PRESENCE OF EFFLUX PUMP MEDIATED ANTIBIOTIC RESISTANCE IN GRAM NEGATIVE BACTERIA ISOLATED FROM PRIMARY SCHOOL PUPILS IN AGO-IWOYE, OGUN STATE, NIGERIA

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ABSTRACT

This study determined the prevalence of efflux pump mediated antibiotic resistance in Gram negative bacteria from primary school pupils in Ago-Iwoye, Ogun State, Nigeria due to the global concerns of such mechanism in antibiotic extrusion. The isolation, characterization and identification of the isolated organisms were performed using standard techniques while the susceptibility patterns of the organisms was carried out following the recommended procedure of NCCLS. The resistant isolates were screened for active efflux pump system in the presence of an efflux pump inhibitor (EPI) (CCCP) and interpreted following standard procedure. Results obtained displays the prevalence rate of the isolated organisms in the following increasing order respectively: *Serratia marcescens* (3.4%), *Enterobacter aerogenes* (5.1%), *Proteus mirabilis* (6.7%), *Klebsiella pneumoniae* (16.9%), *Pseudomonas aeruginosa* (18.5%) and *Escherichia coli* (49.4%) for male subject while that of female subject were *Serratia marcescens* (3.1%), *Enterobacter aerogenes* (4.6%), *Proteus mirabilis* (7.2%), *Klebsiella pneumoniae* (17.0%), *Pseudomonas aeruginosa* (18.6%) and *Escherichia coli* (49.4%). The frequency of the isolated organisms differs according to gender but the variation was found not to be statistically significant (P>0.05). *Pseudomonas aeruginosa, Proteus mirabilis* and *Escherichia coli* demonstrated 100% efflux mediated resistance to all the tested antibiotics. In conclusion, Gram negative bacteria isolated in this study engineered their resistance mostly through efflux pump system.

Keywords: Carbonyl Cyanide m-Chlorophenyl hydrazone (CCCP); Efflux Pump System; (EPS); Gram Negative Bacteria.

INTRODUCTION

Bacterial antibiotic resistance is a growing global concerns adversely affecting health care delivery in both developing and developed countries including Nigeria. This resistance is primarily caused by antibiotic misuse and abuse which could subsequently lead to therapy failure (CDCP, 2013; Read and Wood, 2014). It is not surprising that antibiotic resistance is not an exception for any of the antibiotic classes because several authors have linked bacterial antibiotic resistance to several mechanisms with bacteria having specific mechanism of resistance for each of the antibiotic classes (Livermore, 2004). Among the Gram negative bacteria, efflux pump mediated resistance has been reported as one of the commonest method of bacterial engineered resistance (Nikaido and Takatsuka, 2009; Nikaido, 2009) and this type of resistance is made possible through some transport proteins that function to extrude antibiotic and other toxic compounds from within the cells into the external environment (Webber and Piddock, 2003; Higgins, 2007).

The transport protein called the efflux pump has been reported in both prokaryotes and eukaryotes (Bambeke *et al.*, 2000; Lubelski *et al.*, 2007) and evidences from various research studies connote an alarming rate of resistance in Gram-negative bacteria when compared to Gram-positive bacteria. In Nigeria, several reports on antibiotic resistance has been established (Okeke *et al.*, 2000) and some reports on efflux mediated resistance in different foods has also been reported but there is paucity of information on the prevalence of such resistance in primary school pupils in Ago-Iwoye, Ogun State, Nigeria. The primary school pupils in Nigeria especially the public primary schools are majorly populated by children of the poor with little and/ no access to standard health care delivery, good food and probably debilitated immune system because they can easily be classified as the low socio-economic class. This study was therefore aimed at targeting those vulnerable classes of pupil in order to use the information obtained to advice the society appropriately.

