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ANTIMICIROBIAL SUSCEPTIBILITY PATTERNS OF *Escherichia coli* AND *Shigella sp* ISOLATED FROM DIARRHOEA STOOL OF CHILDREN

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ABSTRACT
A total of 56 and 24 strains of E. coli and Shigella sp. isolated from children less than five years with diarrhoea attending 3 different hospitals in South South Nigeria were screened for their antibiotic resistance patterns. Approximately 80% of E. coli and 70% of Shigella isolates were resistant to tetracycline. The most resistant pattern encountered was with amoxicillin, tetracycline and amoxicillin-clavulanic acid. About 92.9% of E. coli isolates and 87.5% of Shigella isolates were multidrug resistant (MDR). Plasmid curing and conjugation experiment were carried out on 31 selected E. coli donor isolates which were resistant to tetracycline and sensitive to nalidixic acid. Acridine orange was used as the curing agent. Curing of the selected donor isolates showed that tetracycline resistance in 87% of the isolates were plasmid mediated. Transfer rates of 77.4% (24/31) and 29.0% (9/31) were obtained for intra and inter-species tetracycline resistance respectively. The study revealed that tetracycline resistance and transfer among the isolates was quite high yet tetracycline is not commonly used to treat diarrhoea in ages 0-5 yrs. Therefore mechanisms other than selective pressure might exist for maintaining a resistant bacterial pool.
Keywords: Antimicrobial, Escherichia, Diarrhoea, Children, Antibiotic

INTRODUCTION

Diarrhoea is a leading cause of morbidity and mortality in developing countries, particularly in children. Diarrhoea are caused by bacterial, viral and parasitic infections, as well as food intolerance, reaction to medicines, and other physiological disorders (Abrami *et al*., 1998). *Escherichia coli* and *Shigella* species have now been established as aetiological agents of diarrhoea diseases of humans in developing countries (Asghar, 2002; Ekwenye and Kazi, 2007), but remains as an occasional cause of diarrhoea among children in industrialized countries, particularly in settings such as day care. The risk of diarrhoea in developing countries is attributed to deficiencies in environmental sanitation and personal hygiene (Prado *et al*., 1998).

Antimicrobial resistance among enteric pathogens is a serious problem in developing countries. Increased antimicrobial usage is the main driving force leading to evolution of drug-resistant bacteria (WHO, 2001). Tetracycline is not commonly used to treat diarrhoea in children between the ages of 0-5 yrs in Nigeria (Blake *et al*., 2003). In spite of this, tetracycline resistance rate is high. Studies showed that, once evolved, resistance genes could spread through the world's bacterial populations, irrespective of the pattern of antimicrobial use in an area (O'Brien, 2002). Therefore, mechanisms other than selective pressure might exist for maintaining a resistant bacterial pool.

Pathogenic organisms have developed a number of elaborate mechanisms for acquiring and disseminating antibiotic resistance. Antimicrobial resistance can be spread from cell to another by direct cell to cell content. Conjugation, the transfer of DNA between bacteria involving direct contact depends on plasmids. Plasmids allow the movement of genetic material, including antimicrobial resistance genes between bacteria species and genera (Prescott *et al*., 1999; Miranda *et al*., 2000). Multiple antibiotic resistance in bacteria are most commonly associated with the presence of plasmids which contain one or more resistance genes. *E. coli* has become a significant public health problem worldwide with the evolution of multiresistant antibiotic plasmids genes (Armstrong *et al*., 1996; Smith *et al*., 2003). Transfer of resistance genes between *Shigella* sp and *Escherichia coli* has been observed (Hooper, 2000; Tenover, 2006; Shoemarker *et al*., 2001). This study was carried out to investigate antibiotic resistance patterns of *E.*coli and *Shigella species* in pediatric diarrhoeal samples and the possibility of transfer.

