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Chromosome Mediated Fluoroquinolone and Extended Spectrum Beta-lactamase Resistant Genes in *E. coli* of Poultry Origin in Ekiti State

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Authors' contributions

This work was carried out in collaboration between both authors. Author AOO designed the study managed the analyses of the study. Author KOO wrote the protocols, wrote the first draft of the manuscript and managed the literature searches. Both authors read and approved the final manuscript.

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ABSTRACT

Background: One health approach aimed at solving global health crisis links human, animal, and environmental health. This inclusive strategy has contributed to antibiotic classification in both human and animal medicine.
Aims: The aims of this research work are to determine the phylogenetic relationship of *E. coli* isolated from poultry and waste sources. The presence of chromosome mediated fluoroquinolone and extended spectrum beta-lactamase resistant genes will also be detected in the isolates.
Study Design: Experimental design.
Methodology: Data on farming attitudes of poultry farmers were collected using a questionnaire. *E. coli* was isolated from fresh poultry droppings and waste disposal sites using eosine methylene blue agar. The antibiotic sensitivity profile of the isolates was determined using the modified Kirby Bauer disc diffusion method. Phenotypic expression of fluoroquinolone (*qnrS*) and beta-lactamase (*blaCMY*) resistant traits were further detected using Polymerase Chain Reaction. The 16S rRNA

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gene sequencing was carried out followed by sequence alignment of *E. coli* genes with those from GenBank sources to determine the molecular identity of the isolates. Spearman's correlation coefficient (r_s) was run to determine the relationship between antibiotic treatment and resistant profile of the isolates. The phylogenetic relationship of the isolates was determined using Bio edit and Mega 6 software.

Results: Organic poultry farming was practiced by small-scaled, peasant farmers who raised free range birds while antibiotics were widely used on farms that adopted intensive mode of farming. The percentage occurrence of *E. coli* from waste disposal sources was lesser than that from fresh poultry droppings. Highest percentage of antibiotic resistance to the fluoroquinolones was found while the carbapenemase recorded the lowest. Statistical analysis shows that antibiotic treatment in poultry and resistant profile of isolates to antibiotics are directly related. The percentage similarity of gene sequence with those from Gene Data Bank (≥99.29%) validates the identity of the isolates as *E. coli*. About, 60% of the sampled population had the *qnrS* gene with a band size of approximately 322 base pair. Besides, 40% of the sampled isolates possessed the *blaCMY* gene with a band size of approximately 460 base pair. Both genes co-existed in the chromosome of 15% of the sampled isolates sourced from poultry droppings and waste sources. Phylogenetic classification links the origin of isolates from waste disposal sources to poultry production sites. Besides, variant strains of multiple antibiotic resistant *E. coli* from poultry with antibiotic treatment were more diverse compared to those obtained from birds raised without antibiotics.

Conclusion: The *qnrS* and *blaCMY* genes found in multiple antibiotic resistant *E. coli* mediated resistance to critically important antibiotics. The co-existence of these genes in variants strains of *E. coli* occupying different phylogenetic clusters suggests that antibiotics were widely used on the birds. Antibiotic treatment regimen in poultry may be responsible for the expression of antibiotic resistant genes found in the chromosome of the variant strains of *E. coli*.

Keywords: Poultry; antibiotic resistant genes; E. coli.

ABBREVIATION

M.A.R.B. : Multiple Antibiotic Resistant Bacteria

1. INTRODUCTION

The development of one health approach linking the health of humans, animals, and the environment has contributed to antibiotic classification in both human and animal medicine. Critically important antibiotics include the aminoglycosides, third and fourth generation cephalosporins, tetracyclines, sulfonamides, fluoroquinolones, macrolides and penicillins. Veterinary highly important antibiotics are rifampicin, first generation cephalosporin, lincomycins, bacitracin, colistin, and first generation quinolones [1]. Also, there are veterinary important antibiotics, virginiamycin, avilamycin, fusidic acid, novobiocin. Some of the veterinary critically-important antibiotics such as the fluoroquinolone and newer generation of cephalosporins are also considered to be critically important for both humans and animal health. These two classes of antibiotics are not recommended as prophylaxis or for first line treatment in poultry [1]. There are suggestions that antibiotic usage in poultry is responsible for selection and transmission of antibiotic resistant genes. Antibiotics at sub-lethal concentration can promote genetic changes through different pathways involving various stress responses [2]. Bacterial strains harboring genes encoding resistance may be transferred to the microbiota of an animal host without causing an infection. However, these antibiotic resistant bacteria can potentially spread and cause infection to other host [3,4]. Antibiotic resistant genes are often acquired among bacterial population through spontaneous mutation (vertical evolution) and horizontal gene transfer. Vertical evolution involves transfer of changes in genetic composition from parents to their offspring. Horizontal gene transfer entails movement of genetic materials to another organism of different offspring [5].

