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Research Article

Effects of *gyrA* and *parC* Mutations in Quinolones Resistant Clinical Gram Negative Bacteria from Nigeria

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ABSTRACT: The most important of fluoroquinolones resistance mechanisms is the accumulation of mutations in the bacterial enzymes targeted by fluoroquinolones; DNA gyrase and DNA topoisomerase IV. The effect of gyrase and Topoisomerase IV enzymes mutations on quinolones resistance in clinical Gram negative bacteria in Nigeria was extensively investigated. 115 Gram-negative bacteria of 4 species were analyzed for antimicrobial susceptibility, polymerase chain reaction amplifications of quinolone resistance determining regions, mutation detection using denaturing high-performance liquid chromatography or sequencing. These strains were *Klebsiella pneumoniae*, *Escherichia coli*, *Proteus mirabilis*, *Pseudomonas aeruginosa*. Minimum inhibitory concentrations showed that the level of resistance was high with MIC_{50} greater than clinical break point for all drugs. 85 of the 115 isolates carried a mutation in QRDRs. Mutations in *gyrA* were found at positions 83 and 87 of the quinolone resistance determining regions (QRDRs). 4 amino acid substitutions were seen in *gyrA* including double mutations at codons 83 + 87 while 2 substitutions were seen within *parC* at codons 80 and 87. All *parC* mutations were seen in strains which also carried a *gyrA* mutation. Mutation at codon position 83 with amino acid subtitution of leucine for serine resulted in a higher MIC than substitution to threonine at position 83 or double mutations of serine 83 and asparagine 87. In conclusion, quinolone resistance in clinical Gram negative bacteria in Nigeria is also mediated by accumulation of mutations at QRDR.

Key Words: *Quinolones, topoisomerase, mutation, bacteria, Nigeria*

INTRODUCTION

Quinolones seem to confound resistance; they are a class of agents to which mutational resistance is unlikely to develop and against which resistance genes could not be acquired. Fluoroquinolone antibiotics achieve higher serum levels than those of nalidixic acid and are more potent against Gram negative bacteria; drug concentrations 1000-fold more than those required to inhibit growth are routinely achieved (Robicsek *et al.*, 2006). The minimum inhibitory concentration, MIC required for the most resistant *Escherichia coli* has reached 256 µg/ml for ciprofloxacin; this represents an increase in resistance of over 8000-fold (Threlfall *et al.*, 1997; Murphy *et al.*, 1997). Daini et al. had reported high-levels of fluoroquinolone resistance in clinical isolates of Gram negative bacteria isolates in Nigeria (Daini *et al.*, 2006). Similarly, levels of quinolone resistance in clinical *E. coli* isolates have been reported at 40% in Hong Kong (Ling *et al.*, 2006).

Two mechanisms of resistance had been found to determine resistance to fluoroquinolones. The most important of these mechanisms in Gram negative bacilli is the accumulation of mutations in the bacterial enzymes targeted by fluoroquinolones: DNA gyrase and DNA topoisomerase IV (Hooper, 2003). The other described mechanism of resistance operates by decreasing intracellular drug accumulation by

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upregulation of native efflux pumps (Webber and Piddock, 2001; Poole, 2005) either alone or together with decreased expression of outer membrane porins (Ruiz, 2003; Piddock, 2006). Chromosomal resistance to fluoroquinolones (FQs) due to amino acid substitutions in the quinolone resistance-determining regions (QRDRs) of DNA gyrase (GyrA) and/or topoisomerase IV (ParC) has been reported (Jacoby, 2005). Several studies have reported transferable resistance to fluoroquinolones or nalidixic acid caused by plasmid mediated quinolones resistance (PMQR) in clinical isolates of Gram negative bacteria in several countries (Park *et al.*, 2006; Robicsek *et al.*, 2006; Yamane *et al.*, 2007**)** including Nigeria (Soge *et al.*, 2006; Ogbolu *et al.*, 2011). This type of resistance is usually low, higher levels of quinolone resistance arose readily by mutation (Martinez-Martinez *et al.*, 2003; Wang *et al.*, 2004). This study however extensively investigated the effect of gyrase and Topoisomerase IV enzymes mutations on quinolones resistance in clinical Gram negative bacteria from Nigeria.