MATERIALS AND METHODS

A total of 120 diarrhoea stool samples were obtained from children between 0 – 5 yrs of age attending Baptist medical center Eku, General hospital Agbor and University of Benin Teaching Hospital, Benin City. A loopful of the samples obtained was inoculated on nutrient broth, Muller-Hinton agar (MHA), MacConkey agar, deoxycholate citrate agar (all oxoid, England). To enhance the isolation of *Shigella sp*., selenite F broth (oxoid, England) was used. Incubation followed at 37^{o}C for 24hrs (Cheesbrough, 2000). Isolates were then characterized using standard bacteriological methods according to Cowan and Steel (1974).

Antibiotic Susceptibility Testing

Antibiotic susceptibility was determined using the disc diffusion methods as recommended by National Committee for Clinical Laboratory Standard (NCCLS, 1997) using Mueller Hinton agar. The antibiotic disc used contained: amoxicillin (Amx) 300g, amoxicillin-clavulanic acid (Amx-cla) 30µg, nalidixic acid (Nal) 30µg, gentamicin (genm) 10µg, nitrofurantoin (Nit) 300µg, tetracycline (Tet) 30µg. The zones of inhibition were then measured and results recorded as sensitive (S) or resistant (R) based on National Committee for Laboratory Standards (NCCLS, 1997).

Plasmid DNA Curing

Acridine orange was used as curing agent. The method described by Silhavy *et al*., (1984) was used. Sub-inhibitory concentration of 0.10mg/ml of acridine orange was used for plasmid curing. Selected isolates which were resistant to tetracycline and sensitive to nalidixic acid were used as donor, while those resistant to nalidixic acid but sensitive to tetracycline were used as recipient. The selected isolates were grown for 24hr at 37^{o}C in nutrient broth containing 0.10mg/ml acridine orange. A loopful of each were then sub-cultured on MHA plates and incubated at 37^{o}C for 24hrs, after which colonies were screened for antibiotic resistance by disk diffusion method.

Tetracycline Resistance Gene Transfer by Conjugation

Conjugation was carried out on selected isolates according to the methods of Thompson 1989, Kreuzer and Massey, (1996). Donor and recipient strains were incubated separately on nutrient broth at 37^{o}C for 24hrs. Fifty microliter (50µl) of donor and recipient broth culture were transferred to the same spots on the MHA plates supplemented with nalidixic acid (30µg/ml) and tetracycline (30µg/ml). The nalidixic acid and tetracycline incorporated into the MHA will inhibit the growth of donor and recipient cells but will not inhibit the growth of the transconjugants. The plates were left for about 10 mins (to enable cell to cell contact), after which sterile wire loop was used to streak out the inoculum. The plates were incubated at 37^{o}C for 24hrs. Transconjugants were selected on MHA plates supplemented with nalidixic acid (30µg/ml) and Tetracycline (30µg/ml). The transconjugants were further screened for antibiotic resistance as previously described.

RESULTS AND DISCUSSION

In recent years, it has become clear that *E. coli* play an important role in the etiology of acute diarrhoea (Ogunsanya *et al*., 1994; Jindal *et al*., 1995). Diarrhoea in children in developing countries has been reported in 50% to 60% of diagnosed cases (Mertens*et al*., 1990). The results of this study revealed that the prevalence rate of *E. coli* was higher than *Shigella* in all the locations sampled. Agbor had the highest prevalence of *E. coli* (52.5%). The result is shown in table 1. The prevalence of *E. coli* and *Shigella* in this study is similar to those obtained from Yah *et al*., (2007). Several studies have also shown the importance of *E. coli* and *Shigella* as a cause of diarrhoea in children (Nataro *et al*., 1998; Uma *et al*., 2009).

