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Plasmid Analysis of Fluoroquinolone Resistant Commensal *E. coli* from Faecal Samples of Apparently Healthy Cattle in Ado-Ekiti, Ekiti-State

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Abstract: This study was carried out to investigate the prevalence of fluoroquinolone resistance and plasmid carriage among isolates of commensal *E. coli* isolated from faeces of cattle. Fresh faecal samples were collected from apparently healthy cattle and were cultured on eosine methylene blue agar plates from which 500 commensal *E. coli* isolates were recovered and characterised using standard biochemical tests. Using protocol recommended by the Clinical Laboratory Science Institute, all isolates were examined for their susceptibility to five fluoroquinolones: norfloxacine (5 μg), levofloxacine (5 μg), pefloxacine (5 μg), ofloxacine (5 μg) and ciprofloxacine (5 μg). The resistance among isolates against the fluoroquinolones are as follows: pefloxacine, 99 (19.8%); ciprofloxacine, 55 (11.0%); norfloxacine, 39 (7.5%); ofloxacine 26 (5.2%) while the isolates showed least resistance against levofloxacine 23 (4.6%). The organisms also showed considerable multiple fluoroquinolone-resistance and sixteen different fluoroquinolone-resistance phenotypes were observed with the most prominent phenotype observed to be Cip-Nor-Ofx-Pef-Lev. Thirteen representative isolates were selected and examined for the presence of plasmids. Twelve of the representative isolates carried multiple plasmids while one isolate carried a single plasmid. After mating experiments, plasmids were transferred to recipient strains at high frequencies of conjugation. These findings have serious public health implications as fluoroquinolone-resistant bacteria could be shed into the immediate environments, food and drinking water sources.

Key words: Prevalence, fluoroquinolone resistance, plasmid, eosine, phenotype, Nigeria

INTRODUCTION

The fluoroquinolones are a group of synthetic antimicrobials that possess a broad-spectrum of activity against gram-positive and negative bacteria and are one of the most prescribed families of antimicrobials worldwide (Ip *et al*., 2006; Morgan-Linnell *et al*., 2009). Historically, the onset of the use of fluoroquinolones in humans signalled effective prevention and control of infections, most especially pneumonia, intestinal infections, urinary tract infections and other life threatening infections (Collignon and Angulo, 2006). Furthermore, fluoroquinolones were subsequently licensed for use in animal medicine to prevent and control infections, enhance increase in animal weight and maintain overall quality of animal health with consequent increase in productivity (Riddle *et al*., 2000).

In some cases some fluoroquinolones are licensed exclusively for use in poultry and birds, cattle and canines (Iovine and Blaser, 2004) and they have been highly effective for treatment of animal diseases such as mastitis, respiratory tract infections, urinary tract infections and bronchitis (Guardabassi *et al*., 2004). However, the emergence of bacterial pathogens and other commensal bacteria which inhabit the gut and show resistance to fluoroquinolones has compromised the effectiveness and use of the fluoroquinolones and this phenomenon have been attributed to overdependence on flouroquinolones in veterinary and human medicine.

The common mechanism of resistance against quinolones and fluoroquinolones include antibiotic efflux pumps, enzymatic modifications, decreased permeability in the porin channels on bacterial cells and alteration of enzymatic targets in bacterial sells (Ruiz, 2003; Hawkey, 2003). More recently, plasmids harbouring genes that confer resistance against fluoroquinolones have been described as the genetic mechanism for resistance among bacteria from animal origin. In addition, chromosomally

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mediated resistance against fluoroquinolones was believed to be the major mechanism before to the discovery of plasmid-mediated resistance to fluoroquinolones (Nordmann and Poirel, 2005; Venturini *et al*., 2009). Similarly, Fortini *et al.* (2009) reported that novel genetic elements, most especially plasmids are present among bacteria isolated from food producing animals and their presence often constitute serious public health threat due to the relatively high frequency at which the genetic elements are transferred.

In this study, we seek to determine the plasmid profile of commensal *E. coli* isolated from cattle which showed resistance against fluoroquinolones in the study area where meat is considered essential to human diet.

MATERIALS AND METHODS

Collection and processing of samples: Faecal samples were collected from apparently healthy cattle at Igbo-Adere, Ado-Ekiti, Ekiti-State and plated directly onto Eosin Methylene agar (EMB) plates. Five hundred isolates of commensal *E. coli* were recovered and identified on the basis of their characteristic green metallic sheen and biochemical tests as described by Olutiola *et al*. (2001).

