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Full Length Research Paper

Transfer of tetracycline resistance gene (*tet*^{r}) between replicons in some enteric bacteria of diarrhoeal origin from some hospitals in South-South, Nigeria

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From April to June 2005, a total of 120 feacal samples were obtained from diarrheagenic patients (0-5 years) attending Baptist Medical Center, Eku (BMC), Central Hospital, Agbor (CHA) and University of Benin Teaching Hospital, Benin City (UBTH). These were screened for the presence of bacteria that could cause diarrhoea. The enteric organisms isolated included *Escherichia coli, Klebsiella* spp., *Salmonella* spp., *Aeromonas* spp., *Shigella* spp. and *Shigella* spp. Antimicrobial susceptibility testing among the isolates showed resistance to amoxicillin (92%), amoxicillin-clavulanic acid (84.4%), tetracycline (71.4%), gentamycin (43.5%), nalidixic acid (38.3%) and nitrofurantoin (7.9%). *E. coli* showed the highest resistance to most of the antibiotics. Tetracycline resistance gene was detected in about 72% (110) of the total isolates, out of which 76 (69%) were subjected to curing experiment in the presence of 75 µg/ml acridine orange. Sixty (79%) of tetracycline resistant isolates lost their tetracycline resistance markers (*tet*^{r}) indicating that the *tet*^{r} gene was located on a plasmid. Attempt was made to transfer the *tet*^{r} gene from one replicon to the other within the same species and from one genus to the other. The rate of intra-species transfer of *tet*^{r} gene (67%) was significantly higher (< 0.05) than its rate of inter-generic transfer (24%).

Key words: Tetracycline resistance gene (*tet*^{r}), replicon, intra-species, inter-generic gene transfer.

INTRODUCTION

Antibiotic resistance in enteric pathogens is of great impor-tance in the developing world, where the rate of diarrhoeal disease is high. Continued mismanaged selective pressure has contributed towards the emergence of multiple drug resistant bacteria and that has been regarded as an inevitable genetic response to antimicrobial therapy (Smith et al., 2003). *Escherichia coli* and *Klebsiella* spp. are important opportunistic pathogens that have shown an increasing antimicrobial resistance to most antibiotics (Mirand et al., 2004; Sheikh et al., 2003). Intestinal strains of these pathogens are primary causes of urinary tract infections, septicemia, diarrhoea and nosocomial infections. Individuals who are debilitated or have other predisposing factors are at higher risk of infection than healthy persons (Sheikh et al., 2003; Lisa and Rodgers, 1999).

The most common genetic instrument for resistance among bacteria is the R-plasmid (Okeke et al., 1999). Acquisition of these plasmids occurs via all three types of recombination (transformation, transduction and conjugation), although conjugation appears to be the most common and convenient method (Sheikh et al., 2003). Unfortunately, there are still large gaps in our understanding of how new multi-resistance plasmids evolve. Infections caused by *E. coli* have become a significant public health problem world-wide with the evolution of multi-resistant antibiotic plasmid genes (Armstrong at al., 1996; Smith et al., 2003; Mirand et al., 2004). Surveillance studies have demonstrated the emergence of highly resistant *Klebsiella* spp. (Calva et al., 1996). The members of the genus *Klebsiella* have also been linked

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Table 1. Incidence of bacterial isolates from the hospitals (%).

Isolates	EMC	CHA	UBTH	Total (%)
Escherichia coli	19 (33.9)	21 (41.1)	16 (33.3)	56 (36.1)
Klebsiella spp	12 (21.4)	10 (19.6)	15 (31.3)	37 (23.9)
Salmonella spp	06 (10.7)	02 (3.9)	14 (29.2)	22 (14.2)
Aeromonas spp	05 (8.9)	08 (15.6)	02 (4.2)	15 (9.7)
Shigella dysenteriae	07 (12.5)	04 (7.8)	01 (2.1)	12 (7.7)
Shigella flexneri	07 (12.5)	06 (11.1)	NIL (0.0)	13 (8.4)
Total	56 (36.4)	51 (33.1)	48 (31.2)	155 (100)

