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Determination of Quinolone Resistance in *Escherichia coli* Isolates

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Abstract
Quinolone resistance in 102 isolates of Escherichia coli from faecal samples of asymptomatic cattle and chickens in Anyigba, Kogi State, Nigeria was determined by antimicrobial susceptibility testing and Polymerase Chain Reaction (PCR) with qnrA primers. Susceptibility of the isolates to two quinolones (ciprofloxacin and nalidixic acid) was conducted using the disc diffusion method. Diameter of zones of inhibition around antimicrobial susceptibility discs were measured and interpreted using the Clinical Laboratory Standard Institute (CLSI) measurements. Plasmid and genomic DNA extracted from antimicrobial resistant isolates were subjected to Polymerase Chain Reaction with qnrA primers for DNA hybridization. Antimicrobial resistance (AMR) to ciprofloxacin observed for isolates from cattle faeces and chicken faecal isolates was 10.6% and 58.2% respectively. AMR to nalidixic acid was 12.8% and 60.0% respectively. Intermediate susceptibility of isolates to nalidixic acid is higher than intermediate susceptibility to ciprofloxacin (26.5% and 49.1%). There is an observed significant difference ($p<0.05$) in the antimicrobial susceptibility of Escherichia coli isolated from cattle faeces and chicken faeces to these quinolones. Conversely, hybridization of qnrA with plasmid DNA or genomic DNA was not detected in the PCR. The results indicated that Escherichia coli isolates from faeces of these animals are more susceptible to ciprofloxacin than nalidixic acid. Higher resistance observed for isolates from chickens could be a result of more exposure to antimicrobials in feed (as growth promoters and prophylactics) and in treatment of infections. Molecular determinant(s) of resistance in the resistant isolates appears to be genes other than qnrA as may be determined in further studies.

Keywords: *Escherichia coli,* resistance, quinolones, gene, hybridization,

Introduction
Quinolones are broad spectrum antimicrobials that are widely used in both human and animal medicine for the treatment of severe infections. This group of antimicrobials have been increasingly used because of their safety, availability both orally and parenterally and their favourable pharmaco-kinetics (1). Their spectrum of efficacy against a wide range of Gram negative and Gram positive pathogenic bacteria has led to their widespread use. There is increasing concern about the emergence of resistance to this agents.Their wide use has triggered increased bacterial resistance worldwide. Resistance to these agents is multifactorial and can be via one or a combination of target-site gene mutations, increased production of multidrug –resistance (MDR) efflux pumps, modifying enzymes, and /or target protection proteins (2) Mutations in *gyrA*(gyrase) and *parC* (topoisomeraseIV) genes are the most common mechanism involved in high-level quinolone resistance, yet the spread of plasmid- mediated quinolone resistance genes and efflux-pump mutants have also been described(3)Ciprofloxacin-resistant *E. coli* was found in 22.4% of turkey breeding flocks and 60.9% of meat flocks. Two main mutations in *gyrA*, as well as a range of silent mutations, were identified in resistant isolates. Flocks with transferable resistance genes *qnrB, qnrS*, and *aac*(6')-*Ib-cr* were found at a low flock prevalence of 4.2%, 1.6% and 1.0%, respectively; however, under laboratory conditions only transfer of *qnrS* genes could be demonstrated. The work has confirmed the occurrence of ciprofloxacin-resistant *E. coli* strains throughout turkey breeding and meat flocks, with almost one-third of *E. coli* isolates being resistant to ciprofloxacin (1)
Antibiotic use has been known to select for resistance not only in pathogenic bacteria but also in endogenous flora of exposed individuals or population (4).The development of resistance to antimicrobials, especially of the fluoroquinolones (FQs), used in human and veterinary medicine has become an issue of great concern to veterinarians, farmers and antibiotic manufacturing companies and is fast becoming a global health focus. Mitigation of this trend necessitates a thorough knowledge of the mechanisms of resistance by these microbes.
E. coli is incredibly diverse bacterial species with the ability to colonize and persist in numerous niches in the environment and within animal hosts. It is one of the common microbial flora of gastrointestinal tract of human beings and vertebrate animals (5). Apart from being shed in faeces, its presence has also been detected in soil, plants and in water where it could serve as one of the factors affecting animal and human health. Commensal *E. coli* strains are thought to maintain the physiological milieu of the gut and support digestion as well as defend against enteric pathogens (6). Studies have shown that commensal bacteria of humans and animals could serve as good