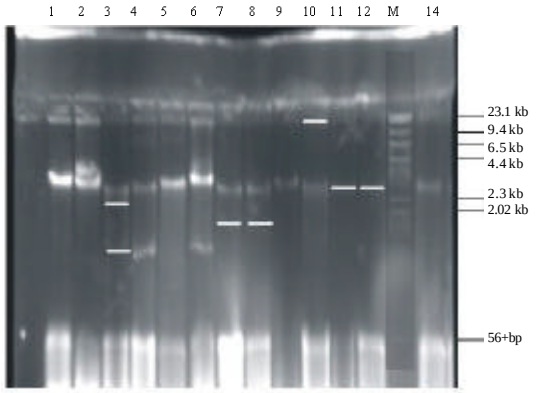

Fig. 1: Plasmid profiles of representative isolates of fluoroquinolone resistant *E. coli* (Lane 1: E464, 22.7 kb, 3.7 kb, 564 bp; Lane 2: E22.7 kb, 3.9 kb, 3.7 kb; Lane 3: E626, 22.7 kb, 3.9 kb, 3.7 kb; Lane 4: E580, 22.7 kb, 3.8 kb, 1.67 kb, 564 bp; Lane 5: E522, 22.7 kb, 3.9 kb, 564 bp; Lane 6: E466, 22.7 kb, 3.9 kb, 1.67 kb; Lane 7: E467, 22.7 kb, 3.9 kb, 1.67 kb; Lane 8: 3.4 kb, 1.98 kb, 564 bp; Lane 9: E406, 3.9 kb; Lane 10: E404, 2.27 kb, 3.4 kb, 564 bp; Lane 11: E295, 3.4 kb; Lane 12: E277, 3.4 kb, 564 bp; Lane M: Marker: Hind III digest; Lane 14: E80, 3.4 kb, 564 bp)

Antibiotic susceptibility testing: All test innocula were inoculated onto Mueller-Hinton broth (0.5 McFarland turbidity) from 24 h cultures. All bacterial isolates were tested for their susceptibility on Mueller-Hinton agar against five fluoroquinolones: norfloxacine (5 μg), levofloxacine (5 μg), pefloxacine (5 μg), ofloxacine (5 μg) and ciprofloxacine (5 μg) (Oxoid, UK). Susceptibility tests were done using the agar disk diffusion method according to the recommendations of the Clinical Laboratory Science Institute (CLSI, 2008). The zones of inhibition were measured and compared with standard interpretative charts.

Plasmid analysis and mating experiment: Thirteen representative isolates were selected on the basis of their multiple fluoroquinolone resistance phenotypes and cultured overnight on Mueller-Hinton broth (Fig. 1). Plasmids DNA were extracted from cultured cells using the modified alkaline lysis protocol method (Birnboim and Dolly, 1979; Johnson, 1998). The plasmid DNA was electrophoresed on 0.8% agarose gel stained with ethidium bromide and visualized by UV-transillumination. Plasmid sizes were estimated by comparing with standard DNA bands obtained from Lambda Hind III digest. The isolates that were confirmed to carry plasmid (s) were further selected and conjugated with recipient strain *E. coli* 25922 and transconjugants were selected on Mueller-Hinton plates containing tetracycline.

RESULTS

Results obtained in this study indicate that resistance against pefloxacine was highest with 99 (19.8%) isolates showing resistance to the antibiotic while resistance against levofloxacine was least (Table 1). Some of the isolates showed considerable multiple fluoroquinolone-resistance and 16 different fluoroquinolone-resistance

Table 1: Susceptibility to fluoroquinolones of commensal *E. coli* isolated from apparently healthy cattle

Antibiotics	Number (%)
Pefloxacine	99 (19.8)
Ciprofloxacine	55 (11.0)
Norfloxacine	39 (7.8)
Ofloxacine	26 (5.2)
Levofloxacine	23 (4.6)

Table 2: Multiple fluoroquinolone-resistance among commensal *E. coli* isolates from apparently healthy cattle

Antibiotics	Number (%) n = 500
Pefloxacine	99 (19.8)
Ciprofloxacine	55 (11.0)
Norfloxacine	39 (7.8)
Ofloxacine	26 (5.2)
Levofloxacine	23 (4.6)