MATERIALS AND METHODS

Bacterial strains

One hundred and fifteen clinical Gram negative bacterial isolates of 4 species were obtained from 585 non duplicate clinical specimens including; aspirates, ear swab, wound swab, throat swab, high vaginal swab, eye swab, sputum, urine, cerebrospinal fluid and blood culture for a period between 2005 and 2007. Single isolates from each specimen were retained. The isolates were from four teaching hospitals in south-western Nigeria, University College Hospital, Ibadan; Obafemi Awolowo University Teaching Hospital, Ile-Ife; Ladoke Akintola University of Technology Teaching Hospital, Osogbo and Olabisi Onabanjo University Teaching Hospital, Sagamu. All isolates were speciated using API 20E strips (BioMerieux, France).

Antimicrobial susceptibility testing

Disc diffusion: All the bacterial isolates were tested by disc diffusion method using standard sensitivity agar plates (Oxoid Iso-sensitest) onto which commercially prepared discs had been asceptically placed as recommended by British Society for Antimicrobial Chemotherapy (BSAC). The oxoid discs and their concentrations are shown in Table 2. The plates were incubated aerobically at 37^{o}C for 24 hours. Inhibition zone diameters were measured to the nearest millimeter using a calibrated transparent ruler. The susceptible inhibition zone diameter breakpoint used throughout the study for each antibiotic to the various organisms was based on BSAC recommendations (http://www.bsac.org.uk/susceptibility_testing/guide_to_antimicrobial_susceptibility_testing.cfm). The diameters of the zone of inhibition were recorded. Growth within the zone of inhibition was recorded as resistance. Sensitivity patterns were compared against control strains *Escherichia coli* (NCTC 10418) and *Pseudomonas aeruginosa* (NCTC 10662).

Agar dilution: The minimum inhibitory concentrations (MICs) of β-lactam antibiotics were determined. MIC testing was performed using the agar dilution method according to the recommendation of BSAC All runs included the control organisms; *Escherichia coli* (NCTC 10418) and *Pseudomonas aeruginosa* (NCTC 10662). A start and finish plate without antibiotic was also included as a growth control.

Genomic DNA preparation

Genomic DNA was required for amplification of various genes encoding topoisomerase enzymes; gyrA, gyrB, parC or parE. An overnight culture of 1.5 ml of Iso-sensitest broth was pelleted by centrifugation at 12,000 rpm for 3 minutes in a micro centrifuge (MSE, U.K.) to harvest bacteria. The resulting supernatant was discarded and pellets then re-suspended in sterile double de-ionised water. The genomic DNA was then extracted using the Invisorb Spin Cell Mini Kit (Invitek, Gesellschaft fur Biotechnik & Biodesign mbH) according to manufacturer's instructions. At the end of the procedure, 5 µl of DNA was visualised after electrophoresis on a 1% agarose gel (see 3.7) at 100 V for 1 h 30 min. The gels were visualised in a SYNGENE Gene Genius image analyser (SYNGENE, Cambridge, U.K.). DNA was stored at -20ºC until needed.

Amplification of quinolone resistance determining regions (QRDRs)

Polymerase chain reaction was used to amplify the quinolone resistance determining regions (QRDRs) of *gyrA, gyrB, parC* and *parE* for mutation detection. The primers sequence, annealing temperatures and product sizes are shown in Table 1. The other PCR conditions were as follows; 1 min denaturation at 95 ^{o}C and 5 min extension at 72^{o}C, for a total of 30 cycles.