MATERIALS AND METHODS

Study population
Urine samples were collected from a total of three hundred and seventy-two (372) primary school children in Ijebu North Local Government Area, Nigeria during the period of September 2012 - March 2013. In the collection of urine from students, the following exclusion criterion included those pupils who have been on antibiotics treatment while the opposite was the case for the inclusion criteria.

Ethical clearance
Verbal consent of the school principal, teachers, parents and guardians of the pupils were obtained after holding an interactive sessions, informing them about the purpose of the study prior to the commencement of the study. Permission was also sought from the Ogun State Ministry of Health through the Ijebu North Local Government Area, Ogun State, Nigeria.

Sample collection, transportation and Microbiological Analysis
Clean mid-stream urine specimens were obtained from primary school children in sterile universal bottles and transported to the laboratory immediately on ice pack bags for urinalysis. Each of the urine samples was ten-fold serially diluted, plated, enumerated and characterized using standard biochemical tests as described by Fawole and Oso (2001).

Bioassay (Agar and broth dilution)
Antimicrobial sensitivity testing of all isolates were performed on Mueller Hinton Agar by the Kirby Bauer disk diffusion method following the guideline of the Clinical and Laboratory Standards Institute (CLSI, 2005). All inoculums were standardized using the McFarland nephelometer method. Minimum Inhibitory Concentration (MIC) of tetracycline, augmentin, ofloxacin, gentamicin, nalixidic acid, nitrofurantoin, cotrimoxazole and amoxicillin against the gram negative isolates were determined using the two-fold serial broth (Mueller –Hinton Broth) dilution method with an inoculum of 1×10^6 CFU/mL. The MIC was taken as the lowest concentration inhibiting visible growth after 18 h incubations at 37 °C.

Screening of efflux pump system in the resistant strains of bacteria
The presence of efflux pump system was investigated in the most resistant strain of Gram negative organisms by the incorporation of carbonyl cyanide m-chlorophenyl hydrazone into minimum inhibitory concentration (MIC) media of all the antibiotics. The presence of efflux pump system was based on the reduction of minimum inhibitory concentration (MIC) value of the tested antibiotics (Okeke *et al.*, 2000).

Statistical analysis
The data obtained from this study was analyzed using frequency distribution in percentages.

Results
Tables 1 and 2 shows the prevalence of Gram negative uropathogens in the examined urine samples of both male and female subjects. In all the 372 pupils, *Escherichia coli* was found to have a very high proportion of 88(49.4%) in male while that of female was 96(49.4%). *Serratia marcescens* has the lowest proportion of 6(3.4%) of all the male samples examined. while of female was 6(3.1%) of all the samples examined.

The antibiotic resistant pattern of Gram negative uropathogens is shown in table 3. It shows that *Pseudomonas aeruginosa, Proteus mirabilis* and *Escherichia coli* had an alarming trend of multi

drug resistant attributes. These bacterial isolates displayed total resistance to all the tested antibiotics. *Klebsiella spp* was however found to be highly sensitive to all the tested antibiotics except tetracycline with 100% resistance. The resistant pattern of *Serratia marcescens* varies between 25% for Gentamicin through 50% for Ofloxacin, Nalixidic acid and Cotrimoxazole to total resistance to Nitrofuratoin and Amoxicillin. However, this isolate was found to be sensitive to Tetracycline and Augmentin. Consequently, 50% of *Enterobacter species* strains investigated for susceptibility to the tested antibiotics were found to be resistant to Ofloxacin, Gentamicin and Nalixidic acid while the whole strains were sensitive to Tetracycline, Augmentin, Nitrofuratoin, Cotrimoxazole and Amoxicillin.

