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QUINOLONES RESISTANCE AND R-PLASMIDS OF SOME GRAM NEGATIVE ENTERIC BACILLI

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Out of the two hundred and sixty bacteria isolates from clinical specimens obtained from different body sites at the University College Hospital Ibadan, 166 belonged to the family of Enterobacteriaceae and Pseudomonaceae. The isolated gram-negative enteric bacilli consist of *Escherichia coli* (22), *Klebsiella species* (65), *Proteus species* (20), *Salmonella typhi* (2), *Pseudomonas aeruginosa* (39) and *Pseudomonas species* (18). Among the antimicrobial agents tested, high resistance was found with ofloxacin 44.0%, followed by pefloxacin 30.1% and ciproflaxacin 21.7%. Ciprofloxacin has the lowest MIC of 2 – 32 µg/ml while ofloxacin has the highest 64 µg/ml. Of the 166 strains, 44 were resistant to most of the antimicrobial agents tested. All the strains that were resistant to any antimicrobial agents were also resistant to ofloxacin. A total of 27 plasmids ranging in molecular sizes from 6.6kb to 17.4kb were extracted from the resistant strains and grouped into 5 plasmid profiles. Transformation experiment revealed that 59.2% of the resistant strains carried a common R-plasmid of size 10.7kb. Plasmid-mediated resistance to ciprofloxacin and pefloxacin was found. *Klebsiella species* harboured the highest number of R-plasmids with 8, followed by *Pseudomonas aeruginosa* with 4.

INTRODUCTION

The fluoroquinolones are a new class of synthetic antimicrobial agents which have greater activity against Gram positive and Gram-negative bacteria than the older quinolone analogue, nalidixic acid and oxolinic acid which are used for urinary infection and occasionally enteric infections (1, 2). In veterinary medicine, fluoroquinolones are used for treatment and metaphylaxis but not as growth promoters.

Quinolone resistance is increasing in clinical isolates and has reached a minimum inhibitory concentration (MIC) in *Escherichia coli* at 265 mg/ml for ciprofloxacin (3, 4, 5). The mechanism for this resistance involve chromosomal mutations that modify DNA gyrase or DNA topoisomerase IV the targets of quinolone action or results in decreased quinolone accumulation (6, 7, 8).

Multiple antibiotic resistance in bacteria is most commonly associated with the presence of plasmids which contain one or more resistance genes, each encoding a single antibiotic resistance phenotype (9-15). Transferable resistance to fluoroquinolones has been found in a clinical isolate of *K pneumoniae* in a broad host range plasmid (16).

Quinolone such as ciprofloxacin and ofloxacin have been introduced in to Nigeria while newer ones like pefloxacin and sparfloxacin are just being introduced in to Nigeria by some pharmaceutical companies under different trade names. These quinolones are used for various diseases ranging from urinary infections, enteric infections, septic wound, septicaemia etc. Some researchers have opined that susceptibility to quinolones may remain high in Nigeria as these drugs are expensive and beyond the reach of most individuals, but their use is increasing and resistance may become problematic in the years to come (17-20).

The recent incidence of emergence of resistance to the quinolones as reported by Oni *et al* (19) has prompted this present study. Thus this paper describes the antimicrobial susceptibility testing and plasmid screening of some gram-negative bacilli commonly isolated from clinical specimens in University College Hospital Ibadan, Nigeria.

MATERIALS AND METHODS

Bacteriology

Sixty-five strains of *Klebsiella spp.*, 22 strains of *Escherichia coli*, 20 strains of *Proteus spp.*, 18 strains of *Pseudomonas spp.*, 39 strains of *Pseudomonas aeruginosa* and 2 strains of *Salmonella typhi* isolated by standard procedures (21, 22) from 260 clinical specimens sent to the diagnostic laboratory of Medical Microbiology and Parasitology Laboratory of University College Hospital, Ibadan from May to December 2002 were studied.

Antimicrobial susceptibility testing

Antimicrobial disc susceptibility tests were carried out on the isolates using stokes disc diffusion technique (23) on freshly prepared Mueller-Hinton agar (Oxoid, England), and standardized by the method of National Committee for Clinical Laboratory Standard (24), using the following antibiotic discs; Pefloxacin 5μg (Peflotab), Ofloxacin 30 μg (Tarivid), Ciprofloxacin 5μg (Ciprotab), Ceftazidime 30 μg (Fortum), Ceftriaxone 30μg (Rocephine), Gentamicin 10μg (Abtek), Amoxicillin 25μg (Abtek), Augmentin 30μg (Abtek), Cotrimoxazole 30μg (Abtek). *Escherichia coli* (NCTC 10418) was used as control. Plates with antibiotic discs were incubated for 24 hours at 37oC and sensitivity pattern was compared with that of the control organism.