Table 3: Multiple fluoroquinolone-resistance phenotypes among commensal *E. coli* isolates from apparently healthy cattle

S/N	No. of fluoroquinolones	------Resistance phenotypes--------					No. of isolates
1	2	Cip	Nor				1
2		Cip	Pef				3
3		Lev	Pef				1
4		Pef	Nor				2
5		Pef	Ofx				3
Total							10
6	3	Cip	Pef	Nor			9
7		Cip	Pef	Ofx			1
8		Lev	Cip	Nor			1
9		Lev	Cip	Ofx			1
10		Lev	Cip	Pef			3
11		Lev	Nor	Ofx			4
12		Pef	Nor	Ofx			1
Total							20
13	4	Cip	Pef	Ofx	Nor		5
14		Lev	Cip	Nor	Ofx		1
15		Lev	Cip	Pef	Ofx		1
Total							7
16	5	Lev	Cip	Pef	Nor	Ofx	23
Total							23

Cip-Ciprofloxacine, Lev-Levofloxacine, Nor-Norfloxacine, Ofx-Ofloxacine, Pef-Pefloxacine

Table 4: Plasmid profile of fluoroquinolone resistant isolates

Lanes	Isolate code	Plasmid bands (Approx.)	Fluoroquinolone resistance phenotypes
1	E646	22.7 kb, 3.7 kb, 564 bp	Pef-Cip
2	E625	22.7 kb, 3.9 kb, 3.7 kb	Pef-Ofx-Nor
3	E626	22.7 kb, 3.8 kb, 2.3 kb, 1.67 kb, 564 bp	Pef-Cip
4	E580	22.7 kb, 3.8 kb, 1.67 kb, 564 bp	Pef-Ofx-Cip-Lev
5	E522	22.7 kb, 3.9 kb, 564 bp	Nor
6	E466	22.7 kb, 3.9 kb, 1.67 kb	Pef
7	E467	3.4 kb, 1.98 kb, 564 bp	Ofx-Nor-Lev
8	E440	3.4 kb, 1.98 kb, 564 bp	Pef
9	E406	3.9 kb	Pef
10	E404	22.7 kb, 3.4 kb, 564 bp	Pef
11	E295	3.4 kb	Cip
12	E277	3.4 kb, 564 bp	Pef-Cip
M	Marker: Hind III Digest	-	-
14	E80	3.4 kb, 564 bp	Ofx

Amp-Ampicillin, Col-Colistin, Cot-Cotrimoxazole, Gen-Gentamicin, Nal-Nalidixic-acid, Nit-Nitrofurantoin, Tet-Tetracycline, Strep-Streptomycin, Pef-Pefloxacine, Ofx-Ofloxacine, Cip-Ciprofloxacine, Lev-Levofloxacine, Nor-Norfloxacine

patterns were observed among isolates that were confirmed to show multiple fluoroquinolone resistance (Table 2). The most predominant resistance patterns were Lev-Cip-Pef-Nor-Ofx (Table 3). Multiple fluoroquinolone-resistant isolates were defined as those isolates that showed resistance against a minimum of two fluoroquinolones. The overall plasmid profiles of the representative isolates revealed that 12 of the 13 representative isolates carried multiple plasmids while the remaining one carried only one plasmid (E295, Lane 11); with molecular weight of plasmids ranging between 564 bp and 22.3 kb (Table 4). Lambda Hind III digest was used to estimate the molecular weight of all bands resolved after electrophoresis. In the mating experiments, plasmids were successfully transferred to the plasmid-free recipient *E. coli* 25922 at varied frequencies of conjugation and MIC of transconjugants for tetracycline at a minimum of 16 mg L^{-1}. One of the isolates carrying plasmids did not transfer it to the recipient (Table 5).