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indicators of antimicrobial selective pressure and reveal the potential for antimicrobial resistance emerging in enteric pathogens (7). The French Institute for Public Health Surveillance has recommended that studying antimicrobial resistance in commensal bacteria from healthy animals would be extremely valuable, as these organisms could serve as a reservoir for genes that encode antimicrobial resistance and, given the right conditions, could transfer these resistance genes to pathogenic bacteria (8). This study was aimed at determining the level of antimicrobial resistance of *Escherichia coli* recovered from faeces of asymptomatic cattle and chickens in Anyigba, Kogi state of Nigeria to two quinolones (ciprofloxacin and nalixidic acid) and to investigate the presence of the *qnr*A gene in the resistant isolates to investigate the molecular mechanisms of resistance to these antimicrobials.

Materials and Methods

One hundred and two faecal samples were collected from cattle faeces (n=47) and Chicken faeces (n=55) in Anyigba, Kogi State for the isolation of *Escherichia coli*. One gram each of faeces was emulsified in 10ml phosphate buffered saline (PBS) and properly homogenized by vortexing for 2 minutes. Serial dilutions of the faeces were done. The pour plate technique was employed in the dilution and inoculation of the samples into the medium (MacConkey agar) (9). Inoculated plates were allowed to gel and then were transferred to the incubator for 24hrs at 35^oC.

Typical reddish pink colonies with cells appearing as Gram negative short rods after Gram staining and microscopic observation were thereafter sub cultured on Eosin Methylene Blue (EMB) agar and incubated for 24hrs (10). Colonies with greenish metallic sheen were sub-cultured on nutrient agar slants prepared in bijou bottles and incubated at 35^oC for 24 hrs. These were used for biochemical tests. Standard biochemical tests conducted included indole, citrate, urease, sugar fermentation and hydrogen sulphide production using Triple Sugar Iron (TSI) agar.

Antibiotic drug susceptibility test was conducted using the disk diffusion method. Inoculum's density of isolates was standardised by suspending loopful of test isolates in sterile normal saline to a concentration that is comparable 0.5 McFarland turbidity standards (11, 12). One ml of the culture of the prepared isolates was inoculated into Mueller Hinton Agar (Oxoid, UK) plates. The inoculum was spread evenly over the entire surface of the plates and was allowed to dry for 5 minutes. Antibiotic discs (Oxoid, UK) of ciprofloxacin (CIP) 5μg, nalixidic acid (NA) 30μg were used. The antibiotic discs were evenly placed on the surface of the plates using sterile forceps. Plates were incubated at 35^oC for 18 hours. Diameter of zones of inhibition was measured in millimeters and were interpreted as Sensitive, Intermediate or Resistant according to the CSLI (Clinical and Laboratory Standard Institute) 2011 breakpoints for the antibiotics. Sterile discs containing no antibiotic were used as negative control, while *E.coli* ATCC25922 was used as positive control (13).

Isolation of Plasmid DNA (14) was carried out by Centrifugation of overnight culture of *E.coli* on LB broth at 8000rpm for 2min was done. Cell pellets were re-suspended in 200µl ice-cold buffer (400mM Tris HCl, pH8) and 400µl of 4% SDS.