Antibiotic resistant genes encode gene products that genetically confer on bacterial cells the ability to grow when antibiotics are administered on them [6]. These antibiotic resistant genes include *qnrA, qnrB, qnrS, AcrA* (fluoroquinolone resistant gene)*; aac*(*3*)*-IV, aac*(*3*)*-II, aac*(*6'*)*-ib-cr, ant*(*3'*)*-I, aph*(*3*)*-II*-aminoglycoside resistant genes; *blaSHV, blaCMY, blaOXA, blaTEM, blaCTX*-beta-lactamase resistant genes*; ermA,*

ermB, ermC, msrA-erythromycin resistant genes; *sul-I, sul-II*-sulfonamide resistant genes*; tetA, tetB, tet C*-tetracycline resistant genes*; dfrA1*-trimethoprim resistant gene; *aadA1*- streptomycin resistant gene; *cmlA*, *floR*, *cat-I* -chloramphenicol resistant genes; *Mcr-I* polymyxin resistant gene [7,8]. The genes that code for antibiotic resistance in bacteria can be plasmid mediated or located on chromosomes [9]. It was observed that some strains of *E. coli* have plasmids with an intact promoter, and genes that encode for resistance to streptomycin, ß-lactamase, sulfonamides and tetracycline. But some of these genes were not expressed due to a chromosomal transcriptional control that silenced the expression of plasmid genes. Also, plasmid borne cells can be eliminated in a bacterial population [10]. Besides, natural plasmids are usually large, maintained at low copy number and prone to loss [11]. Apparently, the bacterial chromosome may be considered a more stable and natural repository of antibiotic resistant genes than any other known source(s).

2. MATERIALS AND METHODS

2.1 Research Tools

A questionnaire was administered to farm managers, and data on poultry management practices were collected.

2.2 Place and Duration of Study

Microbiology Department, Ekiti State University, Ado-Ekiti, from February 2017 to December 2019.

2.3 Study Population and Site

The study population comprises managers and birds such as layers, broilers, turkeys and free range birds from twelve poultry farms in Ido and Usi Ekiti. A total of 204 fecal droppings, 12 feed samples, 12 water samples and 12 samples comprising soil and waste water from disposal sites were collected for culturing.

2.4 Collection of Samples

Fresh fecal droppings from poultry birds were randomly sampled with a sterile swab stick and transferred into a freshly procured, factory-packed, sealed polythene bag. Farm feed, water and soil from disposal sites were also collected in sterile universal containers and immediately transferred to the Microbiology Laboratory, Ekiti State University, Ado-Ekiti [12]. The samples were cultured within 2 hours of collection.

2.5 Isolation Techniques and Biochemical Characterization

Swab sticks containing fecal droppings from poultry birds were suspended in 5 mL of sterile saline water, prepared as 10% suspension. The suspension was streaked on E.M.B agar plates with sterile wire loop and incubated at 37°C for 18-24 hours. Distinct colonies with green metallic sheen and dark centers from the primary culture were preliminarily identified as *E. coli* [13,12]. One gram of the collected soil sample and 1 ml of waste water sample ware serially diluted. About 0.1 mL of the tenth-fold dilution from the eighth tube and a loopful of diluents from test tubes with dilution factor of 10^{-5} and 10^{-6} were cultured using pour plating and streaking methods on EMB agar plates respectively. The plates were incubated at 37°C for 24 hours. Identified distinct colonies were sub-cultured on sterile eosin methylene blue agar in order to obtain a pure secondary culture. They were preserved on nutrient agar slant for biochemical characterization [12].

2.6 Antibiotic Susceptibility Testing

Antibiotic susceptibility test was carried out using the modified Kirby Bauer disc diffusion method. Antibiotic discs (Oxoid) comprising ciprofloxacin (5 µg), Tetracycline (30 µg), ofloxacin (5 µg), Trimethoprim/sulfamethozazole (1.25/23.75 µg), gentamycin (10 µg), amoxicillin-clavulanic acid (20/10 µg), ceftaxidime (30 µg), meropenem (10 µg) and cefriazone (30 µg) were used. Mac Farland standard of 0.5 which gives an inoculum size of 1.5×10^{-8} CFU/mL was used to standardize the density of the suspension. The zones of inhibition were recorded and interpreted as susceptible, intermediate and resistant based on procedures of Clinical and Laboratory Standard Institute, 2013 [14].

