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Multi-antibiotics-resistance plasmid profile of enteric pathogens in pediatric patients from Nigeria

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Abstract

A total of 938 faecal samples of diarrheal stool of pediatric patients attending Madonna University Teaching Hospital (MUTH) from June 2003 to June 2004 were examined. 218 of eight different bacterial strains namely *Escherichia coli* 90(41.3%), *Shigella dysenteriae* 38(17.4%), *Pseudomonas aeruginosa* 20(9.2%), *Salmonella typhi* 18(8.3%), *Staphylococcus aureus* 7(3.2%), *Proteus mirabilis* 5(2.3%), *Enterococcus faecalis* 25(11.5%) and *Klebsiella pneumoniae* 15(6.9%) were isolated. The susceptibility pattern of the isolates to the various antibiotics varied with *Proteus mirabilis* and *Klebsiella pneumoniae* 100% sensitive to peflacine and *Enterococcus faecalis* 100% sensitive to ciprofloxacin and augmentin. Most of the isolates were least sensitive to cotrimoxazole, ampicillin, erythromycin gentamicin, streptomycin and chloramphenicol. The resistance plasmids to the various isolates were very diverse and distributive among the isolates. They were also highly transferable with a high frequency range of $2x10^{-2}$ to $6x10^{-4}$. Some of the isolates had plasmids bands that ranged from ≤0.55kbp to ≥1.14kbp. This indicates that plasmids allow the movement of genetic materials, including antimicrobial resistance genes between bacterial species and strains.

Keywords: Diarrheagenic pathogens, antibiotics resistance plasmids profile

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INTRODUCTION

Intestinal diseases of microbial origin are marked principally by diarrhea and sometimes by ulcero-inflammatory changes in the small or large intestine[13]. It is usually a symptom of gastroenteritis and can be accompanied by severe abdominal pain, urgency, perianal discomfort, and incontinence[13.] Diarrhea posses a very serious problem in developing countries where it is the leading cause of morbidity and mortality among children and adult. It ranks second only to respiratory diseases and is a major cause of morbidity among notifiable diseases in some part of the world[4]. An estimate of about 5 million children (more than 13,600 a day) dies from diarrheal diseases in Asia, Africa and South America. In the U.S, estimates exceed 10,000 deaths per year from diarrhea and an average of 500 childhood deaths are reported per day[22].

Epidemiological studies have also shown that diarrhea is responsible for more than 3.1 million deaths each year among children less than 15 years of age, mostly in developing countries10. It is estimated that U.S adults each year experience 99 million episodes of acute diarrhea. Although mortality due to acute diarrhea in children has decreased both in developed and developing countries in recent years after the introduction of oral dehydration solution. Those associated with persistent diarrhea occur in malnourished children and is usually disproportionately high, accounting for up to 45% of diarrhea deaths in Brazil, Bangladesh and in several African countries[9,22].

In Nigeria, the incidence of acute watery diarrhea is approximately 4.9 episodes per year and there are approximately 200,000 diarrhea related deaths of children aged below five years with an average of 300 deaths per day[8,10]. Ogunsanya *et al.*[19] found that 70-90% of children in Lagos from December 1989 to May 1990 were infected with enteropathogenic bacteria as compare to 28% of control group. According to Federal Ministry of Health,[8] the risk factors include malnutrition, poor personal hygiene, unhygienic food preparation, improper sewage disposal, environmental sanitation problems, inadequate water supply, over crowding. Also, people who visit foreign countries are at risk for traveler's diarrhea, caused by eating food or drinking water contaminated with bacteria, viruses or sometimes parasites[7,25].

Antimicrobial resistance on enteric pathogen is of great public health concerned in the developing world where the rate of diarrhea is highest. Researchers have also reported an increasingly widespread use of antibiotics in food and animals contributing to high dissemination of resistant enteric infections to humans. Most of these life-threatening enteric pathogens include *Escherichia coli, Salmonella, Shigella, Yersinia, Vibrio, Campylobacter* species *Entamoeba histolytica*, and *Giardia lamblia* in developing countries[6,5,22]. In the 1960's *Salmonella* species resistant to ampicillin, chloramphenicol and trimethoprim – sulfamathoxazole have been reported with increasing frequency through out the world. Strains of *Campylobacter jejuni* have been reported as resistant to ampicillin, carbenicillin, clindomycin, gentamicin streptomycin and metronidazole[24,26]. The present study was aimed at assessing the level of antibiotic resistance from diarrheal stool of pediatric children age less than 5 years attending Madonna University Teaching Hospital (MUTH) Elele, River State, Nigeria and also to determine the plasmids transferability of the antibiotic resistance markers among the enteric bacteria population. Plasmids have been found to confer drug resistance to their host bacteria[14,27]. Other studies have shown that they can be transfer from one bacterium to another by conjugation, transformation and by phage – mediated transudation[17,22,29,33].