Tubes were inverted twice at 28 ^{o}C. Addition of ice-cold 3M sodium acetate (300µl) followed and was centrifuged at 3000rpm for 5 min. 700µl chloroform was added and centrifuged at 3000rpm for 1min. one ml of absolute alcohol was added to 500µl of supernatant to precipitate plasmid DNA. Tubes were kept on ice for 1hr and centrifuged at 3000rpm for 30 min. this was followed by washing of the pellets with 70% ethanol and centrifuged for 5min. Supernatant was discarded and tube inverted on paper towel to drain excess ethanol. Finally re-suspended on 10mM Tris (pH8) for gel electrophoresis

Cetyl Trimethyl Ammonium Bromide (CTAB) method of DNA extraction from microbes (15) was adopted for the extraction of genomic (chromosomal) DNA (n=6).

One-ml of *E. coli* culture was dispensed into 1.5 ml Eppendorf tube and centrifuge at 1400 rpm for 30 sec to harvest the cells. Pre-warmed (60^oC) CTAB buffer (400 µl) and 10%SDS (75 µl) were added. This was heated in a water bath at 65^oC for 30 min. The mixture was allowed to cool and 10 µl of proteinase K was added. It was then incubated for 30min at 37^oC. To the mixture, 500 µL of chloroform was added and vortexed for 5 minutes after which it was centrifuged at 10,000 rpm for 10 minutes. The supernatant was collected into another tube and 1 µl RNAse was added. This was further incubated at 37^oC for 30 min. After incubation, 500µl of Isopropanol was added and was kept at -20^oC for 1 hour, centrifugation at 10,000 rpm for 10 minutes followed. The supernatant was decanted and the pellets washed with 70% ethanol. It was gently mixed and further centrifuged at 10,000 rpm for 5 minutes. The supernatant is decanted and then dried for 30 minutes to drain off the ethanol. The resulting DNA was re-suspended in 200 µl of sterile water for gel electrophoresis.

Isolated plasmid and genomic DNA were hybridized with the forward (GGGTATGGATATTATTGATAAAG) and reverse (CTAATCCGGCAG CACTATTTA) primers of the *qnr*A. A cocktail of 10µl is made consisting of 5µl Master mix(made up of Taq polymerase, dNTPs, reaction buffer, $MgCl_2$, KCl and a PCR enhancer/ stabilizer),1µl Forward primer, 1µl Reverse primer, 2µl Extracted DNA and1µl Nuclease free water. The cocktail is dispensed into

eppendorf tubes and loaded into the Thermal cycler (Multigen Optimax, Labnet International Inc.) for hybridization and amplification. Initial denaturation at 94^{o}C for 5 minutes, denaturation at the same temperature for 1 minute, annealing at 44.3^{o}C for 30 seconds, extension at 72^{o}C for 2 minutes, final extension at the same temperature for 4 minutes and hold at 4^{o}C for 10 minutes. Initial denaturation to extension ran for 36 cycles. Amplified samples (PCR products) were analysed by gel electrophoresis in agarose gel and stained with ethidium bromide. A molecular weight marker (PCR ladder) was loaded as a standard. Electrophoresis tank ran at 100 volts, 129mA for 90 minutes. Gel images were viewed with the trans illuminator and captured with a camera.

Agarose gel was prepared by weighing 0.8 g of agarose in 100ml of 1xTAE (Tris Acetate EDTA) and warmed in a microwave at medium temperature. Ten microlitres (10 µl) of ethidium bromide was added before pouring the agarose into the gel caster with the comb inserted. The comb is removed and then the gel is transferred to the electrophoresis tank. DNA ladder (10µl) is dispensed into the 1st well as a standard. Test isolates were mixed with loading dye in a 7 µl:3 µl proportion and then loaded into the appropriate wells. The electrophoresis tank was then connected to the power pack and run at 75 volts for 90minutes. Thereafter the gel was transferred to the UV trans illuminator (Gel Documentation, Cleaver Scientific Ltd.) for the observation of the electrophoresis image and then captured with a camera. The image was saved unto computer.