Minimum inhibitory concentration

The minimum inhibitory concentration (MICs) of ciprofloxacin, pefloxacin and ofloxacin for all the bacterial isolates was determined as described by Goldstein and Acar (3). Serial doubling dilution of the antimicrobials was made for the range of 0.0625 μg/ml to 256 μg/ml. Such stock diluted solution of ciprofloxacin, pefloxacin and ofloxacin was added to universal bottles containing 10ml volumes of nutrient broth to produce equivalent concentration of antibiotic required. Each dilution was inoculated with one drop (0.02ml) of the overnight broth culture containing 10^4 organisms/ ml of the test organisms using different sterile Pasteur pipettes. Five colonies of the species were then suspended in to 5 ml of Mueller Hinton broth and incubated overnight. The culture was then diluted 1 in 1000. The same procedure was repeated for the control organisms *Escherichia coli* NCTC 109418. A tube of broth without antibiotic was also included to show the suitability of the broth for the growth of the organism and unseeded tube of broth with and without antibiotic to serve as controls of the sterility and clarity of the broth. All plates were incubated at 37°C for 18 hours.

Isolation and separation of plasmid DNA

Plasmid DNA was isolated, separated and stained as previously described (4). Plasmid profile groups were constructed by grouping strains possessing the same profile (containing the same number and molecular mass) or part of a profile constituting a core profile. Bacterial strains that carried the plasmid were regarded as constituting one plasmid group.

Genetic transfer

Transformation was done as described by Hanahan (25) using *Escherichia coli* K-12, HB101 (ara-14, galk2, hsd520, lacyl, leu, mtl01, pro A2, recA13, rpsl20, supE44, thii xyl-5) as recipient and plasmid pBR322 as the positive control. Co-transformation of resistant character was determined by testing all transformants against all antibiotics to which the donor strains was resistant, extracts from transformants were obtained as described above and subjected to agarose gel electrophoresis. Transformation was confirmed as positive only when resistant transformants were shown to contain a plasmid (s) of a size similar to that found in the original isolate.

Plasmid curing

The curing of the resistant plasmids of the clinical bacterial isolates was done as described by Vivyan *et al* (35).

RESULTS

The sources of the clinical bacterial isolates are shown in Table 1. The isolates were from various body sites.

Table 1: Sources of the bacterial isolates

Body sites	**Bacterial isolates**						Total
	E. coli	*K. spp*	*P. spp*	*Ps. aeruginosa*	*S. typhi*	*Ps. spp*	
Ear swab	0	8	9	19	0	7	43
Wound swab	13	23	9	13	1	6	65
Throat swab	1	3	1	0	0	0	5
Conjunctival swab	0	1	0	0	0	1	2
High vaginal swab	3	9	1	0	0	2	15
Endocervical swab	1	2	0	0	0	0	3
Sputum	0	7	0	6	0	1	14
Urine	3	8	0	1	0	0	12
Tracheal aspirate	1	2	0	0	0	1	4
Cerebrospinal fluid	0	2	0	0	1	0	3
Total	**22**	**65**	**20**	**39**	**2**	**18**	**166**

Table 3: MICs of a cumulative percentage of isolates with inocula of 10^5 CFU

Organisms (No. of Strains)	**Antimicrobial Agents**	**MIC_{50}**	**MIC range (µg/ml)**	**MIC_{90}**
Escherichia coli (22)	Ciprofloxacin	4	0.0313 – 64	8
	Pefloxacin	8	0.125 – 64	16
	Ofloxacin	8	0.125 – 128	32
Klebsiella spp (65)	Ciprofloxacin	4	0.0625 – 64	16
	Pefloxacin	8	0.25 – 128	32
	Ofloxacin	8	0.5 – 128	32
Proteus spp (20)	Ciprofloxacin	2	0.0313 – 32	4
	Pefloxacin	4	0.0625 – 64	8
	Ofloxacin	4	0.0625 – 64	16
Pseudomonas aeruginosa (39)	Ciprofloxacin	8	0.25 – 128	32
	Pefloxacin	16	0.5 – 128	64
	Ofloxacin	16	0.5 – 256	64
Salmonella typhi (2)	Ciprofloxacin	4	0.0313 – 32	4
	Pefloxacin	4	0.0625 – 32	8
	Ofloxacin	8	0.0625 – 32	16
Pseudomonas spp (18)	Ciprofloxacin	8	0.25 – 128	32
	Pefloxacin	16	0.5 – 128	64
	Ofloxacin	16	0.5 – 256	64