MATERIAL AND METHODS

Samples were collected from 938 diarrheal stools of pediatric patients attending Madonna University Teaching Hospital (MUTH) Elele, River State, Nigeria age less than 5 years. The samples were kept in a clean, dry, disinfectant free container and were free from urine. The samples colleted were well labeled ready for analysis

The samples obtained were inoculated aerobically on sterile blood agar, MacConkey agar, eosin methylene blue, *Salmonella-Shigella* agar, nutrient agar and nutrient broth at 37^0C for 24 hours. The colonies of each representative isolates were then characterized using standard bacteriological method according to Cowan and Steel[3]. Other tests included gram stain, pigment production, hemolysin production, motility indole, urea, citrate and hydrogen sulfide utilization, oxidase, and sugar fermentation were used to isolate the enteric gram negative bacteria. They were further sub culture on nutrient agar slants and stored at 4^0C for further analysis.

Susceptibility Testing

Susceptibility were determined both by overnight broth-micro-dilution and agar disk diffusion methods as recommended by Bauer *et al.*[1] and National Committee for Clinical Laboratory Standard[18] using Oxoid- Mueller Hinton agar (Difco Laboratories, Detroit, Mich). The following antibiotics were used to screen for the resistance of the isolates; ciprofloxacin- CPX (10µg), Peflacine- PEF (10µg), Ofloxacin- OFX (10µg), augumentin-AU (30µg), gentamicin-GN (10µg), streptomycin- S (30µg), chloramphenicol- C (10µg), ampicillin-PN (30µg) and cotrimoxazole- SXT (5µg) (Optun Laboratories Nig Ltd., Nigeria). The zones of inhibition were then measured and the results recorded as sensitive (s) or resistance (R) base on World Health Organization Drug Information[28] and National Committee for Clinical Laboratory Standard[18].

Conjugation and Plasmids profiles

Conjugation experiments were performed using *E coli* strains obtained from Nigerian Institute for Medical Research (NIMR) as recipient as previously described by Olukoya and Oni[20], Yukata *et al.*[29] and Wang *et al.*[17]. The donors and recipients-plasmid -free - rifampicin resistant strains were incubated both on broth culture (nutrient broth 'E'-Antec Diagnostic Products, UK) and on Nutrient agar (nutrient agar-International Diagnostic Group UK) at 37^0C for 18 hours. The transconjugants were selected on nutrient agar medium supplemented with ampicillin 30µg/ml and rifampicin 200µg/ml to inhibit the growth of the donor and recipient respectively. The frequency of transfer of the plasmids was determined by dividing the number of transconjugants by the number of donor cells according to Wang *et al.*[17] and Yukata *et al.*[29]. Curing experiments were carried out according to Miller[16] and Olukoya & Oni[20]

The transconjugants were re-streaked onto fresh selective nutrient plates and their identities were re-confirmed on the basis of their biochemical methods and their antibiotics resistance confirmed. The Birnboim and Doly[2] and Gummic[11] methods were employed for screening plasmids (rapid alkaline extraction) of donors and transconjugants. The plasmids DNA were then electrophoresed on 0.8% agarose gel, stained with 14µl/g ethidium bromide. The DNA was then photographed with Polaroid camera and viewed using UV trans-illumination. The molecular weights and distances were then determined using standard methods according to Meyers *et al.*[15] and Birnboim and Doly[2] using standard DNA molecular weight marker II (0.12-23.1kbp) of bacteriophage lambda HindIII (Roche Diagnostic GmbH).

