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Full Length Research Paper

Identification of GyrA mutations conferring fluoroquinolone resistance in *Pseudomonas aeruginosa* isolated from poultry in Ibadan, Oyo State, Nigeria

A. O. Ogunleye

Department of Veterinary Microbiology and Parasitology, University of Ibadan, Ibadan. Oyo State, Nigeria. E-mail: peculiarj@yahoo.com, ao.ogunleye@mail.ui.edu.ng. Tel: +234-8054461821.

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The quinolone-resistance determining-region of *gyrA* was polymerase chain reaction (PCR)-amplified and sequenced in seven fluoroquinolone-resistant *Pseudomonas aeruginosa* (Nalidixic acid MICs ranging 8 to128 µg/ml, ciprofloxacin MICs ranging 8 to 32 µg/ml and levofloxacin MICs ranged from 32 to 64 µg/ml) isolated from poultry that died of septicaemic clinical diseases in Ibadan, Oyo State, South Western Nigeria. The seven isolates were also multidrug resistant to various combinations of the commonly used antibiotics like streptomycin, ampicillin, tetracycline, kanamycin, neomycin and chloramphenicol. The entire seven isolates possessed the *gyrA* mutation encoding the histidine to tyrosine conversion at amino acid 150 (H150Y). Additional substitutions observed included: aspartic acid to tyrosine substitution at amino acid 87 (D87Y) in two isolates, aspartic acid to glycine D87G substitution in three isolates, another simultaneous seven substitutions: aspartic acid to alanine, D87A; alanine to proline, A62P; tyrosine to isoleucine, Y83I; methionine to leucine, M92L; leucine to methionine, L98M; L128M; and aspertic acid to proline D148P in one of the isolate, whereas one of the seven isolates possessed only the H150Y substitution. The H150Y is typical of the quinolone resistant *P. aeruginosa* isolated from septic poultry in Nigeria, regardless of other resistance- patterns exhibited to other commonly used antibiotics. This study associate *gyrA* mutations with fluoroquinolone resistance in *P. aeruginosa* isolated from septicaemic poultry in Nigeria, where fluoroquinolone use in livestock is not strictly regulated and misuse/abuse of most antibiotics is rampart.

Key words: *Pseudomonas aeruginosa,* resistance, fluoroquinolone, poultry, Oyo state, Nigeria.

INTRODUCTION

Pseudomonas aeruginosa is a clinically important pathogen. It is usually incriminated as nosocomial pathogens (Sherertz and Sarubbi, 1983; Hooper and Wolfson**,** 1991). Infection with it is usually associated with significant morbidity and mortality, especially in patients with cystic fibrosis (Hancock and Speert, 2000; Obritsch et al., 2005).

It has earlier been reported as causative agent of opportunistic nosocomial infections such as wound, urinary and respiratory tract infections (Hancock and Speert, 2000). Fluoroquinolones and aminoglycosides are two important classes of antibiotics used in the treatment of *Pseudomonas* infections (Gorgania et al., 2009). Fluoroquinolones in particular have been widely used for the treatment of nosocomial infection (Hooper, 1998; Yamaguchi et al., 2000). They are bactericidal members of the quinolone family, which acts by inhibiting the bacterial DNA gyrase and topoisomerase IV, thus causing the inhibition of the DNA transcription and replication (Gorgania et al., 2009). DNA gyrase is targeted in Gram-negative organisms, whereas for Gram-positive organisms' topoisomerase IV is the target (Jalal et al., 2000).

P. aeruginosa is intrinsically resistant to a wide range of

antibiotics due to its low outer membrane permeability and presence of several drug efflux systems (Lambert, 2002). These properties contribute in no small measures to the problem of antibiotic resistance encountered in the treatment of infection by this organism (Lambert, 2002). It is equally known to exhibits natural and acquired resistance to many structurally and functionally diverse antibiotics (Wang et al., 2007). Resistance by *P. aeruginosa* to fluoroquinolones could be achieved by the modification of DNA gyrase and topoisomerase IV, decreased permeability of the cell wall, and multidrug efflux systems (Drlica and Zhao, 1997; Hancock, 1998). Out of all these possible resistance mechanisms, alterations in DNA gyrase or topoisomerase IV caused by mutations in the genes that encode DNA gyrase (*gyrA*) and topoisomerase IV (*parC*) within the quinolone-resistance-determining region (QRDR) plays a major role in fluoroquinolone resistance among clinical isolates of *P. aeruginosa* (Yoshida et al., 1990; Cambau et al., 1995; Jalal and Wretlind, 1998; Mouneimne et al., 1999). The unregulated use of fluoroquinolones can generate mutations at the quinolone-resistance determining-region (QRDR) of *gyrA* that encodes for subunit A of DNA gyrase, the enzyme targeted by fluoroquinolones. These mutations eliminate the high affinity binding of fluoroquinolones and GyrA thus compromizing the efficacy of these drugs (Jalal et al., 2000).