In agreement with reports from other developing countries (Uma *et al*., 2009; Lima *et al*., 1995), the present study demonstrated high rates of resistance in *E. coli* and *Shigella* sp to tetracycline and other antibiotics commonly available in Nigeria (Table 2). Approximately 80% of *E. coli* and 70% of *Shigella* isolates were resistant to tetracycline despite the fact that tetracycline is not commonly used to treat diarrhoea in ages 0-5yrs (Blake *et al*., 2003). The high prevalence rate of tetracycline resistance may be explained by the wide use of tetracycline in animal feed and consumption of meat of animals that have received tetracycline. There is also the possibility of transfer of tetracycline resistance from adult who carry the resistant genes to children through contacts. The use of other related broad-spectrum antibiotics for diverse infection in children can result to selection of resistant strains in the normal intestinal flora. It has been reported that antimicrobial resistance genes can be readily transmitted between commensal Enterobacteriaceae and enteropathogens *in vivo* and *in vitro* (Blake *et al*., 2003). Smith *et al*., (2003) reported that *E.coli* isolated from animals haboured plasmids and could disseminate the plasmids in the environment. According to Sahm *et al* (2001), concurrent resistance to antimicrobials of different structural classes have arisen in a multitude of bacterial species and may complicate the therapeutic management of infections. In this study, 92.9% *E. coli* isolates and 87.5% *Shigella* isolates were resistant to three or more antimicrobial agents and were considered as multidrug resistant (MDR). Multidrug resistance has been ascribed in most instances to the presence of plasmids (Yah *et al*., 2007).

Plasmid curing studies with acridine orange showed that out of 31 selected donor isolates, 27 (87%) harboured plasmids. Drug resistance character is most often encoded on plasmids. This explains the high prevalence of plasmid observed in this study. More so, resistance carried on plasmids can easily be transferred among isolates. The results also showed that conjugation was a very convenient method of transferring *tet*^{r} gene among intra species bacteria. Approximately 77.4% (24 out of 31) of tetracycline resistant *E. coli* strains transferred *tet*^{r} gene to recipient *E. coli* C93 (Table 4). *E. coli* and *Shigella* are members of the enteric family and genetic transfer is possible. Several studies have supported the ease of genetic transfer among the Enterobacteriaceae family (Wang *et al*., 2004; Yukata *et al*., 2004; Yah *et al*., 2007). About 29.0% (9 out of 31) of *E. coli* isolates transferred *tet*^{r} gene to recipient *Shigella* C85 (Table 5). Such broad host transferrable plasmids play an important role in the spread of antibiotic resistance. Aluyi and Akortha (2002) also reported inter-generic rate of 33% from enteric bacteria of diarrhoea origin to *E. coli* (UB5201).

According to Nashiran *et al.*, (2005) and Heritage *et al.*, (2003), the transfer of resistance genes between different bacterial species may go unnoticed by traditional infection control and epidemiological methods, thereby undermining hospital infection control policies. Nashiran *et al.*, 2005 concluded that plasmids outbreaks may go unrecognized but also that stringent measures directed against all bacilli can be effective in controlling outbreaks if proper hygiene, wearing of gloves and gowns during patients care is observed.

Table 1: Prevalence of *E. coli* and *Shigella* sp. isolated from diarrhoea patients

Location	**No of samples collected**	**Bacteria isolate**	**No. of isolate (% prevalence)**
Eku	40	*E. coli*	19 (47.5)
		Shigella sp.	13 (32.5)
Agbor	40	*E. coli*	21 (52.5)
		Shigella sp.	10 (25.0)
Benin	40	*E. coli*	16 (40.0)
		Shigella sp.	1 (2.5)
Total	120		80 (66.7)

Table2: Resistance of *E. coli* and *Shigella* sp. isolated from diarrhoea patients

Isolates (No. of isolates)	**No. of isolates resistant to antibiotics (%)**					
	Amx	**Tet**	**Amc-cla**	**Nal**	**Gen**	**Nit**
Escherichia coli (56)	54(96.4)	45(80.4)	51(91.1)	22(39.3)	24(42.9)	6(10.7)
Shigella sp. (24)	24(100)	17(70.8)	19(79.2)	14(58.3)	11(45.8)	1(4.2)
Total (80)	78(97.5)	62(77.5)	70(87.5)	36(45)	35(43.8)	7(8.8)