RESULTS

A total of 938 faecal samples of pediatric patients attending Madonna University Teaching Hospital (MUTH) from June 2003 to June 2004 were examined. Two hundred and eight (218) of eight different bacterial strains namely *Escherichia coli* 90(41.3%), *Shigella dysenteriae* 38(17.4%), *Salmonella typhi* 18(8.3%), *Pseudomonas aeruginosa 20*(9.2%), *Staphylococcus aureus* 7(3.2%), *Proteus mirabilis* 5(2.3%), *Enterococcus faecalis* 25(11.5%) and *Klebsiella pneumoniae* 15(6.9%) were isolated and identified as shown in Table 1. The susceptibility pattern of the isolate to the different antibiotics varied as shown in Table 2. *Proteus mirabilis* and *Klebsiella pneumoniae* were 100% sensitive to peflacine and *Enterococcus faecalis* was also 100% sensitive to ciprofloxacin and augumentin. Most of the isolates were least susceptible to cotrimoxazole; ampicillin, erythromycin, gentamicin, streptomycin and chloramphenicol.

Pseudomonas aeruginosa were the most resistant strains of the pathogens to the various antibiotics with a resistant range of 0.0% to 55% as shown in Table 1.

TABLE 1: Percentage (%) of Organisms Associated with Diarrhea Susceptible to Antimicrobial Agent

Organisms	Number of Isolates	Percentage of Organisms Susceptible To Antibiotics									
		OFX	PEF	CPX	AU	GN	S	SXT	PN	E	C
S dysenteriae	38(17.4%)	52.6	65.8	78.9	26.3	13.2	0.0	0.0	0.0	0.0	0.0
E faecalis	25(11.5%)	60.5	80.0	100	100	60	88	28	31.6	80	72
S aureus	07(3.2%)	28.6	71.4	85.7	14.3	0.0	28.6	0.0	0.0	0.0	42.9
P aeruginosa	20(9.2%)	50.0	35	55	10	0.0	0.0	0.0	0.0	15	10
P mirabilis	05(2.3%)	20.0	100	80	80	0.0	0.0	60	20	20	0.0
E coli	90(41.3%)	88.9	94.4	97.8	88.8	0.0	22.2	24.4	0.0	0.0	0.0
K. Pneumoniae	15(6.9%)	53.3	100	86.7	0.0	60	20	46.7	66.7	6.7	40
S typhi	18(8.3%)	83.3	83.3	77.8	55.6	44.4	16.7	22.2	5.6	11.1	55.6
Total	218(100%)										

KEY: OFX=Ofloxacin, PEF=peflacine, CPX=ciprofloxacin, AU=augmentin, GN=gentamicin, S=streptomycin, PN=ampicillin, E=erythromycin C=chloramphenicol, SXT=cotrimoxazole

Table 2: Antibiotic Resistance and Plasmid Profile of Isolates Obtained from Diarrhea Patients

Isolates	Antibiotics	No with Plasmids	Plasmids size (kbp)	Transferred Plasmids Size (kbp)	Frequency of Transfer
EC	CPX,S,GN,CO,C,E	3	1.14, 0.85, 0.55	1.14, 0.85	$6x10^{-4}$
1	CPX,AU,C,SXT,PN	2	1.08, 0.79	1.08, 0.79	$3x10^{-4}$
2	CPX,GN	0	-	-	-
3	PEF,CPX,SXT,PN	2	1.14, 0.85	1.14	$2x10^{-2}$
5	GN,SXT,PN,CO	2	1.14, 0.85	1.14,	$6x10^{-2}$
6	CO,GN,C,SXT,S	2	1.08, 0.79	1.08, 0.79	$3x10^{-1}$
9	CPX,GN,C	0	-	-	-
15	GN,CPX,E,PN,S	1	1.14	1.14	$6x10^{-2}$
20	GN,OFX,C,S,E	2	1.14, 0.85	0.87	$6x10^{-2}$
40	CO, PN, CPX,SXT	1	1.08	1.08	$2x10^{-2}$
18	PN, GN,S,E,SXT	2	1.08, 0.79	1.08	$3x10^{-4}$
56	SXT, C,S,PN,GN	3	1.08, 0.79, 0.55	0.79, 0.55	$6x10^{-2}$
21	CO, C,GN,PN,SXT,E	3	1.14, 1.08, 0.85	1.14, 0.85	$6x10^{-2}$
48	CO,PN,CPX,SXT,C	1	1.08	1.08	$6x10^{-2}$