In Nigeria, there is abuse and misuse of various groups of antibiotics including quinolones and fluoroquinolones based drugs in food animals as well as in poultry ; and this has been attributed to the isolation and occurrence of multidrug resistant *Escherichia coli* and *Salmonella enterica* that are capable of transferring R factors to commonly used and often abused antibiotics like ampicilin, tetracycline, neomycin, kanamycin, chloranphenicol, nalidixic acid and ciprofloxacin in poultry to *E. coli* 356 K12 resistant to 200 µg/ml of streptomycin used as sensitive recipient (Ogunleye et al., 2010a and 2010b). Quite often, *P. aeruginosa* that are multidrug resistant are isolated from septic poultry in Nigeria apart from *E. coli* and *Salmonella species*. The current study was carried out to characterize seven *P. aeruginosa* strains isolated in Ibadan, Oyo state, Nigeria between 2005 and 2007 from septicaemic poultry (pullets) while screening for *Salmonella species* in septic poultry to investigate their possible involvement in perpetration of drug resistance especially to the fluoroquinolone which is often currently one of the choiced group of drug in the treatment of septicaemic conditions in poultry in Nigeria. All the seven isolates selected for this study were resistant to more than two commonly used antibiotics in poultry like ampicilin, tetracycline, neomycin, kanamycin, chloramphenicol, nalidixic acid streptomycin and ciprofloxacin. Levofloxacin, a fluoroquinolone which is not currently commonly used in Nigeria was also included. The characterizations of the seven isolates were based on morphologic and biochemical characterizations, antibiotic susceptibilities as well as DNA sequencing of the QRDR in *gyrA*.

MATERIALS AND METHODS

Isolation and identification of the isolates

The seven isolates were recovered from liver samples of poultry birds that died of septicaemic conditions in Ibadan, Oyo state Nigeria. Samples were inoculated onto Pseudomonas agar F (MP Biolab) and incubated aerobically at 37℃ for 24 to 48 h. The suspected *Pseudomonas species* were further characterized biochemically and identified as *P. aeruginosa* according to standard methods (Barrow and Feltham, 1993).

Determination of the ampicillin, chloramphenicol, streptomycin, tetracycline, kanamycin, neomycin, nalidixic acid, ciprofloxacin and levofloxacin MICs values

Ampicillin, chloramphenicol, streptomycin, tetracycline, kanamycin, neomycin, nalidixic acid, ciprofloxacin and levofloxacin (all obtained from SIGMA-ALDRICH) MICs were determined using the two-fold micro-broth dilution method as per CLSI standards (CLSI, 2009). MICs were ascribed to the lowest concentration of the antibiotics that inhibited growth of the isolate while *S. enterica* serovar Typhimurium definitive phage type DT104 was used as positive control.

Amplification of the *gyrA* QRDR and DNA sequencing of the PCR product

Chromosomal DNA was produced from the seven isolates by heating overnight LB broth culture at 99℃ for 15 min. A 560 base-pair region of *gyrA* of the chromosomal DNA produced was PCR amplified. The PCR was performed using the FailSafe™ System (EPICENTRE® Biotechnologies) in 50 µl containing 1uM of forward and reverse oligonucleotides (F=5´ATGAGCGACCTTGCGAGAAATACACCG3´, R=5´TTCCAT-CAGCGCCCTTCAATGCTGATGTCTTC3´), 1.25 units of the FailSafe™ Enzyme, FailSafe™ PCR buffer B, and 1µl of crude DNA template.

BIO-RAD MJ Mini personal Thermal cycler was used for the DNA amplification using the following PCR protocol: initial denaturation at 95℃ for 2 min, followed by 40 cycles of 95℃ fo r 1 min, 53℃ for 30 s, and 70℃ for 45 s. Amplified DNA products were resolved using 1% (w/v) agarose gel electrophoresis. PCR products were purified and sequenced at Iowa State University DNA sequencing facilities (Ames, IA, USA).