Denaturing high-performance liquid chromatography (DHPLC)

High through-put mutation detection to identify mutations within genes of interest was achieved using DHPLC. DHPLC was performed with the WAVE DNA fragment analysis system (Transgenomic Inc.) which employs a combination of temperature-dependent denaturation of DNA and ion pair

chromatography. Briefly, DNA specifically amplified from the test strain is mixed with that amplified from the wild-type strain. The amplimers are denatured simultaneously in the same reaction mixture and then allowed to slowly reanneal. Thus, four species of duplex DNA can form. Heteroduplexes will result in double-stranded DNA containing a mismatch bubble at the point mutation site. Under the non-denaturing conditions (~51ºC) used to separate DNA fragments, all four duplexes will have the same retention time during ion pair chromatography. As the temperature increases from 54 to 63ºC (dependent on DNA sequence), the heteroduplex DNA fragments start to denature in the region on either side of the mismatched bases and are eluted from the column ahead of the still intact homoduplexes. It is this combination of ion exchange and partial denaturation that forms the basis of DHPLC. Temperature-dependent resolution of homo- and hetero- duplexes occurs and can range from full separation of all four duplex species to a single peak with a shift in retention time dependent upon analysis temperature. These changes in elution profile are indicative of mutations in the DNA. Subtle differences in elution profile were confirmed by overlay of the profiles in question using NAVIGATOR (Transgenomic) software. This was particularly important for the recognition of multiple mutations as described previously (Eaves *et al.*, 2002).

Polymerase chain reaction products (15 µl) were mixed with an equal amount of DNA amplified from a wild-type standard. DNA mixtures were hybridized to form heteroduplexes by heating to 95ºC for 4 min and then cooled by lowering the temperature down gradually to 35ºC in 1ºC/min steps. The NAVIGATOR utility software was used to determine the correct partial denaturation temperature for mutation scanning based on the sequence of the wild-type DNA from *E. coli* (NCTC 10418) and *P. aeruginosa* (NCTC 10662).

One hundred and fifteen Gram negative bacilli duplex products (*gyrA, B, par C* and *E*) and 1 negative control (water) were analysed using the wave Nucleic Acid fragment analysis system (95 samples and a control were run at a time). Duplex DNA (15 µl) was loaded on the DNASepCatridge (Transgenomic) in 54% eluent A-46% eluent B. The gradient for separation was a 4-min linear gradient from 51% eluent B to 59% eluent B. The flow rate was 0.9 ml min^{-1}. Eluent A contained 0.1 M triethylammonium acetate (TEAA), and eluent B contained 0.1 M TEAA in 25% (vol/vol) acetonitrile. The column was subsequently washed in 100% eluent B for 30s and then re-equilibrated with 54% eluent A-46% eluent B between injections. DNA fragment elution profiles were captured online and visually displayed using Transgenomic NAVIGATOR software.

DHPLC analysis of topoisomerase fragments was initially performed at 61ºC (the predicted average melting temperature over the whole product sizes for the topoisomerase genes fragments was 62ºC) and then at 63ºC to optimize mutation detection at the 3´ region.

Sequencing

PCR products required for sequencing were cleaned using QIAquick PCR purification kits (Qiagen) according to manufacturer's instruction. Gel electrophoresis was used to determine the quality and quantity of purified amplimers and to inform the dilution of DNA for sequencing. If the purified band was weak, the DNA was used without dilution. A strong band was diluted 1 in 2 in sterile distilled water or 1 in 5 and very strong bands were diluted 1 in 10.

Table 1.
Primers used to amplify topoisomerase genes in Gram-negative enteric bacilli.