EMC = Eku Medical Centre, CHA = Central Hospital, Agbor and UBTH = University of Benin Teaching Hospital.

to epidemics of diarrhea because some strains appear to have acquired plasmid from *E. coli* that code for heat labile and heat stable enterotoxins (Wang et al., 2006). Most diarrhoael infected patients in Nigeria treat diarrhea with tetracycline prior to receiving definitive laboratory results. This has provided the selective pressure necessary to maintain and transfer R-plasmids. The aim of this research is to demonstrate the intra and inter-generic transfer of (tetracycline resistance) gene *tet*^{r} among *E. coli* and *Klebsiella* spp., isolated from diarrhoeal stools.

MATERIALS AND METHODS

Laboratory isolation and identification

From April to June (2005), a total of 120 fecal samples were obtained from diarrheagenic patients (0-5 yrs) attending Baptist Medical Center Eku (BMC), Central Hospital Agbor (CHA) and University of Benin Teaching Hospital Benin City (UBTH).

Swabs of stool were inoculated on sterile Mac-Conkey agar, deoxycholate citrate agar, nutrient agar and nutrient broth and incubated at 37^{o}C for 24 h. Non-lactose fermenting colonies were inoculated into selenite F broth at 37^{o}C for 24 h, to enhance the isolation of *Shigella* spp. Isolated colonies were then characterized using standard bacteriological methods of Cowan and Stell (1974). They were further subcultured on nutrient agar slants and stored at 4^{o}C for further analysis.

Antimicrobial susceptibility testing

Antimicrobial susceptibilities test were determined by the disk diffusion method of Cheesbrough (2000) using Oxoid-Muller Hinton agar. The antibiotic disk (May and Baker) used contained the antimicrobial agents; amoxicillin (25 µg), tetracycline (30 µg), amoxicilllin-clavulanic acid (30 µg), nalidixic acid (30 µg), gentamicin (10 µg) and nitrofurantoin (300 µg). The zones of inhibition were measured and the results recorded as sensitive (S) or resistance (R).

Plasmid curing and conjugation

Representative strains were subjected to acridine orange mediated plasmid curing (Sheikh et al., 2003) using Oxoid-Muller Hinton agar. The colonies were screened for antibiotic resistance by disk diffusion method as earlier described.

Conjugation was performed by the methods of Anyanwu (1983). Donor isolates (*E. coli* and *Klebsiela* spp.) which are resistant to tetracycline and sensitive to nalidixic acid were used. The trans-conjugants were selected on Oxoid-Muller Hinton agar medium supplemented with antibiotics to inhibit the growth of the donor and recipient, respectively. All plates were incubated at 37^{o}C for 24 h. The transconjugants were re-streaked onto fresh selective nutrient agar plates and their identities were re-confirmed on the basis of biochemical methods and their antibiotic resistance patterns. Intra-species and inter-genetic transfer of tetracycline resistant gene were carried out on 2 species, *E. coli* and *Klebsiella* spp., out of the 6 species identified.

RESULTS AND DISCUSSION

Table 1 shows the total number of each type of isolates obtained from various hospitals. The number of bacterial isolates from the different hospitals were as follow; 56 (36%) from Eku Medical Centre (EMC); 51 (33%) from Central Hospital Agbor (CHA) and 48 (31%) from University of Benin Teaching Hospital Benin City (UBTH). The isolates were found to be resistant to amoxicillin (92%), amoxicillin-clavulanic acid (84.4%), tetracycline (71.4%), gentamicin (43.5%), nalidixic acid (38.3%) and nitrofurantoin (7.7%; Table 2). The results of antibiotic susceptibility test also indicated that *E. coli* showed higher rates of resistance to most of the antibiotics. A total of 110 (71.4%) isolates were resistant to tetracycline. Tetracycline resistant gene *tet*^{r} was observed in 45 (80.3%) out of 56 *E. coli* isolates, 31 (83.7%) out of 37 *Klebsiella* spp. isolates; 14 (70%) out of 22 *Salmonella* spp.; 3 (20%) out of 15 *Aeromonas* spp., 9 (56%) out of 12 *Shigella* spp. and 10 (6.2%) out of 12 *Shigella* spp. isolates. *Klebsiella* spp was the most resistant to tetracycline while *Aeromonas* spp. was the least resistant. No resistance to nitrofurantoin was observed in *Salmonella* and *Shigella* spp. Seventy-six tetracycline resistant isolates were subjected to curing. Of these, 60 (79%) were cured of tetracyline resistance gene (*tet*^{r} Table 3). Seventeen (54.8%) out of 31 tetracycline resistant *E. coli* isolates transferred their gene into *E. coli* CHA101 (Table 4). The result also showed that *E. coli* UBTH I31 probably had the *tet*^{r} on the chromosome, yet transfer was observed. Fourteen 14 (66.6%) out of 24 *Klebsiella* spp. transferred their *tet*^{r} gene. Tetracycline