RESULTS

The seven *Pseudomonas species* conformed to the biochemical characteristics of *P. aeruginosa*. MIC values for the seven isolates for ampicillin, chloramphenicol, streptomycin, tetracycline, kanamycin, neomycin, ciprofloxacin and levofloxacin are shown in Tables 1 and 2. The positive control *S. enterica* serovar Typhimurium definitive phage type DT104 was resistant to all the tested antibiotics at the MICs of 256, >256, 64, 256, 256, 256, 128, 64 and 64 µg/ml for ampicillin, tetracycline,

Table 1. Minimum inhibitory concentrations of the *Pseudomonas aeruginosa* isolates to commonly used antibiotics.

S/N	*Pseudomonas aeruginosa* isolate	Strep (µg/ml)	Amp (µg/ml)	Tet (µg/ml)	Kan (µg/ml)	Neo (µg/ml)	Chl (µg/ml)
1	Isolate 1	16	64	16	32	64	8
2	Isolate 2	64	128	128	64	64	128
3	Isolate 3	32	64	64	64	64	256
4	Isolate 4	8	16	8	16	64	8
5	Isolate 5	8	64	6	64	64	8
6	Isolate 6	4	32	4	64	64	8
7	Isolate 7	16	16	4	32	64	32

Amp= ampicillin, Str=Streptomycin, Chl= Chloramphenicol, Kan= Kanamycin, Neo= Neomycin, Tet= Tetracycline.

Table 2. Minimum inhibitory concentrations for quinolone/ fluoroquinolones and respective Mutations in the quinolone resistant determining region of the *Pseudomonas aeruginosa* isolates.

S/N	*Pseudomonas aeruginosa* isolate	Nalidixic acid MIC values (µg/ml)	Ciprofloxacin MIC values (µg/ml)	Levofloxacin MIC values (µg/ml)	Mutation(s) in the QRDR of *gyrA*
1	Isolate 1	16	16	64	H150Y, D87A, A62P, Y83I, M92L, L98M, L128M, D148P
2	Isolate 2	128	32	64	H150Y, D87G.
3	Isolate 3	8	8	64	H150Y, D87 G.
4	Isolate 4	32	8	32	H150Y, D87G
5	Isolate 5	16	8	128	H150Y, D87 Y
6	Isolate 6	16	16	32	H150Y,D 87 Y.
7	Isolate 7	16	16	32	H150Y

chloramphenicol, streptomycin, kanamycin, neomycin, nalidixic acid, ciprofloxacin and levofloxacin respectively.

As shown in Table 2, all the seven isolates possessed gyrA mutations encoding the histidine to tyrosine substitution at position 150 (H150Y). In addition, isolates 5 and 6 have additional aspartic acid to tyrosine substitution at position 87 (D87Y), while isolates 2, 3 and 4 contained the aspartic acid to glycine D87G substitution in addition to the H150Y. Isolate 7 had only the H150Y substitution, whereas isolate 1 had the following additional simultaneous seven substitutions: aspartic acid to alanine, D87A; alanine to proline, A62P; tyrosine to isoleucine, Y83I; methionine to leucine, M92L; leucine to methionine, L98M; L128M; and aspertic acid to proline D148P substitution. Figure 1 shows the gel picture of the amplified quinolone resistant determining regions of the gyrA of four of the *P. aeruginosa* isolates, where lane two was loaded with isolate 5, lane 4 with standard 1Kb DNA ladder, lane 5 with positive control *S. enterica* serovar Typhimurium definitive phage type DT104, lane 6 contained isolate 1, isolate 7 in lane 7 and isolate 3 in lane 9.