Table 5: Result for transconjugants selected among fluoroquinolone-resistant *E. coli*

Lanes	Isolate code	Frequency of conjugation (Transconjugants/donor cells)	MIC (mg L^{-1})
1	E646	1.0×10^{-4}	32
2	E625	1.4×10^{-3}	32
3	E626	1.9×10^{-4}	>64
4	E580	2.2×10^{-5}	32
5	E522	1.6×10^{-4}	16
6	E466	2.5×10^{-2}	32
7	E467	2.3×10^{-5}	64
8	E440	9.0×10^{-4}	64
9	E406	00	64
10	E404	2.8×10^{-4}	32
11	E295	2.7×10^{-5}	16
12	E277	1.6×10^{-3}	64
14	E80	1.9×10^{-3}	64

DISCUSSION

This study has demonstrated that the incidence of fluoroquinolone-resistance among commensal *E. coli* isolated form apparently healthy cattle is relatively high with 20% (99/500) of the multiple fluoroquinolone-resistant isolates showing resistance to the antibiotic. This could be related to the use of fluoroquinolones and other related brooad-spectrum agents in veterinary practice in the study location which exposes the bacteria to a selection pressure that favours the emergence of fluoroquinolone-resistant bacteria in the gut of the animals prior to slaughter. There are previous reports that have shown the increasing incidence of fluoroquinolone resistance among bacteria of animal origins and this study appears to be in accordance with such studies (Nordmann and Poirel, 2005; Collignon and Angulo, 2006). Food animals particularly cattle, swine sheep and birds are routinely fed with low doses of antibiotics through food and water to promote growth and expedite weight gain (Phillips *et al*., 2004; Scheider and Garrot, 2010).

Commensal *E. coli* in cattle and other animals is often used as an indicator organism to access the extent and type of resistance in the gastrointestinal tract since it plays a dynamic role in the ecology of multi-drug resistance bacteria and have been proven to be a reservoir of resistance (Van Donkersgoed *et al*., 2003; Sharma *et al*., 2008). The incidence of resistance against pefloxacine was highest while the organisms demonstrated the least resistance against levofloxacine among other antibiotics that were tested. In most cases of resistance shown by

bacteria against antimicrobials, the relative frequency of resistance to antibiotics is quite often is an indication of the extent of usage of antibiotics (Sharma *et al*., 2008).

Multiple antibiotic resistance is defined as resistance against a minimum of two fluoroquinolones. Some of the isolates showed multiple-antibiotic resistance and studies have shown increasing incidence of fluoroquinolone resistance among enteric bacteria of animal origin (White *et al*., 2000). This is worrisome in view of the limitations that may be imposed on the options of antibiotics that may be used not only in animal medicine but also in human medicine.

A very high level of detection of plasmids was observed in this present study as all representative isolates harboured detectable plasmids with sizes between 564 bp and 22.3 kb (Fig. 1). Twelve representative fluoroquinolone-resistant isolates harboured multiple plasmids while only one isolate carried one plasmid. The high rate of detection of multiple plasmids among the bacteria could be responsible for the high incidence of fluoroquinolone resistance among the isolates as observed in this study. A similar study by Smith *et al.* (2003) showed that multiple fluoroquinolone-resistant commensal *E. coli* isolated from apparently healthy animals also carry multiple plasmids.

Sherley *et al.* (2004) confirmed that an unusually high incidence of antibiotic resistance among enteric bacteria of animal origin is usually predicated on the presence of multiple-plasmids which usually encode genes for antimicrobial resistance. Recent studies have also characterised plasmids that encode fluoroquinolone-resistance among bacteria particularly *E. coli* and Salmonella from clinical and veterinary sources (Fortini *et al*., 2009; Gutierrez *et al*., 2009; Ma *et al*., 2009; Cerquetti *et al*., 2009).

The presence of plasmids in fluoroquinolone resistant isolates is a worrisome phenomenon and it points towards a serious public health threat within the immediate human population (Venturini *et al*., 2009). Fluoroquinolone resistance genes have been found on plasmids carried by ehteric bacteria isolated from chicken and other food animals (Kehrenberg *et al*., 2006; Avsaroglu *et al*., 2007).

Most plasmids that encode fluoroquinolone-resistance are conjugative and self-transmissible-a characteristic that ensures that such plasmids spread among bacterial population. By implication, such fluoroquinolone-resistant genes sometimes carried on plasmids are constantly shed into immediate environment; contaminating food and drinking water meant for humans (Khachatourians, 1998).

CONCLUSION

In summary, this study has confirmed that the prevalence of fluoroquinolone resistance among *E. coli* isolated from cattle is relatively high and the incidence of carriage of plasmids in such isolates is correspondingly high and such plasmids are transferred at high frequency. This observation calls for proactive actions by government, health and animal care professionals to regulate consumption of antibiotics in animals and set up extensive surveillance to monitor the occurrence of fluoroquinolone-resistance among bacteria in food animals.

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