2.7 DNA Extraction and Molecular Identification of Isolates

The DNA samples of twenty selected isolates were sub-cultured in 3 mL of Luria Bertani broth and incubated at 37°C for 18 hours. The bacterial DNA samples were extracted using the phenol chloroform extraction protocol [15]. The quality of the extracted DNA was estimated in 1.5% agarose gel electrophoresis and the DNA

bands were visualised in UV light imaging system. The DNA fragments were amplified by PCR for bacterial identification using previously reported universal 16S rRNA gene primers 27 F 5′- AGAGTTTGATCMTGGCTCAG-3′- and 1525R 5′-AAGGAGGTGATCCAGCC-3′ [16]. PCR was carried out in a GeneAmp 9700 PCR System Thermal cycler (Applied Biosystem Inc., USA). The PCR cocktail contained 10 µL of 5x GoTaq colourless reaction, 3 µL of 25 mM $MgCl_2$, 1 µL of 10 mM of dNTPs mix, 1 µL of 10 pMol each 27F 5′-AGAGTTTGATCMTGGCTCAG- 3′ and 1525R 5′-AAGGAGGTGATCCAGCC-3′ primers and 0.3 units of Taq DNA polymerase made up to 42 µL with sterile distilled water and 8 µL DNA template [17]. The PCR profile consists of an initial denaturation temperature at 94 °C for 5 min; followed by a 30 cycles of 94 °C for 30 s, 50 °C for 60 s, 72 °C for 1 minute 30 seconds; and a final termination at 72 °C for 10 mins [16,18]. The integrity of the amplified 1.5 Mb gene fragment was checked on a 1.5% agarose gel electrophoresis. It was visualized by ultraviolet trans-illumination, photographed and the sizes of the PCR products were estimated [19,15]. The amplified fragments were sequenced using a Genetic Analyzer 3130 XL sequencer from Applied Biosystems using the manufacturers' manual while the sequencing kit used was that of Big Dye terminator. Sequence editing and phylogenetic analysis was done using bio-edit and mega 6 software. The bacterial sources of DNA were identified by matching with validated sequences of highest maximum identity score from the GenBank database [20].

2.8 Molecular Detection of *qnrS* and *blaCMY*-2 Genes in Strains of *E. coli* via PCR

Molecular detection of *qnrS* coding gene in the isolates was by simple PCR on the extracted DNA template using *qnrS* coding regions specific primers. Previously reported primers targeting *qnrS* coding genes, *qnrS*- F: 5´-GCAAGTTCATTGAACAGGGT 3´ and *qnrS*-R: 5´TCTAAACCGTCGAGTTCGGCG 3´ with an amplicon size of about 428 bp were used [21]. The reaction cocktail at a volume of 25 µL was used based on the manufacturer's prescription. The reaction cocktail included 12.5 µL of 2X PCR Master mix with dNTPS (400 µM) and $MgCl_2$ (3 mM) at final concentration of 1X and pH of 8.5, 2.5 µL each of forward and reverse primers (1.0 µM), 5 µL of DNA template (100 ng), nuclease-free water was added to make up the reaction volume [17]. The PCR profile used for the amplification of *qnrS* coding genes included an initial denaturation at 94°C for 45 s followed by 35 cycles each of denaturation temperature of 94°C for 45 s, annealing at 53°C for 45 s, extension (72°C for 5 min) and final extension at 72°C for 5 min [22]. Similarly, molecular detection *blaCMY*-2 coding gene in the isolates was also by simple PCR on the extracted DNA template using *blaCMY*-2 coding regions specific primers. Previously reported primers targeting *blaCMY*-2 coding genes were obtained from Promega, U.S.A. The Primers' coding genes were *blaCMY*-F5 ' -GGCGTGTTGGCGGCGATG-3 and *blaCMY*- R 5´CAGCGGAACCGTAATCCA 3´with an amplicon size of about 364 bp [23]. The reaction cocktail of volume 25 µL used for PCR was based on the manufacturer's prescription. It comprises12.5 µL of 2X PCR Master mix with dNTPS (400 µM) and $MgCl_2$ (3 mM) at final concentration of 1X and pH of 8.5, 2.5 µL each of forward and reverse primers (1.0 µM), 5 µL of DNA template (100 ng), nuclease-free water was added to make up the reaction volume [17]. The PCR profile used for the amplification of *blaCMY*-2 coding genes includes an initial denaturation at 95°C for 2 minutes. Subsequently, 35 cycles each of denaturation temperature of 95°C for 45 s, annealing at 48°C for 45 s, extension at 72°C for 1 mins, followed by final extension at 72°C for 5 mins were observed [23]. The PCR products were analyzed in 1.5% agarose gel [17,19].

