prepared and single colonies were picked from freshly streaked isolates on eosin methylene blue plate and inoculated into 5 ml Luria and Bertani (LB) broth medium and incubated overnight at 37°C for 18-24 h. Bacterial cells were then harvested by centrifugation at 4°C, 8000 rpm (6800 xg) in a refrigerated microcentrifuge for 30 seconds in an Eppendorff's tube. The supernatants, which contain only the LB broth were decanted and cell pellets were harvested.

Genomic DNA extraction and polymerase chain reaction: Genomic DNA extraction to detect the presence of *tolC* gene with 100 base pair (Table 1) was carried out using the method described by Zymo Research mini prep fungi/bacteria extraction protocol. Amplification of resistant DNA fragments was 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 [20].

Cell wall extraction and evaluation using sodium dodecyl sulphate: Cell wall extraction was carried out using the method described by Shu et al. [21]. The MDR *E. coli* isolates were cultured in a 5 ml nutrient broth for 24 h at 37°C using a static incubator, and using a spectrophotometer, 1 optical density of cell turbidity was measured for all the cultured isolates at 600 nm. The bacteria cells were harvested by centrifugation at 3000 xg for 20 min at 4°C, the supernatant was discarded carefully, and the last few drops of liquid removed with a micropipette. The cell pellets were re-suspended gently in 1 mL of Tris-sucrose-EDTA (TSE) buffer (200 mM Tris-HCl, pH 8, 500 mM sucrose, 1 mM EDTA) using a wire loop and incubated on ice for 30 min. After this the digested aliquot was transferred into a 1.5 ml microcentrifuge tube and centrifuged at 16000 Xg for 30 min at 4°C. The supernatant which constitute the cell envelope extract was further transferred into a new microcentrifuge tube using a micropipette. The cell envelope was visualized on Model 45-2020 PEGLAB Biotechnologie GmbH machine using one-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli [22] method on a vertical slab gel (Mini-PROTEAN II Electrophoresis Cell). This was done to separate the protein bands using a 205 kDa ladder (Sigma Chemical Co.) as control marker to estimate the molecular weight of the protein subunits: A 12% gradient separating (acrylamide/bisacrylamide) gel and a 4% (w/v) stacking gel containing 0.1% SDS was used to form a gel slab in which the electrophoresis was run. Fifty microliters aliquots (50 µl) of each sample was applied onto each of the 15 gel wells and run at constant voltage of 200 V for 8 h until the tracking bromophenol blue dye migrated to the bottom of the gel. At the end of the run, the gels were stained with Coomasie brilliant blue R250 in methanol/water/acetic acid (4:5:1 v/v/v) and destained with methanol/water/acetic acid (4:5:1 v/v/v) over night and then vitualized under UV light.

Results

A total of 132 presumed *E. coli* isolates were obtained from the various hospitals sampled, of which 86 isolates were confirmed as *E. coli* using Microgen GNA kit. Out of the confirmed *E. coli* isolates 21 of the isolates were observed to be hyper resistant to the antibiotics tested. The distribution of the isolates is given in Table 2.

The isolates were 100% resistant to amoxicillin, ceftaxime, cefpodoxime, ciprofloxacin and ofloxacin, 95.2% resistant to cefpirome, 85.7% resistant to tetracycline and sulphamethonidazole-trimethroprim, 76.2% resistant to gentamicin, 66.7% to chloramphenicol, 61.9% to aztreonam, 57.1% to ceftriaxone. The isolates were 100% susceptible to imipenem and amikacin but 81.4% susceptible to nitrofurantoin (Table 3).