DISCUSSION

P. aeruginosa is often associated with multiple antibiotics resistance usually mediated by complex resistance mechanism (Wang et al.,

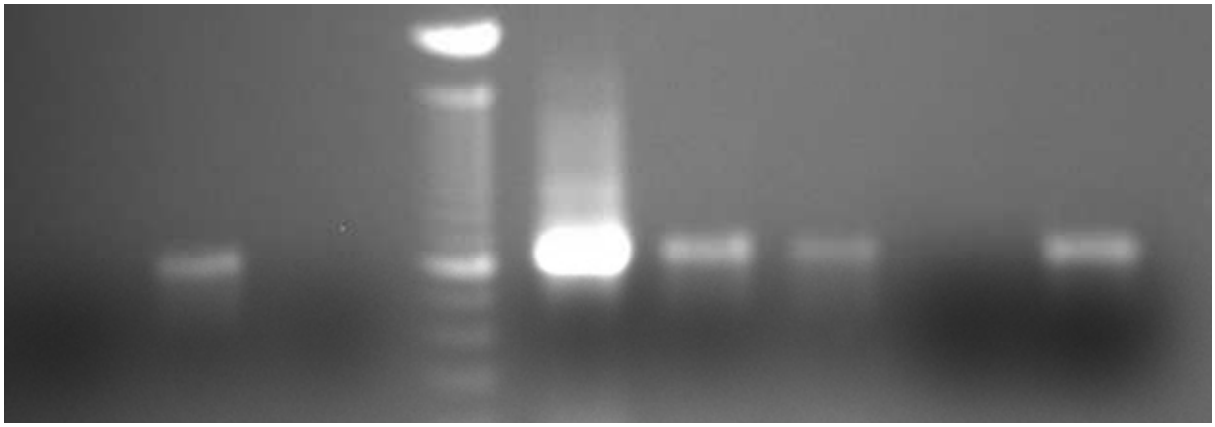

Figure 1. Gel picture of the amplified quinolone resistant determining regions of the gyrA of four of the *Pseudomonas aeruginosa* isolates; lane 2 contained isolate 5, lane 4 for the standard 1Kb DNA ladder, lane 5 for positive control *Salmonella enterica* serovar Typhimurium definitive phage type DT104, lane 6 for isolate 1, lane 7 for isolate 7 and lane 9 for isolate 3.

2007). In Nigeria, it had been earlier reported that extensive use and often abuse/misuse of antimicrobial agents in livestock has resulted in emergence of resistant strains of bacteria in which some were found to habour R factors to commonly used antibiotics (Adetosoye, 1980). In the study, it was reported that all the five *P. aeruginosa* isolated from piglet faeces in Nigeria harboured transmissible R-factors for tetracycline, ampicilin, streptomycin and sulphonamide to sensitive *E. coli* K12 (Adetosoye, 1980). The practice of abuse/ misuse of antibiotics in livestock and sometimes in human have not changed significantly over the years in Nigeria. As a matter of fact, it seems to be on the increase more than ever. This current study describes the identification of gyrase mutations in fluoroquinolone-resistant isolates of *Pseudomonas aeruginosa* recovered from septicaemic poutry in Ibadan, Oyo State, Nigeria. All the seven *P. aeruginosa* contained a typical histidine to tyrosine substitution at amino acid 150 (H150Y) which is homologous to that observed in fluoroquinolone resistant *S. enterica* serotypes Give and Kentucky isolated from apparently healthy pigs, as well as in serotypes 9,12:Nonmotile, Give, Ituri, and Kentucky isolated from septic poultry in Nigeria (Ogunleye et al., 2011). As shown in Table 1 and 2, isolate one with high quinolone/ fluoroquinolone resistance (with respective MICs of 16, 16 and 64 µg/ml for Nalidixic acid, ciprofloxacin, and levofloxacin), was also resistant to ampicillin and neomycin at MICs of 64 and 64 µg/ml respectively. The same isolate however have relatively lower MICs of 16, 16, 32, and 8 µg/ml for streptomycin, tetracycline, kanamycin and chloramphenicol, respectively. This particular isolate had additional seven simultaneous mutations in the form of aspartic acid to alanine, D87A; alanine to proline, A62P; tyrosine to isoleucine, Y83I; methionine to leucine, M92L; leucine to methionine, L98M; L128M; and aspertic acid to proline D148P substitution at gyrA subunit of the quinolone resistance determining region. There are however, the possibilities of mutations in the other subunits going by the high MICs of the isolates studied in this work to the nalidixic acid, ciprofloxacin and levofloxacin.