Table 4 shows the Minimum Inhibitory Concentrations of some antibiotics in relation to Gram negative Bacteria. The Minimum Inhibitory Concentration of Tetracycline with the Gram negative Bacteria was found to be above 250µg/ml for all the isolates which indicated that Tetracycline possessed no inhibitory effect on the tested organisms above these concentrations. However, Augmentin, Ofloxacin, Nalixidic acid, Nitrofuratoin and Amoxycillin had their MIC with *Klebsiella species* to be 62.5µg/ml while these antibiotics possessed MIC greater than 250µg/ml

for *Pseudomonas aeruginosa*, *Proteus mirabilis* and *Escherichia coli* except for Ofloxacin which had a definite MIC with *Pseudomonas aeruginosa* (125µg/ml). Also, Gentamicin and Cotrimoxazole possessed MIC of 31.3µg/ml with *Klebsiella species*.

Table 5 shows the Minimum Inhibitory Concentration of the tested antibiotics in the presence of carbonyl cyanide m-chlorophenyl hydrazone (CCCP) with Gram negative Organisms. The presence of efflux pump system was investigated in the most resistant strain of the Gram negative organisms tested. They engineered their resistance to Tetracycline, Ofloxacin, Gentamicin, and Nalixidic acid by the pumping out of these *Pseudomonas aeruginosa* antibiotics from their cells. This is shown in the table above, as the incorporation of carbonyl cyanide m-chlorophenyl hydrazone (CCCP) into the MIC medium led to the reduction in the MIC Value. However, this is not so for Augmentin, Nitrofuratoin, Cotrimoxazole and Amoxicillin. This same observation was found to be the same for *Proteus mirabilis* and *Escherichia coli*. The strains of *Klebsiella species* investigated in this study, were mostly found to engineer their resistance via other means apart from the use of efflux pump system except with Ofloxacin and Tetracycline.

Tables 1. Prevalence of Gram negative uropathogens in male subjects relative to age in Ijebu North, Ogun State, Nigeria.

Organism	Age group			Total(N)	%
	2-6	7-11	12-16		
Pseudomonas aeruginosa	16	14	3	33	18.5
Proteus mirabilis	6	4	2	12	6.7
Escherichia coli	43	26	19	88	49.4
Klebsiella pneumoniae	15	9	6	30	16.9
Serratia marscencen	4	2	0	6	3.4
Enterobacter aerogenes	5	2	2	9	5.1
Prevalence	89	57	32	178	100

Tables 2. Prevalence of Gram Negative Uropathogens in female subjects relative to age in Ijebu North, Ogun State, Nigeria.

Organism	Age group			Total(N)	%
	2-6	7-11	12-16		
Pseudomonas aeruginosa	18	15	3	36	18.6
Proteus mirabilis	8	5	1	14	7.2
Escherichia coli	50	26	20	96	49.4
Klebsiella pneumoniae	17	9	7	33	17.0
Serratia marscencen	3	2	1	6	3.1
Enterobacter aerogenes	6	2	1	9	4.6
Prevalence	102	59	33	194	100

Table 3. Antibiotic resistant pattern of Gram negative Uropathogens isolated from primary school pupils in Ijebu North, Ogun State, Nigeria.

ORGANISMS	ANTIBIOTICS								
	n	TET	AUG	OFL	GEN	NAL	NIT	COT	AMX
Pseudomonas aeroginosa	69	69(100)	69(100)	69(100)	69(100)	69(100)	69(100)	69(100)	69(100)
Proteus mirabilis	26	26(100)	26(100)	26(100)	26(100)	26(100)	26(100)	26(100)	26(100)
Escherichia coli	184	184(100)	184(100)	184 (100)	184(100)	184 (100)	184(100)	184 (100)	184 (100)
Klebsiella species	63	63(100)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
Serratia marcescens	12	0(0)	0(0)	6(50)	3(25)	6(50)	12(100)	6(50)	12(100)
Enterobacter specie	18	0(0)	0(0)	9(50)	9(50)	9(50)	0(0)	0(0)	0(0)

Table 4. Minimum inhibitory concentrations of some antibiotics against the Gram negative bacteria.