Results

Antimicrobial Susceptibility of *Escherichia coli* isolates to ciprofloxacin is presented in Table 1. Resistance to ciprofloxacin as well as intermediate susceptibility to this antimicrobial was higher in isolates from faecal matter of chickens (58.2% and 27.3%) than in isolates from cattle faeces (10.6% and 25.5%) respectively. Results showed that resistance to nalixidic acid was greater than resistance to ciprofloxacin in faecal isolates from chickens (60.0%) and cattle faecal isolates (12.8%). The intermediate susceptibility of all the isolates tested to nalixidic acid (48.0%) is greater than the number of sensitive and resistant isolates (13.7% and 38.2%) as shown in table 2.

Table 1: Antimicrobial susceptibility of *E. coli* isolates to ciprofloxacin

Test Microorganisms	**Susceptibility patterns (ciproflox.)**			**Total**
	Sensitive ≥ 21mm	Intermediate 16 -20mm	resistant ≤15mm	
Chicken *E. coli*	8	15	32	55
	14.5%	27.3%	58.2%	100.0%
Cattle *E. coli*	30	12	5	47
	63.8%	25.5%	10.6%	100.0%
Total	38	27	37	102
	37.3%	26.5%	36.3%	100.0%

P< 0.05

Table 2: Antimicrobial susceptibility of *E. coli* isolates to nalidixic acid

Test Microorganisms	**Susceptibility to Nalidixic Acid**			**Total**
	Sensitive ≥19 mm	Intermediate 14-18 mm	Resistant ≤13 mm	
chicken *E. coli*	3	19	33	55
	5.5%	34.5%	60.0%	100.0%
Cattle *E. coli*	11	30	6	47
	23.4%	63.8%	12.8%	100.0%
Total	14	49	39	102
	13.7%	48.0%	38.2%	100.0%

P< 0.05

E.coli isolates from chicken faeces (n=10) harboured plasmids while 3 of the isolates from cattle faeces (n=10) harboured plasmids (Fig 1 and 2). Gel electrophoresis image of genomic DNA (n=6) is shown in Fig. 3. PCR products of plasmid DNA (n=13) and genomic DNA (n=6) of resistant isolates did not show the presence of *qnr*A.