2.9 Statistical Analysis

Statistical analysis was carried out using SPSS version 20.0 for the analysis of Spearman's correlation coefficient, percentages and frequency.

3. RESULTS AND DISCUSSION

The antibiotic treatment profile of *E. coli* examined from poultry birds in Ido-Ekiti is shown in Table 1. Data from farm records show that eleven antibiotics comprising enrofloxacin, erythromycin, tetracycline, gentamycin, streptomycin, neomycin, colistin, cotrimoxazole, metronidazole, tylosine and chloramphenicol were selectively used on the farms. Based on the different antibiotic classes administered on the farm, sulfonamides were administered most (50%), while nitroimidazole and amphenicol were the least administered (8%). Diaveridine, an anticoccidiostat was administered in many of the

farms (50%). Carbapenem, penicillin and cephalosporin were not administered.

The level of occurrence of *E. coli* in the samples is shown in Table 2. *E. coli* was found in both poultry droppings and waste disposal sources respectively.

The antibiotic percentage resistant profile of *E. coli* isolated is shown in Table 3. Multiple antibiotic resistant *E. coli* were isolated from 4 poultry waste disposal sites with poultry droppings across the 12 farms. The average resistance of the isolates to ciprofloxacin was the highest (87%), followed by ofloxacin and cotrimoxazole.

The Spearman correlation coefficient (r_s) of variables is shown in Table 4. There was a strong, positive correlation between antibiotics used for treatment and resistant profile of the isolates, which was statistically significant ($r_s(3) = 0.866$, $p = .333$). Besides, there was also a strong positive correlation between antibiotics on sale from market survey and resistant profile of the isolates, which was statistically significant ($r_s(3) = 0.500$, $p = .667$).

The **s**equence alignment of isolates with strains from Gene data bank is shown in Table 5. The percentage similarity of gene sequence with those from Gene Data Bank falls within the range of 99.29% - 100%. The DNA sequences show that all the sampled isolates were strains of *E. coli.* The DNA sequences obtained in this research were deposited in GenBank under the accession numbers: SUB5294851KENECF3MK606083, SUB 5294851KENECJ11MK606084, SUB5294851KEN ECG3MK606085, SUB5294851KENECI1MK60608 6, SUB5294851KENECL6MK606087, SUB529485 1KENECH4MK606088, SUB5294851KENECJ12M K606089, SUB5294851KENECI2MK606090, SUB 5294851KENECL5MK606091, SUB5294851KENE CI14MK606092, SUB5294851KENECA2MK60609 3, SUB5294851KENECD1MK606094, SUB529485 1KENECH8MK606095, SUB5294851KENECK7M K606096, SUB5294851KENECE3MK606097, SUB 5294851KENECC4MK606098, SUB5294851KENE CB5MK606099, SUB5294851KENECK19MK6061 00, SUB5294851KENECK5MK606101, and SUB5 294851KENECG9MK606102.

The agarose gel electrophoresis of PCR product of amplified *qnrS* resistant genes from *E. coli* isolates is shown in Plate 1. Results from Polymerase Chain Reaction and agarose gel electrophoresis of the PCR products show that *E. coli* in lane 1, 2, 4, 6, 7, 8, 10, 14, 17, 18, 19 and 20 comprising 60% of the sampled population had the *qnrS* (fluoroquinolone resistant) gene with a band size of approximately 322 bp on the chromosome.