KEY: EC=*E coli*, 1=*S dysenteriae*, 2=*E coli*, 3=*S dysenteriae*, 5=*E coli*, 6=*K pneumoniae*, 15= *E coli*, 20=*E coli*, *40=E coli*, 18 = *P aeruginosa*, 56 = *P mirabilis*, 21 = *S typhi*, 48 = *S typhi*

All the isolates were very sensitive to ofloxacin, pefloxacin and ciprofloxacin above 50% except *Pseudomonas aeruginosa* that had a percentage sensitivity of 35% to pefloxacin. The resistance plasmids to the various isolates were very diverse and distributive among the isolates as shown in Table 2. These plasmids were highly transferable with a high frequency range of $2x10^{-2}$ to $6x10^{-4}$. Some of the isolates had plasmids bands that ranged from ≤0.55kbp to ≥1.14kbp.

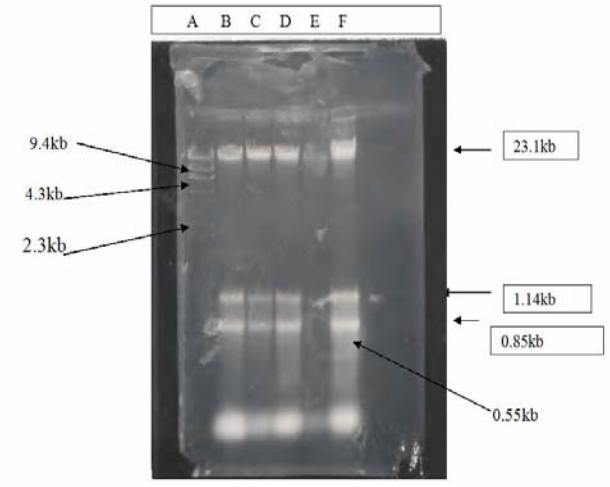

Fig1: Separation of DNA molecular weight on agarose gel stained with ethidium bromide. Line A = Standard bacteriophage Lambda DNA fragment, Lines B, C, D, E and F are DNA fragments of test isolates.

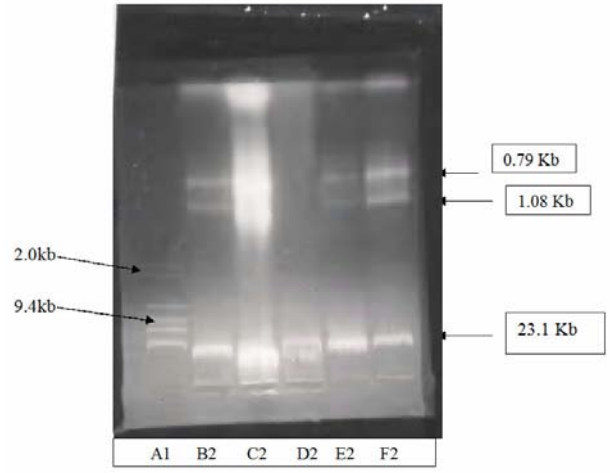

Fig. 2: Separation of DNA molecular weight on agarose gel stained with ethidium bromide. Line A2 = Standard bacteriophage Lambda DNA fragment, Lines B2, C2, D2, E2 and F2 are DNA fragments of test isolates.

Twenty-six percent of the isolates were plasmids mediated, 42% chromosome mediated while 32% of the pathogens were resistant to antibiotics could not be ascertained. *E coli* had the highest frequent plasmids distributive occurrence. The results indicate that the plasmids were able to move genetic antibiotics resistance materials among the various bacterial strains. Some of the isolates had plasmid bands that ranged from ≤0.55kbp to ≤1.14kbp (Figures 1 and 2)

DISCUSSION

This report describes the high rate of antimicrobial resistance among diarrheal stools isolates and their plasmids profile obtained from MUTH pediatric patients. The result indicated that diarrhea posses a very serious problem in developing countries where it is the leading cause of morbidity and mortality among children[19]. The prevalence of *Escherichia coli* 90(41.3%), *Shigella dysenteriae* 38(17.4%), *Salmonella typhi* 18(8.3%) in this study were slightly higher to those obtained by Olukoya and Olasupo[21] ten years ago. The prevalence of *Shigella* species isolated were also proportional to those obtained by Olukoya and Oni[20] and Tjaniadi[26]. The increase in prevalence observed in this study is probably due to lack of education and public awareness on hygienic conditions.