S. No.	Isolates	Antibiotics Resistance	NART	CART	NCART	LR
1	THU1	OFX, ATM, CN,CIP, CPD, CRO, CPO, CTX, SXT, C, AML, TE	12	FLU, MON, AMIN, CEPH, MISC, PEN, TE	7	MDR
2	THU2	OFX, CIP, CPD, CPO, CTX, SXT, C, AML	8	FLU, CEPH, MISC, PEN	4	MDR
3	THU10	OFX, ATM, CN, CIP, CRO, CPD, CPO, CTX, SXT,F, AML, TE	12	FLU, AMIN, MON, CEPH, MISC, PEN, TE	7	MDR
4	THU13	OFX, ATM, CIP, CRO, CPD, CPO, CTX, C, AML, TE	10	FLU, MON, CEPH, MISC, PEN, TE	6	MDR
5	THU19	OFX, ATM, CIP, CRO, CPD, CPO, CTX, SXT, AML, F, TE	11	FLU, AMIN, CEPH, MISC, PEN, TE	6	MDR
6	THU25	OFX, ATM, CN, CIP, CRO, CPD, CPO, CTX, SXT, C, AML, F, TE	13	FLU, MON, AMIN, CEPH, MISC, PEN, TE	7	MDR
7	THU27	OFX, CN, CIP, CRO, CPD, CPO, CTX, SXT, AML, F, TE	11	FLU, AMIN, CEPH, MISC, PEN, TE	6	MDR
8	THS2	OFX, ATM, CIP, CRO, CPD, CPO, CTX, C, AML	9	FLU, MON, CEPH, MISC, PEN	5	MDR
9	THS8	CN, OFX, ATM, CIP, CRO, CPD, CPO, CTX, SXT, C, AML, TE	12	FLU, MON, AMIN, CEPH, MISC, PEN, TE	7	MDR
10	THS12	CN, OFX, ATM, CIP, CRO, CPD, CPO, CTX, SXT, C, AML, TE	12	FLU, MON, AMIN, CEPH, MISC, PEN, TE	7	MDR
11	THS15	CN, OFX, CIP, CPD, CPO, CTX, SXT, AML, TE	9	FLU, AMIN, CEPH, MISC, PEN, TE	6	MDR
12	SBS1	CN, OFX, CIP, CPD, CPO, CTX, SXT, C, AML, TE	10	FLU, AMIN, CEPH, MISC, PEN, TE	6	MDR
13	SBU2	CN, OFX, CIP, CPD, CPO, CTX, SXT, AML, TE	9	FLU, AMIN, CEPH, MISC, PEN, TE	6	MDR
14	SBU12	CN, ATM, OFX, CIP, CRO, CPD, CPO, CTX, SXT, C, AML, F, TE	13	FLU, MON, AMIN, CEPH, MISC, PEN, TE	7	MDR
15	SBU13	CN, ATM, OFX, CIP, CRO, CPD, CPO, CTX, SXT, C, AML, TE	12	FLU, MON, AMIN, CEPH, MISC, PEN, TE	7	MDR
16	SBU15	CN, OFX, CIP, CPD, CPO, CTX, SXT, C, AML, TE	10	FLU, AMIN, CEPH, MISC, PEN, TE	6	MDR
17	SBU16	CN, ATM, OFX, CIP, CPD, CPO, CTX, SXT, C, AML, TE	11	FLU, MON, AMIN, CEPH, MISC, PEN, TE	7	MDR
18	SLU10	OFX, CIP, CPD, CPO, CTX, SXT, C, AML	8	FLU, CEPH, MISC, PEN	4	MDR
19	HGS5	CN, ATM, OFX, CIP, F, CPD, CPO, CTX, SXT, AML, TE	11	FLU, MON, AMIN, CEPH, MISC, PEN, TE	7	MDR
20	HGU1	CN, OFX, CIP, CPD, CPO, CTX, SXT, AML, TE	9	FLU, AMIN, CEPH, MISC, PEN, TE	6	MDR
21	HGU16	CN, ATM, OFX, CIP, CRO, CPD, CTX, C, AML, TE	10	FLU, MON, AMIN, CEPH, MISC, PEN, TE	7	MDR

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: Ceftriaxon; CPO: Cefpirome; CTX: Ceftaxime; SXT: Sulphamethonidazole-Trimethroprim; C: Chloramphenicol; IPM: Imipenem; AML: Amoxicillin; MDR: Multidrug-resistant; NART: Number of Antibiotics Resistant; CART: Class of Antibiotics Resistant; NCART: Number of Classes of Antibiotics Resistant to; LR: Level of Resistance.

Table 3: Antibiotics susceptibility profile of hyper multidrug resistance *E. coli* from UTI and diarrhoeic patients in Zaria, Nigeria.

Detection of *tolC* gene using polymerase chain reaction

The detection of *tolC* gene after the Polymerase Chain Reaction was done by using the gel electrophoresis. Figure 1 shows the bands of *tolC* gene amplified from *E. coli* isolates.

Evaluation of OmpF and OmpC in outer membrane extracts of MDR ESBL producing *E. coli u*sing SDS-PAGE

Extended spectrum betalactamase (ESBL) are betalactam inactivating enzymens, which also confers resistance to other nonstructurally related antibiotics, encouraging a wide resistance profile of the inherent bacteria. This study evaluated a variation in the outer membrane protein of multidrug resistant (MDR) E. coli such as the ESBL encoding E.coli, to substaintiate if a variation in size correlates with resistance property (membrain permiability). The outer membrane proteins study revealed that there is no difference in the size of the OmpC outer membrane protein in all the MDR ESBL producing *E. coli* isolates and that of the ATCC 25922 used as control but the OmpF and OmpA varied significantly from one another (Figure 2).

Discussion

This study showed the possibility of isolating hyper antibiotic resistant isolates among UTI and diarrhoeaic patients in Zaria, Nigeria and still showed that imipenem, amikacin and nitrofurantoin are still effective for the treatment of this superbug *E. coli* strains. This high

resistance might be linked to increased irrational consumption of antibiotics and transmission of resistant isolates between people [23], which could contribute significantly to increased mortality and morbidity among the populace. *tolC* efflux pumps gene, which has been reported to extrusion toxic substrates (including virtually all classes of clinically relevant antibiotics) from within cells into the external environment [24] were observed in all the hyper MDR *E. coli* isolates evaluated. This might contribute to the observed environmentally induced and adaptive resistance without observable changes in the genotype in the *E. coli* isolates as this gene in collaboration with OmpC and OmpF have been reported to cause a reduction in cell envelop permeability, which induces the formation of capsule or regulation in the cell envelop [25,26].