Bacterial isolates	MIC (µg/ml)							
	TET	AUG	OFL	GEN	NAL	NIT	COT	AMX
Pseudomonas aeruginosa	-	-	125	-	-	-	-	-
Proteus mirabilis	-	-	-	-	-	-	-	-
Escherichia coli	-	-	-	-	-	-	-	-
Klebsiella pneumoniae	-	62.5	62.5	31.3	62.5	62.5	31.3	62.5
Uninoculated broth	-	-	-	-	-	-	-	-
Inoculated broth without antibiotic	+	+	+	+	+	+	+	+

Table 5. Minimum inhibitory concentration of the tested antibiotics in the presence of Carbonyl Cyanide m-chlorophenyl Hydrazone (CCCP) against Gram negative organisms

Antibiotics	Organism / MIC (µg/l)			
	PA	PM	EC	KP
Tetracycline	62.5	125	125	62.5
Augmentin	-	-	-	-
Ofloxacin	31.3	62.5	62.5	31.3
Gentamicin	125	62.5	62.5	62.5
Nalixidic acid	62.5	62.5	62.5	62.5
Nitrofurantoin	-	-	-	62.5
Cotrimoxazole	-	-	-	125
Amoxycillin	-	-	-	62.5

Key: PA- *Pseudomonas aeroginosa*, PM- *Proteus mirabilis*, EC- *Escherichia coli*, KP- *Klebsiella pneumonia*

DISCUSSION AND CONCLUSION

This study was undertaken to determine the prevalence of Gram negative uropathogens in primary school pupils as well as to evaluate the presence of efflux pump system in these isolates. Of the species of bacteria recovered from the urine samples in this study, *Escherichia coli* was the most prevalent followed by *Pseudomonas aeruginosa*, *Klebsiella spp*, *Proteus mirabilis*, *Enterobacter spp* and *Serratia marcescens* which was in agreement with the report of Beyene and Tsegaye (2011). In this study, most of the gram negative uropathogen were resistant to almost all the antibiotic tested except for *Serratia marcescens* which had varying resistance and *Klebsiella spp* which was sensitive to the entire tested antibiotic. This is contrary to the report of Mamuye (2016). The ability of *Pseudomonas aeruginosa* to resist all the tested antibiotics may not be unconnected to it remarkable capacity to resist antibiotics, either intrinsically (because of constitutive expression of b-lactamases and efflux pumps, combined with low permeability of the outer-membrane) or following acquisition of resistance genes, over-expression of efflux pumps or decreased expression of porins (Bagge *et al.*, 2002). The multidrug resistance *Escherichia coli* observed in this study may have arisen in poultry from susceptible progenitors and transmitted to human via the food chain (Johnson *et al.*, 2007; Okonkwo *et al.*, 2010). In the present study, resistant rate to antimicrobial agents among uropathogenic *isolates* varied. It showed high resistant rates which are similar to many reports published by other works (Li and Nikaido, 2004; Poole, 2005). The result of the MIC of some antibiotics in relation to the Gram negative bacteria showed that tetracycline at 250µg/ml possessed no inhibitory effects on the tested organism. However, Augmentin, Nalixidic acid, Nitrofuratoin and Amoxycillin had their MIC with *Klebsiella species* to be 62.5 µg/ml while the MBC showed that all the tested antibiotics showed no cidal effect on all the Gram negative bacteria except for *Klebsiella species* which had its minimum cidal concentration at 125, 250, 250, 250, 250 (µg/ml) for Cotrimoxazole, Augmentin, Gentamicin, Nalidixic acid, Nitrofuration and Amoxicillin respectively. This observed resistance to these drugs is a probable indication of earlier exposure of the isolates to these drugs, which may have enhanced resistant development (Ehinmidu, 2003). Minimum Inhibitory Concentration of the tested antibiotics in the presence of carbonyl cyanide m-chlorophenyl hydrazone (CCCP) with Gram negative organisms showed that they engineered their resistance to Tetracycline, Ofloxacin, Gentamicin, and Nalixidic acid by the pumping out of these antibiotics from their cells while there was a varying degree of inhibition in other antibiotics .

