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Identification of GyrA mutations conferring fluoroquinolone resistance in *Salmonella* isolated from poultry and swine from Ogun and Oyo State, Nigeria

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ABSRACT

The quinolone-resistance determining-region of *gyrA* was PCR-amplified and sequenced in eight fluoroquinolone-resistant *Salmonella* (levofloxacin MICs ranging from 32-64 µg /mL) isolated from apparently healthy swine (n=2 isolates) and poultry (n=6 isolates) that died of septicaemic clinical diseases in Ogun and Oyo State, Nigeria. All of the eight isolates possessed the *gyrA* mutation encoding the histidine⇒tyrosine conversion at amino acid 150 (150His⇒Tyr). Additional substitutions included: 83Tyr⇒Ser in one *Salmonella enterica* serotype Give isolate (pig), 87Asp⇒Tyr in one *Salmonella enterica* serotype Kentucky isolate (pig); 83Tyr⇒Phe in one isolate characterized as 9,12:Nonmotile (poultry); and 87Asp⇒Gly in three poultry isolates including *Salmonella enterica* serotypes Kentucky (n=2), and Ituri (n=1). The 150His⇒Tyr is novel while the other mutations have been previously reported in *Salmonella* spp. This is the first study to associate *gyrA* mutations with fluoroquinolone resistance in Nigeria, where fluoroquinolone use in livestock is not tightly regulated.

INTRODUCTION

Considering the possibility of zoonotic transmission of infectious pathogens, transmission of drug resistant pathogens from animal to man could pose a great risk to public health in terms of treatment failure. For instance, 95% of the estimated 1.4 million cases of salmonellosis per year in United States of America were acquired through food-borne transmission (Mead *et al.*, 1999). Food animals are often incriminated as playing significant role in the development and spread of drug resistant pathogens (Threlfall *et al.*, 2000; Walker *et al.*, 2000). Development and transmission of genotypic traits encoding antimicrobial resistance is thought to be enhanced through agricultural and veterinary uses of antimicrobials as therapeutic and subtherapeutic agents (Allen and Poppe.2002).

Fluoroquinolones are the drug of choice against systemic salmonellosis (WHO 2010) but, unfortunately, high level fluoroquinolone resistance is increasingly prevalent in *Salmonella.* The unregulated use of these drugs 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 marginalizing the efficacy of these drugs.

The current study was carried out to characterize eight fluoroquinolone-resistant *Salmonella* strains isolated from swine and poultry in Nigeria. These isolates were subjected to serotype analysis and DNA sequencing of the QRDR in *gyrA*. Isolates were obtained from healthy swine and septicemic poultry.

MATERIALS AND METHODS

Isolation and identification of the isolates:

The two swine isolates were obtained from faecal samples collected from apparently healthy pigs in the University of Ibadan Teaching and Research farm. Samples were inoculated onto MacConkey and deoxycholate citrate agars and incubated aerobically at 37°C for 24-48hours. The yellowish glistening colonies were Gram-stained, and examined for motility and microscopic morphology. Tentative non-lactose fermenting *Salmonella* were further identified biochemically and serologically according to the Kauffman-White Scheme using polyvalent antiserum (Wellcome Research Laboratories, UK.) as *Salmonella species* (Edwards and Ewing, 1972; Barrow and Feltham, 1993). The other six *Salmonella* isolates were obtained from previous studies (Ogunleye *et al.*, 2010a,b).

Determination of the levofloxacin MIC values

Levofloxacin (Sigma Chemicals) MICs were determined using the two-fold micro-broth dilution method (CLSI. 2009). MICs were ascribed to the lowest concentration of levofloxacin that inhibited growth.

Serotyping of the isolate:

The two pig isolates and six poultry isolates were sub-cultured into TSA agar and submitted to National Veterinary Service Laboratories in Ames, Iowa, USA for serotyping. Serotyping was performed as per the Kauffman-White Scheme.

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

A 560 base-pair region of *gyrA* was amplified from chromosomal DNA obtained by heating the isolate at 99°C for 15 minutes. PCR was performed using the FailSafe™ System (EPICENTRE® Biotechnologies) in 50µl containing 1uM of forward and reverse oligonucleotides (F=5'ATGACGCGACCTTGCGAGAAATACACCG3', 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°C for 2 minutes, followed by 40 cycles of 95°C for 1 minute, 53°C for 30 seconds, and 70°C for 45 seconds. 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

Two *Salmonella* serotypes (Give and Kentucky) were isolated from the healthy pigs as shown in Table 1. The six poultry isolates belong to four different serotypes: Give, Kentucky, Ituri and 9,12:Nonmotile.

MIC values were essentially indistinguishable (32-64 μg/mL) given the nature of the assay. All eight isolates possessed gyrA mutations encoding the histidine to tyrosine substitution at amino acid 150 (150His⇒Tyr). One pig isolate (serotype Give) had an additional tyrosine to serine substitution at amino acid 83 (83Tyr⇒Ser), while the second pig isolate (serotype Kentucky) had contained the 87Asp⇒Tyr substitution. Three poultry isolates (two with the serotype Kentucky and one serotype Ituri) displayed the 87Asp⇒Gly alteration. The 9,12:Nonmotile poultry isolate bore a 83Tyr⇒Phe substitution. Two of the poultry isolates (9,12:Nonmotile and serotype Give) exhibited only the 150His⇒Tyr mutation.

DISCUSSION

The study herein describes the identification of gyrase mutations in fluoroquinolone-resistant isolates of *Salmonella* from Nigeria. *Salmonella enterica* serotypes Give and Kentucky were isolated from apparently healthy pigs while serotypes 9,12:Nonmotile, Give, Ituri, and Kentucky were isolated from septic poultry. All eight strains contained a novel histidine to tyrosine substitution at amino acid 150 while the 9,12:Nonmotile poultry isolate also expressed a novel tyrosine to phenylalanine substitution at amino acid 83. All other substitutions have been identified previously (Luque *et al.*, 2009; Kehrenberg *et al.*, 2007; Lee *et al.*, 2004; Ling *et al.*, 2003). The serotype Kentucky isolates from poultry may be clonal since they have identical genotypes and phenotypes in regards to the parameters studied herein.

The study also describes the novel isolation of *Salmonella enterica* serotype Ituri from septic chickens. This pathogen does, however, have a history as an avian pathogen since it was isolated from ducks in the Belgian Congo (Kauffman and Fain, 1953). Pathogenicity experiments will be conducted with this isolate using poultry as host in a future study.

The mutations described herein are stable and thus transferred to successive generations in the presence or absence of selective pressure. The observation in this study is therefore of public health concern, because of the possibility of transmission of drug resistance from broad host range serotypes from poultry and the apparently healthy pigs to man, thereby constituting a potential threat to treatment of *Salmonella* infections in humans and poultry.

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Table 1: Characterization of *Salmonella* isolates studied.

Animal Source	Serotype	Levofloxacin MIC values (µg /mL)	Mutation(s) in the QRDR of *gyrA*
Pig	Give	32	150His⇒Tyr, 83Tyr⇒Ser
Pig	Kentucky	32	150His⇒Tyr, 87Asp⇒Tyr
Poultry	9,12:Nonmotile	32	150His⇒Tyr, 83Asp⇒Phe
Poultry	9,12:Nonmotile	32	150His⇒Tyr
Poultry	Give	32	150His⇒Tyr
Poultry	Kentucky	64	150His⇒Tyr, 87Asp⇒Gly
Poultry	Kentucky	64	150His⇒Tyr, 87Asp⇒Gly
Poultry	Ituri	32	150His⇒Tyr 87Asp⇒Gly