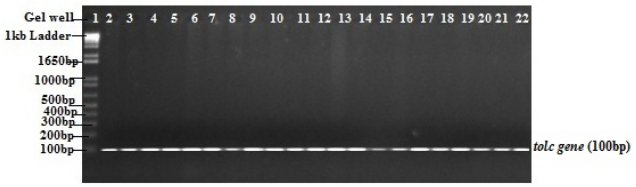

Figure 1: Electrophoretic gel of *tolC* genes amplified from *E. coli* isolates (Lane 1: 1kb DNA Ladder; Lane 2: THU1; Lane 3: THU;Lane 4: THU10; Lane 5: THU13; Lane 6: THU19;Lane 7: THU25; Lane 8: THU27; Lane 9: THS2; Lane 10: THS8; Lane 11: THS12; Lane 12: THS15; Lane 13: SBS1; Lane 14: SBU2; Lane 15: SBU12; Lane 16: SBU13; Lane 17: SBU15; Lane 18: SBU16; Lane 19: SLU10; Lane 20: HGS5; Lane 21: HGU1; Lane 22: HGU16).

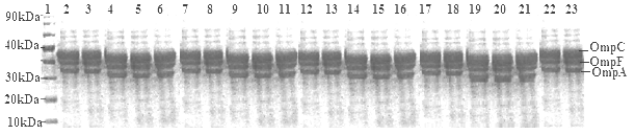

Figure 2: OmpF (37kDa) and OmpC (38kDa) in outer membrane protein of MDR ESBL Producing E. coli. (Lane 1: 1kDa protein ladder; Lane 2: THU1; Lane 3: THU2; Lane 4: THU10; Lane 5: THU13; Lane 6: THU19; Lane 7: THU25; Lane 9: THS2; Lane 10: THS8; Lane11: THS12; Lane 12: THS15; Lane 13: SBS1; Lane 14: SBU2; Lane 15: SBU12; Lane 17: SBU15; Lane 18: SBU16; Lane 19: SLU10; Lane 20: HGS5; Lane 21: HGU1; Lane 22: HGU16; Lane 23: ATCC25922).

Adaptive resistance/mutation on the OmpC and OmpF are known to cause a non-specific solute transport out of the cell in a process that does not involve the alteration or degradation of the drugs [27]. On comparing the expression of OmpC and OmpF in the outer membrane protein of the MDR *E. coli* using SDS-PAGE with a sensitive ATCC 25922 typed culture, the result showed that there were no significant difference between the size of outer membrane protein (OmpC) in all the MDR *E. coli* isolates and that of the ATCC 25922 used as control while the OmpF and OmpA varied significantly from one another.

The identification of OmpA and OmpF was based on their relative hydropathy, as more hydropathic proteins are expected to migrate at a faster rate, from least to most hydropathic (OmpC, OmpF, and OmpA) [11]. This study concurs with the study of Kim et al., [28] and Olga et al., [8] who reported that, a decrease in OmpF influenced an increase in resistance to various antibiotics. Also, the finding of Hany et al., [29] who reported that there is possibility of total cellular protein contents of *Pseudomonas aeruginosa* [30] to decrease after antibiotic treatment while its outer membrane protein (OmpC) contents remain approximately constant for both tetracycline and ciprofloxacin treated and untreated cells.

Brenda et al. [31] also reported that there were no detectable consistent effects of antibiotic or temperature on outer membrane protein (OmpC) expression for either species of *Mannheimia haemolytica* and *Haemophilus somnus* treated with chlortetracycline (CTC) and chlortetracycline-sulfamethazine. However, Cloete [32] had reported that OMPs play an important role in antibiotic resistance, in which, hydrophilic antibacterial agents e.g. aminoglycosides could be prevented from entering through the outer membrane by the effect of the Omp lipopolysaccharide layer and its phospholipids. Also hydrophobic agents could be excluded by outer membrane protein while hyper-susceptibility to antibiotics might occur when the lipopolysaccharide of Omp is altered or constant. Further study has also showed that OmpC has a smaller channel size relative to OmpF, increase in OmpC will exclude passage of larger hydrophilic antibiotics capable of fitting thorough OmpF but not OmpC [11].

Conclusion

This study isolated *E. coli* which are resistant to commonly prescribed antibiotics within Zaria, Nigeria. It identified that *tolC* gene was present in all the superbugs evaluated and could be the major efflux pump genes responsible for this MDR characteristic observed this isolates. Also this study noted changes in cell envelope in the MDR isolates, which may also play significant roles in limiting permeability of antibiotics into the drugs target sites. Therefore, immediate and holistic empirical surveillance, high hygiene practices, stoppage of over the counter drugs and use of antibiotics in poultry is recommended to combat the rising trend and spread in antibiotics resistant properties.

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