Key MIC_{50} Minimal inhibitory concentration for 50 percent of strains
MIC_{90} Minimal inhibitory concentration for 90 percent of strains
CFU = Colony Forming Unit

Of the 166 clinical bacterial strains isolated, 44 were resistant to most of the antimicrobial agents tested (Table not shown). The frequency of susceptibility to ceftazidime was the highest (83.1%) while the sensitivity to fluoroquinolones was, ciprofloxacin (78.3%), pefloxacin (69.9%) and ofloxacin (56.0%) (Table2).

Table 2: Antibiotic sensitivity pattern of some Gram negative enteric bacilli

Antibiotics	Number sensitive	% sensitive	% Resistant
Ciprofloxacin	130	78.3	21.7
Pefloxacin	116	69.9	30.1
Gentamicin	73	44.0	56.0
Amoxicillin	6	3.6	96.4
Augmentin	11	6.6	93.4
Cotrimoxazole	3	1.8	98.2
Ceftazidime	138	83.1	16.9
Ofloxacin	93	56.0	44.0
Ceftriaxone	124	74.7	25.3

All the strains that were resistant to any antimicrobial agents were also resistant to ofloxacin. The MIC expressed as MIC_{50}, MIC_{90} and range are shown in Table 3. Considering the Gram-negative bacilli, ciprofloxacin has the best sensitivity result, followed by pefloxacin and then ofloxacin. A total of 27 different plasmids with molecular mass ranging from 6.6kb to 17.4kb were observed in the antibiotic resistant strains. Plasmids were not detected in 17 of the resistant strains indicating that their resistance was probably chromosomally borne. Five different plasmid profile groups for the antibiotic resistant strains could be defined. The number of strain per plasmid profile group vary from 1-24 (Table 4).

Table 4: Plasmid profile groups of antibiotic resistant bacterial strains

Plasmid Profile	No. of strains	Molecular mass (kb) of plasmids
0	17	No plasmids
1	24	10.7
2	1	10.7, 6.6
3	1	10.7, 17.4
4	1	11.7

The most common antimicrobial resistance pattern was CipPefOfxGenAmxAugCotCazCro. This was followed in decreasing order of occurrence by the R-types resistance patterns; CipPefOfxGenAmxAugCotCazCro,PefOfxGenAmxAugCotCazCro,CipPefOfxGenAmxAugCot,OfxAmxAugCotGen,CipPefOfxGenAmxAugCotCro, and CipOfxGenAmxAugCotCazCro (Table 5).

Table 5: Antimicrobial resistance patterns of 44 clinical bacteria strains in relation to plasmid contents

Antimicrobial Resistance patterns	No	%	No with plasmids
Cip Pef Ofx Gen Amx Aug Cot Caz Cro	9	20.5	6
Pef Ofx Gen Amx Aug Cot Caz Cro	8	18.2	3
Cip Pef Ofx Gen Amx Aug Cot Caz Cro	1	2.3	1
Cip Pef Ofx Gen Amx Aug Cot Caz Cro	11	25.0	7
Cip Pef Ofx Gen Amx Aug Cot Caz Cro	4	9.1	4
Cip Pef Ofx Gen Amx Aug Cot Caz Cro	6	13.6	4
Ofx Amx Aug Cot Gen	5	11.4	2

Key: Cip = Ciprofloxacin, Pef = Pefloxacin, Ofx =Ofloxacin, Gen = Gentamicin, Amx = Amoxicillin, Aug = Augmentin, Cot = Cotrimoxazole, Caz = Ceftazidime, Cro =Ceftriaxone

Strains showing the resistance pattern, CipPefOfxGenAmxAugCotCazCro, harboured the highest number of plasmids while the lowest number was found in the single strain (*Klebsiella spp*) with the resistance pattern, CipPefOfxGenAmxAugCotCazCro.

Transformation experiment showed that 59.2% of the resistant strains that harboured plasmids were able to transfer their resistance plasmids to *E. coli* k-12 HB 101. Plasmid-determined resistance to ciprofloxacin and pefloxacin was found. It is noteworthy that all the R-plasmids isolated in this study have a common molecular size of 10.7Kb (Table 6).