Forward Primer	Sequence (5´to3´)	Reverse Primer	Sequence (5´to3´)	Annealing Temp (º	Product Size (bp)
EcgyrA 4	TCGTTGGTGACGTAATCGGT	*EcgyrA*5	TCCGTGCCGTCATAGTTATC	55	253
EcgyrB1	CAGACTGCCAGGAACGCGAT	*EcgyrB2*	AGCCAAGCGCGGTGATAAGC	59	203
*PagyrA*1	CTATCTCGACTACGCGATGA	*PagyrA*2	ATCTGCTCGGTGCCATCGTA	57	399
PagyrB1	AATTCAGGTCGCAGACCAAG	*PagyrB2*	ATGATGTTGTGGTAGCGCAG	57	489
*EcparC*1	AACCTGTTCAGCGCCGCATT	*EcparC*2	ATGCGGTGGAATATCGGTCG	56	434
EcparE1	TACCGAGCTGTTCCTTGTGG	*EcparE2*	GGCAATGTGCAGACCATCAG	57	237
*PaparC*1	GAGCAGGCCTATCTGAACTA	*PaparC*2	ACGGCACCTCGGAATAG	57	355
*PaparE*1	CGGCGTTCGTCTCTCGGGCG	*PaparE*2	TCGAGGGCGTAGTAGATG	57	591

Six microlitres of this diluted DNA was added to 4 µl of primer at a concentration of 0.8 pmol/µl. Final 10 µl reaction mixtures containing 5 ng PCR products were used as template for *Taq*Cycle Sequencing using ABI Prism Big Dye Terminator version 3.0 cycle sequencing kits (Applied Biosystems). Cycle sequencing products were subsequently analysed on an ABI PRISM 3700 DNA analyser (Functional Genomics Laboratory, University of Birmingham). For each PCR product an amplimer from a control strain was also sequenced in order to confirm amplification of the expected product.

RESULTS

Identification of Bacterial species

Four Gram negative bacteria were identified and they consisted of *Klebsiella pneumoniae*, *Escherichia coli*, *Proteus mirabilis*, *Pseudomonas aeruginosa*. The strains were obtained from diverse clinical sources in order to capture as much diversity as possible.

Susceptibility testing

All the strains examined showed resistance to one or more of the eleven antibiotics used for disc susceptibility study. The results depict a pattern of multiple and high level resistance; more than 75% of isolates were resistant for each antibiotic. The fluoroquinolones showed slightly lower level of resistance than the rest of the antibiotics including the third generation cephalosporins. More isolates were sensitive to ciprofloxacin than all the antibiotics tested (23.5%). The least active drugs were nalidixic acid and tetracycline with only 1.7% of isolates sensitive to each (Table 2).

Table 2: Antibiotic Susceptibility Pattern of 115 Bacterial Strains

Antibiotic (µg/ml)	No. of Strains Sensitive	% Sensitive Strains	% Resistant Strains
Ciprofloxacin (5)	27	23.5	76.5
Pefloxacin (5)	22	19.1	81.9
Ofloxacin (30)	26	22.6	77.4
Sparfloxacin (5)	23	20.0	80.0
Nalidixic acid (30)	2	1.7	98.3
Ceftazidime (30)	16	13.9	86.1
Ceftriazone (30)	14	12.2	87.8
Gentamicin (10)	17	14.8	85.2
Augmentin (30)	14	12.2	87.8
Amoxicillin (25)	8	7.0	93.0
Tetracycline (30)	2	1.7	98.3

Table 3. MICs of species to fluoroquinolone antibiotics

Organisms (no of strains)	Antimicrobial Agent	MIC_{50}	MIC_{90}	Range	Resistance (%)[a]
		(µg/ml)			
Escherichia coli	Ciprofloxacin	256	256	0.015-256	24 (85.7)
N = 28	Pefloxacin	256	256	0.25-256	17 (60.7)
	Ofloxacin	128	256	0.25-256	22 (78.6)
	Sparfloxacin	256	256	0.25-256	26 (92.9)
K. pneumoniae	Ciprofloxacin	256	256	0.015-256	48 (76.2)
N = 63	Pefloxacin	256	256	0.25-256	39 (61.9)
	Ofloxacin	256	256	0.25-256	58 (92.1)
	Sparfloxacin	256	256	0.25-256	62 (98.4)
Proteus mirabilis	Ciprofloxacin	128	256	0.015-256	10 (90.9)
N = 11	Pefloxacin	256	256	0.25-256	10 (90.9)
	Ofloxacin	128	256	0.25-256	10 (90.9)
	Sparfloxacin	256	256	0.25-256	11 (100)
Pseudomonas aeruginosa	Ciprofloxacin	256	256	0.015-256	12 (92.3)
N = 13	Pefloxacin	256	256	0.25-256	11 (84.6)
	Ofloxacin	256	256	0.25-256	11 (84.6)
	Sparfloxacin	256	256	0.25-256	11 (84.6)