Isolates 2, 3 and 4 on the other hand exhibited similar additional substitution in form of aspertic acid to glycine D87G regardless of the observable variations in their respective resistance to the other commonly used antibiotics. For instance, the isolate 3 with MICs 8, 8 and 64 µg/ml for nalidixic acid, ciprofloxacin, levofloxacin also showed resistance to ampicillin, tetracycline, kanamycin neomycin and chloramphenicol at MICs of 64, 64, 64, 64 and 256 µg/ml respectively. Isolate 2 with higher quinolone/fluoroquinolone level of resistance (128, 32, and 64 µg/ml for nalidixic acid, ciprofloxacin and levofloxacin respectively) also showed resistance to more of the commonly used antibiotics tested at MICs of 64, 128, 128, 64, 64 and 128 µg/ml for streptomycin, ampicillin, tetracycline, kanamycin, neomycin, and chloramphenicol respectively. Isolate 4 with MICs 32, 8 and 32 µg/ml for nalidixic acid, ciprofloxacin and Levofloxacin was resistant also to neomycin (64 µg/ml), while its MICs for streptomycin of 8 µg/ml, ampicillin (16µg/ml), tetracycline (8 µg/ml), kanamycin (16 µg/ml) and chloramphenicol (8 µg/ml) were relatively low.

Similarly, isolates 5 and 6 had identical additional substitution D87Y, aspartic acid to tyrosine regardless of the pattern of resistance manifested by the isolates to the other commonly used antibiotics. Isolate 5 for instance (MICs 16, 8 and 128 µg/ml for nalidixic acid, ciprofloxacin,

levofloxacin) was also resistant to ampicillin, kanamycin, and neomycin at MICs 64, 64 and 64 µg/ml respectively, but sensitive to streptomycin, tetracycline, and Chloramphenicol at MICs 8, 6, and 8 µg/ml respectively. Whereas, isolate 6 (MICs 16, 16 and 32 µg/ml for nalidixic acid, ciprofloxacin, levofloxacin) was resistant also to ampicillin, kanamycin, and neomycin at 32, 64 and 64 µg/ml but sensitive to streptomycin, tetracycline and chloramphenicol at 4, 4 and 8 µg/ml respectively. Isolate 7 on the other hand with MICs 16, 16 and 32 µg/ml for nalidixic acid, ciprofloxacin and levofloxacin and additional resistance to kanamycin, neomycin and chloramphenicol at MICs 32, 64 and 32 µg/ml respectively exhibited only the H150Y substitution.

From this work, DNA gyrase A subunit mutation at amino acid 150 in form of Histidine to tyrosine thus seem to play a typical role in resistance to quinolone/fluoroquinolone particularly at MICs ranges of 8 to 128 µg/ml in *P. aeruginosa* from poultry sources in Nigeria regardless of the additional resistance patterns to other commonly used antibiotics. The aspartic acid to glycine D87G substitution exhibited by three of the *P. aeruginosa* isolate from Nigeria had been earlier attributed to quinolone resistance among clinical isolate of *Pseudomonas aeruginosa* recovered in Japan in 1989 and 1993 (Yonezawa et al., 1995). The other mutations observed in the current work include: D87A; alanine to proline, A62P; tyrosine to isoleucine, Y83I; metionine to leucine, M92L; leucine to methionine, L98M; L128M; and apertic acid to proline D148P substitution. These mutations are stable and are capable of being transferred to successive generations in the presence or absence of selective pressure, more so going by the practice of abuse/misuse of antibiotics in most parts of Nigeria, including the area of study. The observation in this study is therefore of public health concern, because of the possibility of transmission of drug resistance from *P. aeruginosa* to other poultry pathogens, and to human because it is an ubiquitous environmental organism capable of causing opportunistic infections in human (Yamaguchi et al., 2000; Akasaka et al., 2001).

Should these multiple resistant strains of *P. aeruginosa* isolated from septic poultry in Nigeria contaminate food or meat products for human consumption, or water and feed for animal consumption, or contaminate wounds, ear or eye in humans; these may result in serious health hazard, in terms of treatment failure, thus constituting a potential threat to treatment both in poultry infections, other livestock as well as human in Nigeria that could have otherwise be freely treated with quinolone based drugs and other commonly used antibiotics. The observation in this study whereby some of the isolates studied were found to be sensitive to a few of the commonly used antibiotics such as streptomycin, tetracycline, and chloramphenicol despite their resistance to quinolone/fluoroquilonone based drugs underscores the value of antibiotic sensitivity testing to make the right judgement on the course of treatment in Nigeria.

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