Table 6: Characteristics of some of the clinical bacterial R-plasmids

Bacterial strain	Plasmids molecule size (kb)	Antibiotic gene transferred to *E. coli* Hb101	Transformant R-plasmid size (kb)
MmB8 (*K. spp*)	10.7	Pef	10.7
MmB10 (*Ps. aeruginosa*)	10.7	Cip	10.7
MmB11 (*K. spp*)	10.7	Cip Pef	10.7
MmB17 (*Ps. aeruginosa*)	10.7	Cip Pef	10.7
MmB30 (*K. spp*)	10.7	Cip Pef	10.7
MmB32 (*K. spp*)	10.7	Cip Pef	10.7
MmB36 (*K. spp*)	10.7	Pef	.0.7
MmB37 (*Pr. Spp*)	10.7	Cip Pef	10.7
MmB41 (*Ps. aeruginosa*)	6.6, 10.7	Pef	10.7
MmB42 (*K. spp*)	10.7	Cip Pef	10.7
MmB43 (*Ps. aeruginosa*)	10.7	Pef	10.7

It is also significant that strain MmB4, *Pseudomonas aeruginosa* was able to transfer its 10.7Kb R plasmid to *E. coli* HB101. All the strains harbouring R-plasmids were cured of their plasmids upon treatments with sodium dodecy sulphate (SDS), with resultant loss of their plasmid-associated properties. This indicates that the antibiotic resistant genes of the bacterial strains used in this study were plasmid borne.

DISCUSSION

Most of the Gram-negative bacilli especially *Klebsiella species* and *Pseudomonas species* are intrinsically resistant to most antibiotics, a situation which favour their continued existence in hospital environment (13, 17, 18, 20, 27). This fact greatly contributes to the high incidence of these agents among the patients. The comparative disc sensitivities shown in Table 2 is similar to that obtained by Oni *et al* (19) and Ozumba (20). The increasing resistance of enterobacteriaceae to fluoroquinolones: ciprofloxacin (21.7%), pefloxacin (30.1%) and ofloxacin (44.1%), is in agreement with the studies of Threfall *et al* (4), Nema *et al* (28), Gara *et al* (29). In this study *Pseudomonas aeruginosa* had 85% sensitivity to ciprofloxacin in contrast to the report of Odugbemi *et al* (18) which documented a 100% sensitivity of *Pseudomonas aeruginosa* to ciprofloxacin in 1994 in Lagos, Nigeria.

Most strains have MICs > 4 μg/ml with values as high as 64 μg/ml for ofloxacin. Comparatively ciprofloxacin has the lowest MIC of the fluoroquinolones used in this study. This is similar to the study of Threfall *et al* (4) in which high level resistance to ciprofloxacin in *Escherichia coli* with the MIC's range of 4-8 μg/ml. The antimicrobial resistance pattern revealed a total of seven patterns. This indicates the emergence of resistance to the quinolones in our environment. Also these patterns depict the occurrence of multiresistant strains. This is similar to that obtained by Zafar (30), Wallace *et al* (31) and Livermore *et al* (1). All the strains that were resistant to any antimicrobial agents were also resistant to ofloxacin.

Resistance to high levels of antibiotics has been ascribed in most instances to the presence of plasmids (9, 11,

12, 14, 15). Thus the presence of plasmids among the resistant strains is quite different from the report of Martinez-Martinez *et al* (16). The most common plasmids encountered were 10.7Kb in size. This is in agreement with the findings of Moller *et al* (32), Daini *et al* (9), Wang *et al* (33), and Ogunledun et al (13). 59.2% of the drug-resistant strains carried R-plasmids. Plasmid determined resistance to ciprofloxacin and pefloxacin was found. The emergence of R-plasmids in this study could be due to the indiscriminate and widespread use caused by the over-the counter availability of antibiotics as well as the higher exposure of people to enteric flora in places with poor sanitation (9, 10, 13, 18). A different plasmid profile could be seen for each of the 16 R-plasmids and plasmids of the same molecular weight could be found in different strains. Thus the plasmid profile of these strains was diverse in nature.

Plasmid profiling analysis distinguished more strains than the antimicrobial susceptibility patterns in agreement with the findings of Daini *et al* (9), Levy *et al* (12) and Senerwa *et al* (34). Plasmid profiling has been shown to be a good epidemiological tool in investigating epidemics or outbreaks of bacterial diseases (35, 36). The transformation experiment enabled us to detect non-self transmissible plasmids while curing of the resistant strains of the R-plasmids with SDS showed that their antimicrobial-resistant genes were plasmid-borne (9, 26).

The results of our study highlighted diverse plasmid profiles and widespread antimicrobial resistance patterns of some Gram negative enteric bacilli isolates from Nigeria and we hope that this information from this locality would be a useful baseline for further epidemiological studies.

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