[a] *Percentage resistant according to clinical breakpoint*

Determination of precise MIC values confirmed the numbers of isolates resistant to clinical breakpoint concentrations for fluoroquinolone antibiotics using BSAC guidelines. The MIC results also showed that the level of resistance was extremely high with MIC_{90} values of 256 µg/ml or higher for all drugs. The mean percentage of strains resistant to each of the β-lactam drug was 85% (Table 3).

Mutation in QRDR is Responsible for Quinolone Resistance

Mutations in the quinolone target enzymes, DNA gyrase (*gyrA, gyrB*) and Topoisomerase IV (*parC, parE*) were detected by PCR amplification of the QRDRs of these genes (Figures 1, 2) followed by DHPLC to detect mutations. Any change from the single-peak profile characteristic of wild-type (Figure 3) indicated formation of heteroduplexes and therefore at least one mutation within the test DNA fragment. Eighty-five of the 115 isolates carried a mutation in QRDR, no mutation was detected in *gyrB* or *parE* from any of the isolates studied. However, DHPLC detected 10 different profiles in *gyrA*, single profile for *Pseudomonas aeruginosa* and 9 profiles for the rest of the isolates. Similarly, 1 profile for *Pseudomonas aeruginosa* and 4 different profiles were found within *parC* amplified from the rest of Gram negative bacteria (Figure 4). All samples containing the same single-base substitutions had identical profiles when overlayed using the NAVIGATOR (Transgenomic) software.

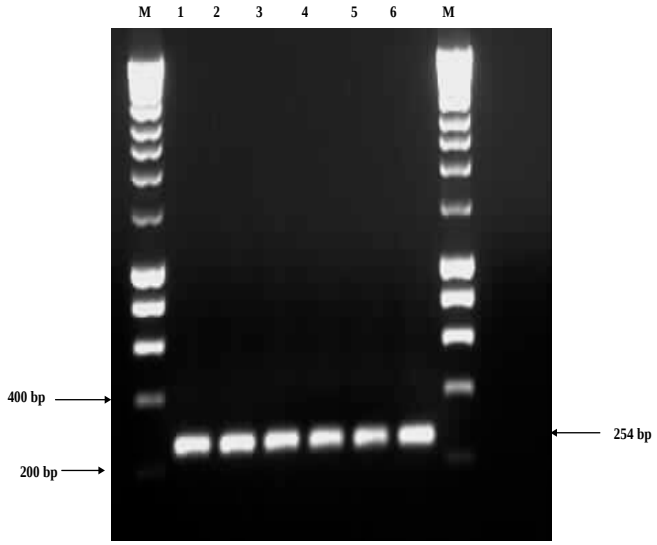

Figure 1
PCR amplification of QRDR of DNA gyraseA for *Enterobacteriaceae*. Lane M: Hyperladder I; 14 bands from 200 – 10,000 bp. Lanes 1 – 6: Test strains. Expected product size is 254 bp.