Conclusively, reducing the prescription of a particular antibiotic and informing patients about appropriate drug use can decrease the resistance to various antibacterial agents of common uropathogens causing urinary tract infections in children. Also, research should focus on advancing on drugs that are less likely to be substrate for MDR efflux pumps.

REFERENCES

Awah, F. (2007). T test (2-tailed), analysis of variance (ANOVA) and Pearson's coefficient. The statistical package for social science (SPSS) for windows.

Bagge, N., Ciofu, O., Hentzer, M., Campbell, J. I., Givskov, M. & Hoiby, N. (2002). Constitutive high expression of chromosomal b lactamase in *Pseudomonas aeruginosa* caused by a new insertion sequence (IS1669) located in ampD. *Antimicrob Agents Chemother* 46, 3406–3411.

Bambeke, V. F., Balzi, E. & Tulkens, P. M. (2000). Antibiotic efflux pumps. *Biochemical Pharmacology* 60, 457–70.

Beyen, G. & Tsegaye, W. (2011). Bcterial uropathogens in urinary tract infection and antibiotic susceptibility pattern in Jamma university specialized hospital, South west Ethiopia. *Journal of Ethiop J Health Sci*. 21(2).

Centers for Disease Control and Prevention. (2013). Office of Infectious Disease Antibiotic resistance threats in the United States.

Clinical and Laboratory Standard Institute. (2005). Performance standards for antimicrobial disk susceptibility tests. NCCLS documents M 100 S15. Wayne, PA, USA: Clinical and Laboratory Standard Institute.

Ehinmidu, J. O. (2003). Antibiotics

susceptibility patterns of urine bacterial isolates in Zaria, Nigeria. *Tropical Journal of Pharmaceutical Research*; 2: 223-228.

Fawole, M.O. & Oso, B.A. (2001). Laboratory manual of microbiology. 5th edition. Ibadan:Spectrum Books Ltd.;15-22.

Higgins, C.F. (2007). Multiple molecular mechanisms for multidrug resistance transporters. *Nature*. ;446(7137):749–757.

Johnson, J.R., Sannes, M.R., Croy, C., Johnston, B., Clabots, C., Kuskowski, M.A., Bender, J., Smith, K.E., Winokur, P.L. & Belongia, E.A (2007). Antimicrobial Drug Resistant *Escherichia coli* from Humans and Poultry Products. *Emerging Infectious Disease*. 13(6): 838-846.

Li, X.Z. and Nikaido, H. (2004). Efflux-mediated drug resistance in bacteria. *Drugs*; 64(2):159–204.

Livermore, D.M. (2004). The need for new antibiotics. *Clin. Microbiol. Infect.* 10 (Suppl 4):1–9.

Lubelski, J., Konings, W.N. & Driessen, A.J. (2007). Distribution and physiology of ABC-type transporters contributing to multidrug resistance in bacteria. *Microbiol Mol Biol Rev*. ;71(3):463–76.

Mamuye, Y. (2016). Antibiotic resistance pattern of common Gram negative uropathogens in St. Paul Hospital millennium medical college. *Ethiop J Health Sci*. 26 (2).

Nikaido, H. & Takatsuka, Y. (2009). Mechanisms of RND multidrug efflux pumps. *Biochim Biophys Acta* .;1794(5):769–81.

Nikaido, H. (2009). Multidrug resistance in bacteria. *Ann Rev Biochem*.;78:119–46.

Okeke, I.N., Fayinka, S.T. & Lamikanra, A. (2000). Antibiotic resistance trends in Escherichia coli from apparently healthy Nigerian students (1986-1998). *Emerg Infect Dis*;6:393-396.