Key: Amx = Amoxicillin, Tet = tetracycline, Amx-Cla = Amoxicillin-Clavulanic acid, Nal = Nalidixic acid, Gen = Gentamycin, Nit = Nitrofurantoin

Table 3: Resistance patterns of donor isolates

Resistance patterns	**No. of strains**
Amx, tet	2
Amx, Tet, amx-cla	13
Amx, tet, gen	3
Amx, tet, amx-cla, gen	11
Amx, tet, amx-cla, gen, Nit	2

Table 4: Intra-species transfer of tet by conjugation from *E. coli* to *E. coli* recipient (C93)

S/N	***E. coli* isolate No.**	**Donor resistance profile**	**Transconjugants phenotype**
1	E_7	amx, tet, amx-cla, gen	tetnal
2	E_8	amx, tet, amx-cla	tetnal
3	E_9	amx, tet, amx-cla, gen	tetnal
4	E_{15}	amx, tet, amx-cla	tetnal
5	E_{19}	amx, tet, amx-cla	tetnal
6	E_{20}	amx, tet, amx-cla, gen	tetnal
7	E_{29}	amx, tet,	tetnal
8	E_{35}	amx, tet, amx-cla, gen	tetnal
9	E_{41}	amx, tet, amx-cla	tetnal
10	E_{42}	amx, tet, amx-cla	tetnal
11	E_{51}	amx, tet, amx-cla	tetnal
12	C_{64}	amx, tet, amx-cla, gen	tetnal
13	C_{67}	amx, tet	tetnal
14	C_{68}	amx, tet, amx-cla	tetnal
15	C_{73}	amx, tet, gen	tetnal
16	C_{83}	amx, tet, amx-cla, gen	tetnal
17	C_{100}	amx, tet, amx-cla, gen	tetnal
18	C_{102}	amx, tet, amx-cla, gen	tetnal
19	U_{19}	amx, tet, amx-cla	tetnal
20	U_{128}	amx, tet, amx-cla	tetnal
11	U_{131}	amx, tet, amx-cla	tetnal
22	U_{133}	amx, tet, amx-cla	tetnal
23	U_{137}	amx, tet, amx-cla, gen	tetnal
24	C_{56}	amx, tet, amx-cla	tetnal

Table 5:Inter-generic transfer of tet^r by conjugation from *Escherichia coli* to *Shigella* recipient (C85)

S/N	*E. coli* isolate No.	Donor resistance profile	Transconjugant phenotype
1	E_{35}	amx, tet, amx-cla, gen	tetnal
2	E_{41}	amx, tet, amx-cla	tetnal
3	E_{42}	amx, tet, amx-cla	tetnal
4	E_{51}	amx, tet, amx-cla	tetnal
5	C_{64}	amx, tet, amx-cla, gen	tetnal
6	C_{83}	amx, tet, amx-cla, gen	tetnal
7	C_{100}	amx, tet, amx-cla, gen	tetnal
8	C_{102}	amx, tet, amx-cla, gen	tetnal
9	U_{137}	amx, tet, amx-cla, gen	tetnal

CONCLUSION

This study has shown that there is an increase in prevalence of *tet^r* genes among *E. coli* isolates of pediatric diarrhoea in the sampling locations, yet tetracycline is not used in children to treat diarrhoea. Majority of the *tet^r* genes may probably be due to the acquisition of resistance genes and or transfer of *tet^r* gene between commensal enterobacteriaceae and enteropathogens. Conjugal transfer of plasmids had greatly contributed to the rapid spread of antibiotic resistance among *E. coli* and *Shigella* isolates. Therefore, controlled administration of antimicrobial agents in adults and animals and their restricted usage only in definitely indicative situations are essential to control the emergence of MDR isolates.

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