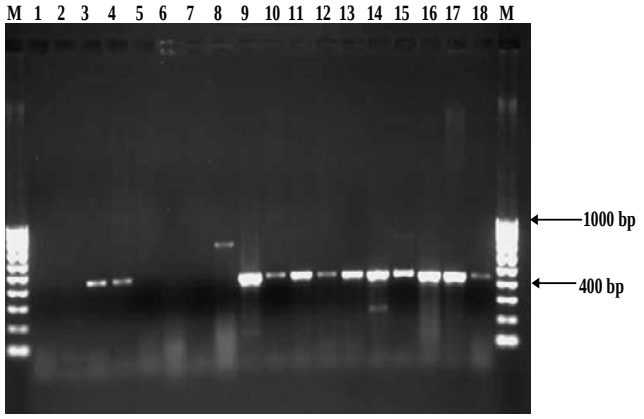

Figure 2
PCR amplification of parC from *Enterobacteriaceae*. Lane M: Hyperladder I; 14 bands from 200 – 10,000 bp. Lanes 1 – 17: Test strains, lane 18: positive control. Expected product size is 434 bp.

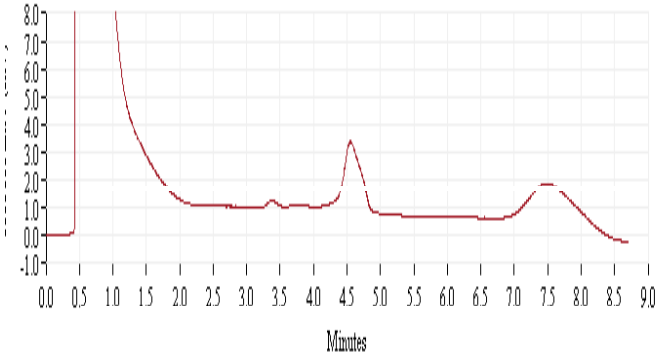

Figure 3.
The wild type strain, NCTC 10418 showing the characteristic single peak of gyrA with no mutation.

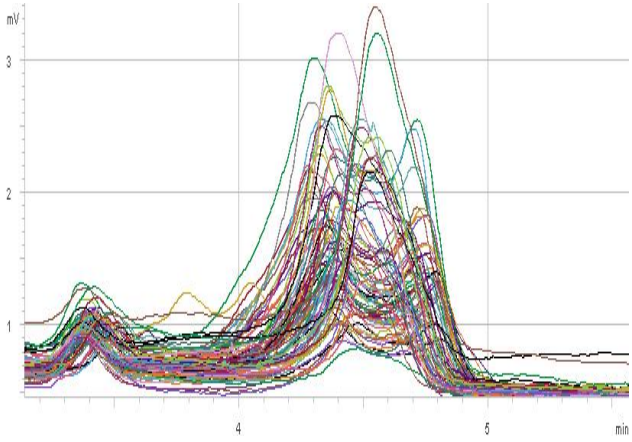

Figure 4
Mutation detection of gyrA genes using Wave Navigator^{R} software. Each pattern represents one strain, identical patterns reflect identical genotypes.

Table 4:
Summary of the mutations detected in the DNA gyrase and Topoisomerase IV genes of the isolates

Gene	Codon position	Nucleotide sequence	Substitution	Strain	Quinolone MIC range (µg/ml)					
					N	NAL	CIP	OFL	PEF	SPX
gyrA	Serine 83	TCG-TTG	Leucine	*K. pneumo*	40	>256	8-256	4-256	32-256	8-256
				E. coli	15	32-256	2-256	1-256	2-256	2-256
	Serine 83	TCG-ATC	Isoleucine	*K. pneumo*	6	>256	256	32-256	256	256
	Serine 83	TCG-TTG	Leucine	*P. mirabilis*	7	>256	128-256	128-256	256	256
	Threonine 83	ACC-ATC	Isoleucine	*P. aerug.*	7	>256	0.5-256	2-256	2-256	2-256
	Serine 83/ Aspartate 87	TCG-TTG/ TGA-TAA	Leucine/ Asparagine	*K. pneumo*	10	>256	256	32-256	256	256
parC	Serine 80	AGC-ATC	Isoleucine	*K. pneumo*	27	>256	256	16-256	256	256
				E. coli	7	>256	256	64-256	256	256
	Serine 80	AGC-ATC	Isoleucine	*P. mirabilis*	5	>256	128-256	128-256	256	256
	Serine 87	TCG-TTG	Leucine	*P. aerug.*	7	>256	0.5-256	2-256	2-256	2-256

n: frequency, MIC: minimum inhibitory concentration
NAL: nalidixic acid, CIP: ciprofloxacin, OFL: ofloxacin, PEF: pefloxacin, SPX: sparfloxacin