Also, the agarose gel electrophoresis of the PCR products of extended spectrum beta lactamase gene *blaCMY*-2 amplified from the DNA of the isolates is represented in Plate 2. The results from molecular analysis show that *E. coli* in lanes 3, 7, 10, 13, 14, 16, 17 and 19 comprising 40% of the sampled population possessed *blaCMY*-2 (the extended spectrum beta-lactamase gene) with a band size of approximately 460 bp on the chromosome. The *blaCMY*-2 gene was present in the two isolates from free range birds obtained from the same farm without a record of antibiotic. The gene was present in two isolates obtained from layers. They were from two different farms. The gene was also present in one isolate each from cockerel and broiler. Also, two isolates from different waste disposal sites harbored the *blaCMY*-2 gene*.* Besides, both the *qnrS* and *blaCMY*-2 genes co-existed in the genome of 15% of the isolates from free range birds, layers, and soil (disposal sites).

The phylogenetic analysis of the sequenced bacterial genes is represented in Fig. 1. The phylogenetic tree constructed was a well rooted tree with *Lactobacillus plantarum* NR_042254.1 used to create an out group (negative control) while *E. coli* MG913260.1 and *Escherichia fergusonii* NR_027549.1 from Gene Data Bank were used as positive controls at species and genus levels respectively. Isolate I2 out-grouped itself from the rest of the samples. The constructed tree has 2 major clades. Isolates B5, C4, H4, I1, G3, L6, F3, A2, and I14 formed a sub-cluster of the major clade. Also, isolates J12, H8, K5, K7, D1, E3, J11, K19, L5, G9, I14 and A2 formed the second sub-cluster of the major clades. In addition, isolates from free range birds without antibiotic treatment were restricted to a sub-cluster in one of the major clades while isolates from birds with antibiotic therapy and those from disposal sites were distributed across the two major clades.

The findings of this research show that antibiotics are widely used in the business of poultry

management. The practice of organic farming that limits antibiotic-use in poultry production was only adopted among small-scaled, peasant poultry farmer(s) with free range birds. Wide-spread use of antibiotics in poultry management is common with large scale poultry management. Periodic use of antibiotics on the farm may be attributed to compromised bio-security measures and failure of routine immunization programs on poultry farms. Besides, antibiotics were used majorly to check high mortality rate, disease outbreak and declining egg production. Knowledge based resources on organic farming may not be available in the area of study due to prior belief in antibiotics for infection control in poultry management. The current level of antibiotic-use in poultry management can be reduced by raising disease resistant birds and implementing viable vaccination schedules.

E. coli was isolated from poultry droppings obtained from different birds. Fecal samples from free range birds, without antibiotic treatment recorded the highest presence of *E. coli*. Also, the presence of the bacteria in soil and waste water samples obtained from disposal sites is an indication of bio-pollution. Antibiotic resistant bacteria constitute a bio-pollutant and the environment is a reservoir of these hazardous agents. The level of occurrence of the bacteria varies in different farms. Comparatively, *E. coli* was more in fresh poultry droppings than in soil and water sourced from the waste disposal sites. Abiotic conditions in the environment may limit the survival rate of the bacteria. However, the observed bacterial presence contrasts that of [24] who recorded an isolation rate of 83% in fecal droppings from poultry sources. The highest level of *E. coli* in samples from free range birds shows that heavy dose of antibiotic-therapy on the sampled farm might be responsible for the observed low isolation rates.

The presence of multiple antibiotic resistant *E. coli* in environmental (soil/water) samples from disposal sites shows that the environment can be a secondary reservoir of multiple antibiotic resistant bacteria. Also, there was spread of multiple antibiotic resistance on the farms than resistance to single antibiotic. Antibiotic therapy eliminates susceptible bacterial population and selects resistant strains [25]. Infection with multiple antibiotic resistant bacteria that evade treatment may pose a threat to humans and health institutions if the sources of spread are not checked.

The highest percentage of antibiotic resistance to the fluoroquinolones was observed. This observation might be associated with wide-use of enrofloxacin on farms. In contrast, resistance to meropenem was the lowest and this may be due to non-use of the carbapenemase class of antibiotics for therapy in poultry. Geographically, this result contrasts that of [26] that the percentage resistance of *E. coli* from human origin to carbapenemese in most European countries was less than 1% [27]. Majority of the isolates were resistant to multiple classes of antibiotics. The resistance shown to antibiotics such as meropenem, amoxycillin-clavulanate and the cephalosporins that were not administered on the farms may be due to the use of an analogue to these antibiotics for therapy. However, meropenem may be an antibiotic of choice presumably because of proactive antibiotic formulation policy limiting its usage for poultry management.

The DNA sequences show that all the sampled isolates were*E. coli* bacteria related to strains with potentials for pathogenicity.