Table 2. Antibiotics resistance of Gram-negative bacteria isolated from diarrhoeal patients from 3 different hospitals.

Isolate (No. of isolates)	No. of isolates resistance to antibiotics (%)					
	Amoxicillin	Tetracycline	Amoxicillin - clavulanic acid	Nalidixic acid	Gentamycin	Nitrofurantoin
Escherichia coli (56)	54 (96.4)	45 (80.3)	51 (33)	22 (14.2)	24 (15.5)	6 (3.8)
Klebsiella spp (37)	33 (89.1)	31 (83.7)	33 (21.4)	10 (6.4)	15 (9.7)	49 (2.5)
Salmonella spp (22)	15 (78.9)	14 (70)	13 (8.4)	11 (7.1)	8 (5.1)	0 (0.00)
Aeromona spp (15)	16 (88.8)	3 (15)	14 (9.0)	2(1.2)	9 (5.8)	1 (0.6)
Shigella dysenteriae (12)	12 (100)	9 (75)	10 (6.4)	5 (3.2)	5 (3.2)	1 (0.6
Shigella flexneri (12)	12 (100)	8 (66)	9 (5.8)	9 (5.8)	6 (3.8)	0 (0.00)
Total (154)	142 (92.2)	110 (71.4)	130 (84.4)	59 (38.4)	67 (43.5)	12 (7.7)

Table 3. Occurrence of tetracycline resistance (R) plasmids among the bacterial isolates (%).

Bacterial isolates	No. of tetracycline resistant isolates tested	No. cured of tetracycline resistant gene
Esherichia coli	33	28 (90)
Klebsiella spp.	21	18 (86)
Salmonella spp.	08	06 (85)
Aeromonas spp.	03	01 (50)
*Shigella dysenteria*e	05	04 (80)
Shigella flexneri	06	03 (50)
Total	76	60 (79)

Table 4. Occurrence of tetracycline resistance (*tet*^{r}) transconjugants (%).

Donor isolate (*tet*^{r}, *nal*^{s})	No. of donor tested	Recipients (*tet*^{r}, *nal*^{r})	No. of *tet*^{r} transipients
Escherichia coli	31	*E. coli* CHA101	17 (55)
Klebsiella spp.	21	*Klebsiella* EMC49	14 (67)
Escherichia coli	31	*Klebsiella* EMC49	14 (45)
Klebsiela spp.	21	*E. .coli* CHA101	5 (24)

resistance gene (*tet*^{r}) was transferred from *Klebsiella* to *E. coli* CHA101 and from *E. coli* to *Klebsiella* EMC49. Fourteen (45.1%) *E. coli* transferred their *tet*^{r} gene into *Klebsiella* EMC49. Five (23.8%) *Klebsiella* spp. transferred their *tet*^{r} gene into *E. coli* CHA101.