Single mutations at the same site but which incorporated a different substitution were easily seen as different DHPLC profiles. DHPLC was both sensitive and reproducible, with even small shifts in retention times (accurate to 0.08 min) indicating possible mutations. Mutations were confirmed by DNA sequencing representatives of each pattern and mutations in *gyrA* were found at positions 83 and 87 of the quinolone resistance determining region (QRDR). Four amino acid substitutions were seen in *gyrA* including double mutations at codons 83 + 87 while 2 substitutions were seen within *parC* at codons 80 and 87. Of the 115 clinical isolates, 85 had mutations in *gyrA* (*K. pneumoniae*; 56 out of 63, *Escherichia coli*; 15 out of 28, *P. mirabilis*; 7 of 11 and 7 of the 13 *Pseudomonas aeruginosa*), 46 in *parC* (*K. pneumoniae*; 27 out of 63, *Escherichia coli*; 7 out of 28, *P. mirabilis*; 5 out of 11, including 7 of *Pseudomonas aeruginosa*). All *parC* mutations were seen in strains which also carried a *gyrA* mutation. The MIC range of nalidixic acid to strains with a *gyrA* mutation was >256 µg/ml except for *E. coli* with 32-256 µg/ml. Mutation at codon position 83 with amino acid subtitution of leucine for serine resulted in a higher MIC than substitution to threonine at position 83 or double mutations of serine 83 and asparagine 87 (Table 4).

DISCUSSION

In this study, there was high level resistance of the Gram negative bacteria isolated from separate hospitals to the fluoroquinolones and other antibiotics. Most strains had MICs values as high as 256 µg/ml to the fluoroquinolones. The MIC break points for defining ciprofloxacin susceptibility have been proposed by Threfall *et al.* (1997) as 1 µg/ml, susceptible; 2 µg/ml, moderately susceptible; 4 µg/ml, resistant and BSAC gives clinical break points as 0.5 µg/ml, susceptible; 1 µg/ml, intermediate; >1 µg/ml, resistant for fluoroquinolones (http://www.bsac.org.uk/susceptibility_testing/guide_to_antimicrobial_susceptibility_testing.cfm/2011). This is a high level of resistance, similar to that seen in the study of Threlfall *et al.* (1997) and Daini *et al.* (2006) who reported high-level resistance to ciprofloxacin in *E. coli* and *Enterobacteriaceae* respectively. These MIC values have shown a tremendous increase in reduced susceptibility to these agents over the course of the years, as evident in MIC values of 0.125-2 µg/ml obtained by Oni *et al.* (2001) for ciprofloxacin in the same environment.

Mutations in *gyrA* are the most common mechanisms of fluoroquinolone resistance in different bacterial species, usually at either codon 83 or 87, and/or the *parC* gene (Piddock, 2002; Ling *et al.*, 2003; Pazhani *et al.*, 2008). We found mutations in *gyrA* occurring at codon 83; replacement of serine with leucine being the most common. This was followed by serine to isoleucine and threonine to isoleucine. Serine 83 has been suggested as an important site for fluoroquinolone resistance (Weigel *et al.*, 1998; Ling *et al.*, 2003). The mutations (amino acid changes) found for ser83 in this study was not uniform as found in the study of Pazhani *et al.* (2008), also was not ser83→phe; ser83→tyr as described by Ling *et al.* (2003). A few of the strains had a second mutation at position 87, with replacement of aspartic acid with asparagine (Ling *et al.*, 2003; Pazhani *et al.*, 2008). Forty strains of *Klebsiella pneumoniae* and 15 of *E. coli* had an amino acid substitution of serine to leucine at codon position

83. While 7 strains of *Pseudomonas aeruginosa* had substitution of Threonine to Isoleucine also at position 83, 10 strains of *K. pneumoniae* had a mutation at position 87 with an amino acid change of aspartic acid to asparagine in addition to the common change of serine to leucine at codon position 83. This kind of resistance seen with Gram-negative bacilli is usually against all quinolones.