Diarrhea may be infective and non-infective but the fact remains that most of them are self-limiting and require adequate rehydration. In all doubtful cases, a stool examination should be done for ova; cyst, blood and hanging drop if cholera is suspected. Presence of leukocytes on blood- stains, usually suggests infection with *Salmonella, Shigella*, invasive *E. coli*, *Yersinia,* or *E. histolytica*.

Use of antimicrobial therapy in diarrheal diseases would include patients with high fever, bloody diarrhea, severe dehydration, systemic toxicity, and immunosuppressed patients, and outbreak of food poisoning. From the result the susceptibility pattern of the isolate to the various antibiotics varied seriously with *Proteus mirabilis* and *Klebsiella pneumoniae* 100% sensitive to peflacine, *Enterococcus faecalis* 100% sensitive to ciprofloxacin and augmentin. Most of the isolates were least sensitive to cotrimoxazole, ampicillin, erythromycin gentamicin, streptomycin and chloramphenicol.

The widespread resistance observed in the study could be due to the indiscriminate use and the over the counter availability of antibiotics as well as the higher exposure of people to enteric flora in places with poor hygienic conditions.

The survey also demonstrated that isolates from diarrhea samples in MUTH especially strains of *Escherichia coli*, *Shigella typhi, Pseudomonas aeruginosa, Proteus mirabilis* and strains of *Klebsiella pneumoniae* harbored conjugative plasmids which may confer resistance to some of these antibiotics. Most of the plasmids screened were in agreement with those obtained by Olukoya and Olasupo[21] who revealed that plasmid profiles differentiated specifically among *Shigella* isolates from Nigeria. The plasmids obtained in this work were smaller in sizes than those obtained by Olukoya and Olasupo[21]. These multiple copies of plasmid bands might have resulted from covalently close circular, open circular or linear forms of the same plasmid that might migrated at different rates on agarose gel electrophoresis. There was also a high degree of plasmids relatedness among the bacteria isolates from the various patients because of the presence of similar size plasmids especially 0.55, 0.79, 0.85, 1.08 and 1.14kbp. Traditionally, the role of antibiotics in antibiotic- induced antimicrobial resistance is to provide selective pressures to resistant clones. In 1990, Chakrabarty *et al*.[13] re-ported the detection of nucleic acids in various antibiotics and the capacity of such nucleic acids to transform bacteria to drug resistance. Subsequently, Webb and Davies[32] demonstrated that DNA encoding antibiotic resistance genes that are present in bacteria used in the production of antibiotics can be recovered in antibiotic preparations. It was proposed that anti-microbial resistance genes might be co-administered with antibiotics to humans or animals and taken up by bacteria in the hosts, contributing to the rapid development of antibiotic resistance[30]. The results also showed that conjugation was a very convenient method of transferring drug resistant genetic markers among intra and inter bacterial populations. This was shown by the high frequency of transfer among the isolates which was in accordance with the results of Wang *et al.*[17] and Yukata *et al.*[29.] This wide spread transfer of antibiotics resistant markers has failed to eradicate microbial infections of diarrhea origin despite their benefits. Although there have been few previous reports focusing on diarrheal diseases, the high frequency with which antibiotics are used empirically to treat diarrheal diseases suggest that there might also be high rate of failure associated with enteric infections[23].

The use of antimicrobial agents in the treatment of diarrhea diseases has greatly improved the quality of life among residents and travelers in developing countries. However, the problems associated with microbial resistance in diarrhea patients still pose a challenge to public health works[4,12].

This challenge can be minimized if governments and associated public health providers can improve the hygienic condition. Also, a call to regulate the use of antimicrobial may be necessary to reduce the resistance to drugs. Government should also encourage the development of new vaccines to help reduce he incidence of emerging diarrheal diseases.

Finally, the government and other health agencies should highlights the necessity of sanitary control system as well as monitor and regulate the use/distribution of antibiotics. These factors may help reducing childhood diarrheal infections of bacterial agents in developing countries, among diarrheal pathogens associated with decreased susceptibility to commonly prescribed antibiotics due to R-plasmids.

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