An important property of enteric pathogens is their frequent resistance to multiple antibiotics (Wataro and Kaper, 1998). In the present study, the isolates showed multiple antibiotic resistances to the antibiotics used. George (1996) reported low resistance rate towards cephalotin, gentamicin, nitrofurantoin and nalidixic acid. Similarly in this study, the isolates were observed to show low resistance rates towards gentamicin, nalidixic acid and nitrofurantoin. Calva et al. (1996) reported that nitrofurantoin and norfloxacin represented 2% of antibiotic usage. Studies in other developed countries have shown that the trend in enteric pathogen is toward increasing antibiotic resistance (Kruse et al., 1995). Previous studies on clinical isolates in Turkey (Erden et al., 2005) recorded resistance to ampicillin (60%), tetracycline, (40%) and amoxycillin-clavulanic acid (40.0%). Mitsuhashi (1965) reported the highest level of resistance to be found in tetracycline (21%) and ampicillin (19%). Okeke et al. (1999) observed in a study carried out on Nigerian students from 1986-1998 that five drugs (ampicillin, sulfonamides, streptomycin, chloramphenicol, and tetracycline) in which there were considerable rise in resistance were extensively used in Nigeria and other developing countries. The selective pressure generated by overall explains the relative high prevalence in isolates. This study reported 110 (71.4%) tetracycline resistant clinical isolates out of 154 isolates. The prevalence of tetracycline resistance in the isolates in this study was significant ($P < 0.05$). In the same survey, Okeke et al. (1999) observed that resistance to tetracycline increased from 34.9% in 1986 to 100% in 1998. Egah et al. (2001) also reported a high level of resistance to tetracycline (75%).

Comparatively, the results of tetracycline resistance observed by Okeke et al. (1999) and Egah et al. (2001)

which was in South-West Nigeria and Northern part of Nigeria respectively and the result of the present study conducted in South-South Nigeria, shows that tetracycline resistant isolates were higher than the figure obtained in other developed countries of Spain (21%), Taiwan (41.0%) and Turkey (60%) (Erden et al., 2005). This is probably due to the indiscriminate use of tetracycline to treat diarrhea along with poor hygiene which is highly prevalent in Nigeria and other developing countries (Hark and Karonk, 1998; Okeke et al., 1999).

Plasmid conjugation is an important mechanism of disseminating drug resistance among bacterial populations. Intra species tetracycline resistance transfer from *Klebsiella* spp. (donor) to *Klebsiella* EMC49 (recipient) was 66%. Transfer from *E. coli* to *E. coli* CHA101 was 54%. The *E. coli* tetracycline resistance strains were found in 80% of the isolates while for *Klebsiella* spp. the tetracycline resistance strains were found in 83%. The results of the current findings were in accordance with those of Yah et al. (2004) while carrying out bacteriological studies on infected kerosene burn wounds in Benin City. They found that majority of *Klebsiella pneumoniae* from University of Benin Teaching Hospital (UBTH) harbour plasmids. This work not only found that *Klebsiella* spp. harbour plasmids but transfer plasmids to *Klebsiella* EMC49.

Transfer was not successful in all strains, although positive during curing procedures. The plasmids or the genetic capabilities may have been lost during repeated successive sub-cultures of the isolates. Though tetracycline resistance transfer was highly mediated by plasmids, transfer of tetracycline resistance in one *E. coli* isolate was chromosomally mediated.

E. coli and *Klebsiella* spp. are members of the enteric family and genetic transfer is common. In this connection, inter-generic transfer of tetracycline resistance gene between *E. coli* and *Klebsiella* spp. was performed. The transfer was quite low when compared with those of Wang et al. (2006) who observed the emergence of plasmid mediated quinolone resistance associated with the quinolone gene in *Klebsiella pneumoniae*. Sheikh et al. (2003) found that plasmid borne antibiotic resistance factors among indigenous *Klebsiella* spp. can be transferred from *Klebsiella* to *E. coli* MD40 (recipient). They also found that a number of the conjugative plasmids carry potentials to disseminate resistance markers to distant recipient cells. The low rate of inter-generic transfer in this study could be as a result of fertility inhibition and plasmid borne resistance markers being non-conjugative/non-transferable.

The study revealed the presence of plasmid DNA in most *E. coli* and *Klebsiella* spp. tetracycline resistant strains and also showed that resistance transfer was more successful between species than between genera.

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