Okonkwo, I.O., Nkag, E.A., Fajobi, O.K., Mejeha, A.O., Udeze, B.O., Motayo, A.A., Ogun, T.A. & Ogunnusi, T.A. (2010). Incidence of Multi-Drug Resistant (MDR) Organisms in Some Poultry Feeds Sold In Calabar Metropolis, Nigeria. *Electronic Journal of Environmental, Agricultural and food chemistry*. 9(3): 514-532.

Read, A.F. & Woods, R.J. (2014). Antibiotic resistance management. *Evol Med Public Health*.; 2014(1):147.

Webber, M.A. & Piddock, L.J.V. (2003). The importance of efflux pumps in bacterial antibiotic resistance. *Journal of antimicrobial chemotherapy*.; 51,9-11.

RADIOLOGICAL IMPACT ASSESSMENT DUE TO NATURAL RADIONUCLIDES IN THE SOILS FROM ALABATA, SOUTHWESTERN NIGERIA

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ABSTRACT

The radioactivity levels of ^{40}K, ^{226}Ra and ^{232}Th and their corresponding gamma absorbed dose rate, effective doserate, and hazard indices were determined in soil samples from a soft-sand collection field at Alabata Abeokuta for building construction purposes. Soil samples were collected randomly from twenty locations in the area and prepared for spectrometric analysis. The activity concentrations of the natural radionuclides in the samples was measured using a single crystal 51mm x 51mm NaI (Tl) detector coupled to a multichannel analyser for spectrometric analysis. The activity concentrations of the radionuclides obtained were used to determine the radiological hazard indices. The mean activity concentrations of ^{40}K, ^{226}Ra and ^{232}Th in the soil samples were 829.1±260.6 Bqkg^{-1}, 61.7±21.7 Bqkg^{-1} and 50.1±16.6 Bqkg^{-1} respectively. The mean gamma absorbed dose rate was 71.45±15.58 nGyh^{-1} while the mean effective dose rate was 0.09±0.02 mSvy^{-1}. The mean radium equivalent, external hazard index, internal hazard index and gamma index were 197.2±36.8; 0.53±0.10, 0.70±0.14 and 0.73±0.14 respectively. The absorbed gamma and effective dose rates were 21.1% and 28.6% higher than the world average values of 59 nGyh^{-1} and 0.07 mSvy^{-1} respectively. The radiological hazard indices were lower than 1.0 world's recommended limit.

Keywords: Impact assessment, activity concentration, hazard indices, natural radionuclides, Alabata- Abeokuta

INTRODUCTION

Natural or artificial ionizing radiation is useful for medical, scientific and technological purposes but some health risk is associated with radiation exposure. The human environment is often subjected to radiation exposures originating from natural background sources comprising terrestrial and extra-terrestrial; and artificial or man-made sources (Shiva *et al.,* 2008).

The natural background radioactivity accounts for 96.1% of the total radiation dose to the world population while the man-made sources account for the remaining 3.9% (Jibiri *et al.,* 2009).

The terrestrial background radiations are mainly from the primordial radionuclides that include the decay series radionuclides (^{226}Ra and ^{232}Th) with their progenies and non-decay series radionuclide, ^{40}K,. Out of the total natural background radiation dose that the world population receives about 85% is from terrestrial sources (IAEA, 1996) and about 23% of the average annual dose to human from all radiation sources (NCRP, 1987).

The great global interest in the study and survey of naturally occurring radiation and environmental radioactivity had been essentially based on the importance of using the results from such studies for the assessment of public radiation exposure rates and the performance of epidemiological studies (UNSCEAR, 2000).

The understanding of the radionuclide inventory and factors that determine radionuclide availability and the health effects to members of the public will help both government and individual to device methods to protect themselves against radiation exposure (Vines and Beard, 2012).The presence of radionuclides in the soil resulting from rocks' weathering is one of the main sources of the natural radioactivity (Taskin *et. al.,* 2009). The level of natural radioactivity in the soil and surrounding environment in a region depends mainly on the