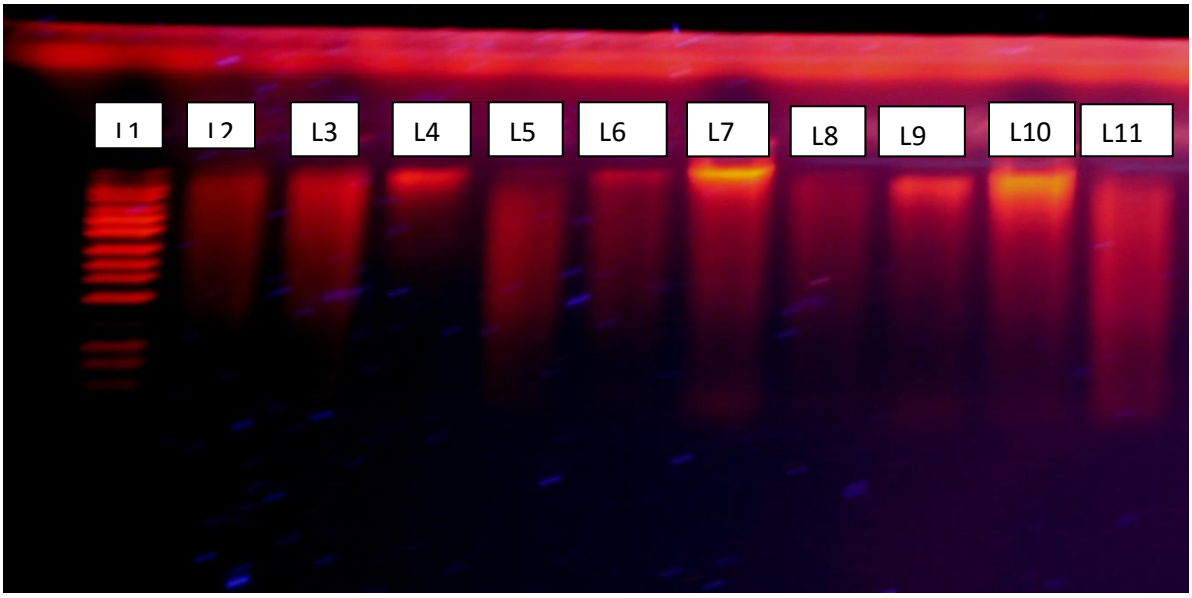

Figure 1: plasmid DNA isolated from antimicrobial resistant *Escherichia coli* in chicken faecal samples.

Legend:

L1 = DNA ladder *L2 = isolate 1* *L3 = isolate 2*
L4 = isolate 6; *L5 = isolate 15* *L6 = isolate 17*
L7 = isolate 26 *L8 = isolate 30* *L9 =isolate 32*
L10 = isolate 45 *L11 = isolate 52*

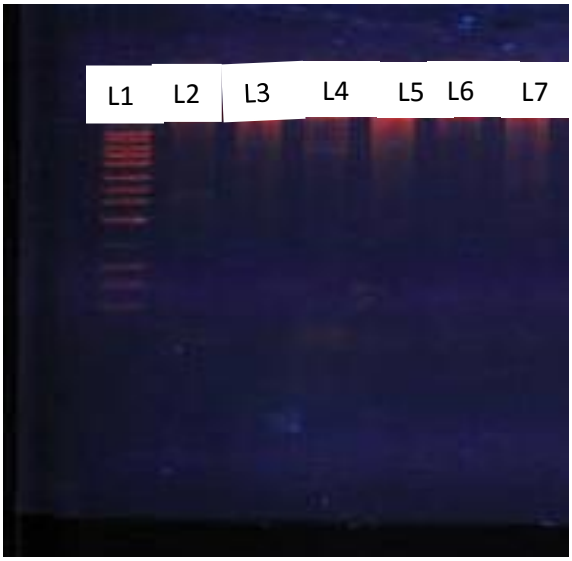

Figure 2: Genomic DNA isolated from antibiotic resistant *Escherichia col*i in cattle and chicken faecal samples.

Legend:

L1 = DNA ladder

Cattle Escherichia coli isolates

L2 = isolate 9
L3 = isolate 11
L4 = isolate 13

Chicken Escherichia coli isolates

L5 = isolate 1
L6 = isolate 2
L7 = isolate 15

Discussion

The significantly higher resistance of isolates from chickens to ciprofloxacin and nalidixic (56.4% and 60.5%) acid could be as a result of increased exposure and usage of antibiotics in chickens than in cattle. There has been a report of AMR for ciprofloxacin (77%) and nalixidic acid (57.4%) among clinical *E. coli* isolates in Ibadan (16). Increasing emergence of resistance to first-line antimicrobials elevated including flouroquinolones have also been reported (17, 18 and 1). There appears to be an increase in the use of fluoroquinolones for the treatment of bacterial infections and for prophylactic purposes both in human and animal medicine. The increase in the use of these antimicrobial agents has resulted in greater resistance due selective pressure. Furthermore, the injudicious use of these agents at insufficiently high doses, or for inappropriate duration of therapy encourages development of resistance.

The non-detection of *qnr*A resistance determinant in antimicrobial resistant isolates is consistent with the reports of some previous studies (1, 3). The molecular basis of resistance of the isolates tested may be as a result of other resistance determinants such as *qnr*B, *qnr*S or *aac* (6')-*lb-cr* a plasmid mediated quinolone resistance determinant. Assessing antimicrobial resistance in non-pathogenic bacteria and understanding the molecular mechanisms of resistance can assist in the control of antimicrobial resistance and the formulation of policies towards it. Human and veterinary medicine practitioners should prescribe and administer antibiotics more conscientiously to minimise the development of resistance.

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