A single mutation in *gyrA* of *E. coli* has been shown to be sufficient to cause high-level resistance to nalidixic acid (Ozeki *et al*., 1997) but additional mutations in *gyrA* and/or the topoisomerase IV genes, occurring in a step-wise manner, have also been shown to be a pre-requisite for high-level fluoroquinolone resistance (Ozeki *et al*., 1997; Weigel *et al.*, 1998). Nucleotide substitutions at codon 83 was found to be the most common *gyrA* mutations of *E. coli* and other enterobacteria be it from clinical, veterinary or laboratory strains (Chen *et al.*, 2001; Ruiz *et al.*, 2002). Serine is commonly substituted by a leucine with a common feature of high-level resistance to nalidixic acid and decreased susceptibility to fluoroquinolones. The strains with secondary mutation of aspartic acid at codon 87 to asparagine are found to have a slightly higher resistance to fluoroquinolones (Wiegel *et al.*, 1998; Saenz *et al.*, 2003). This study equally confirmed resistance phenotypes generally associated with mutations in the QRDR of *gyrA* is characterized by higher MICs of nalidixic compared with those for the fluoroquinolones such as ciprofloxacin (Tavio *et al.*, 1999). Different amino acid substitutions at the same position resulted in different quinolone susceptibility levels, indicating that the final MIC is a function of the specific substitution. This fact is probably due to the mechanism of interaction between the quinolones and their targets. It has been suggested that amino acid 83 of *gyrA* interacts with the radical in position 1 of quinolones, whereas amino acid 87 of *gyrA* interacts with the radical in position 7. Thus, different amino acid substitutions at these points would affect in different ways the affinity for the quinolone molecule (Ruiz, 2003).

In *parC*, mutations only occurred at codon 80 and 87 with substitutions of serine for isoleucine and leucine. All the strains with *parC* mutations equally had mutations in *gyrA* of the QRDR as a primary target for quinolones in Gram negative. Topoisomerase IV mutants do not by themselves confer resistance (Chen *et al.*, 1996). Thus, *parC* mutations confer low-level resistance to ciprofloxacin, with an additional *gyrA* mutation increasing resistance. The most common amino acid change in *parC* is the substitution of serine for isoleucine at codon 80 (Komp *et al.*, 2003; Fendukly *et al.*, 2003; Pazhani *et al.*, 2008) and another substitution which is also similar to that found in this study is replacement of serine for leucine (Pazhani *et al.*, 2008). Previous studies have shown that topoisomerase IV is not as sensitive to quinolones as DNA gyrase (Peng and Marians, 1993; Hopkins *et al.*, 2005) and is therefore, a secondary target for quinolones in Gram negative bacteria. To date, mutations in *parC* and *parE* of *E. coli* have always been found together with mutations in *gyrA* (Komp *et al.*, 2003; Fendukly *et al.*, 2003), suggesting that the mutations in topoisomerase IV will not occur unless the sensitivity of the DNA gyrase to fluoroquinolones has been reduced to at least that of topoisomerase IV by DNA gyrase mutations (Everett *et al.*, 1996). Bachoual *et al.* determined that a mutation in *parC* was the second step leading to high-level fluoroquinolones resistance (Bachoual *et al.*, 1998). Mutations in *parC* and *parE*, therefore, play an important role in the formation of highly resistant strains.

In conclusion, we have further confirmed high level multidrug resistance in Gram negative bacilli bacteria to fluoroquinolones mediated by accumulation of mutations at positions 80, 83 and 87 QRDR of *gyrA* and *parC* in Nigeria.

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