The statistical analysis suggests a direct relationship between antibiotic therapy on the farm and bacterial resistance to antibiotics. This shows a direct proportional relationship between the two variables. Both data on antibiotic treatment and that from market survey are related to antibiotic resistant profile of isolates. However, data on antibiotic treatment on the farm are more related to resistance of bacteria than those obtained from market survey. This result contrasts the findings of [28] who recorded a negative Spearman correlation coefficient, r_s (8) = -0.243 between antibiotic treatment and antibiotic resistant profile of isolates. This may be attributed to the differences in the number of antibiotics used for both treatment and antibiotic sensitivity test. Thus, the phenomenon of bacterial resistance to antibiotics is linked to antibiotic-use for therapy in poultry.

Table 1. Antibiotic treatment profile of poultry birds examined

Characteristics		Frequency (%)
Exposure to antibiotics	Yes	11 (91.7)
	No	1(8.3)
Purpose of usage	Disease prevention	3 (27.3)
	Treatment	7 (63.6)
	Enhance egg production	1 (9.1)
	None	1 (9.1)
Method of administration	Water	11 (100)
	Feed	0 (0)
	Injection	0 (0)
Age of birds when first exposed	2 weeks	5 (45.5)
	1 month	3 (27.3)
	2 months	2 (18.2)
	3 months plus	1 (9.1)
Routine chart for antibiotic use	Yes	3 (25)
	No	9 (75)
Frequency of administration of antibiotics	Cotrimoxazole	6 (50)
	Tetracycline	5 (41.7)
	Gentamycin	3 (25)
	Enrofloxacin	2 (16.7)
	Erythromycin	2 (16.7)
	Streptomycin	2 (16.7)
	Neomycin	2 (16.7)
	Colistin	2 (16.7)
	Metronidazole	1 (8.3)
	Chloramphenicol	1 (8.3)
	Tylosine	1 (8.3)
Frequency of administration of classes of antibiotics	Sulphonamide	6 (50)
	Tetracycline	5 (41.7)
	Aminoglycoside	5 (41.7)
	Fluoroquinolone	2 (16.7)
	Macrolide	2 (25)
	Polymyxin	2 (16.7)
	Nitroimidazole	1 (8.3)
	Amphenicol	1 (8.3)
Product availability from market survey	Tetracycline	2 (100)
	Cotrimoxazole	1 (50)
	Neomycin	2 (100)
	Gentamycin	2 (100)
	Erythromycin	2 (100)
	Penicillin	1 (50)
	Enrofloxacin	1 (50)
	Streptomycin	1 (50)
	Colistin	1 (50)
	Chloramphenicol	1 (50)
	Tylosine	1 (50)

***** *Numbers in parenthesis are percentage values*

Table 2. Isolation rate of *E. coli* from poultry in Ido-Ekiti

							Waste sources/samples examined		
Poultry site	**No of Poultry droppings examined**	**No positive (%)**	**Poultry feed examined**	**No positive (%)**	**Poultry water examined**	**No positive (%)**	**Waste water samples examined**	**Soil samples examined**	**No. positive (%)**	**Total samples examined**
A (Pullet)	17	5 (29.4)	1	-	1	-	-	1	-	20
B(Layer)	17	3 (17.7)	1	-	1	-	-	1	-	20
C(Broiler)	17	3 (17.7)	1	-	1	-	-	1	-	20
D(Broiler)	17	5 (29.4)	1	-	1	-	-	1	-	20
E(Turkey)	17	6 (35.3)	1	-	1	-	-	1	-	20
F(Turkey)	17	5 (29.4)	1	-	1	-	-	1	-	20
G(Layer)	17	8 (47.1)	1	-	1	-	-	1	1	20
H(Layer)	17	11 (64.7)	1	-	1	-	-	1	-	20
I(Layer)	17	13 (76.5)	1	-	1	-	-	1	1	20
J(Cockerel)	17	12 (70.6)	1	-	1	-	-	1	-	20
K(Local)	17	14 (82.4)	1	-	1	-	1	-	1	20
L(Layer)	17	5 (29.4)	1	-	1	-	-	1	1	20
Total (%)	204	90 (44.1)	12	-	12	-	1	11	4 (33.3)	240 (100)

** Numbers in parenthesis are percentage value*

Table 3. Antibiotics percentage resistant profile of *E. coli* isolated

		Cephalosporin			Fluoroquinolone		Aminoglycosid	Caberpenem	Sulfonamide	Tetracycline	
Source code	**N**	**CRO (%)**	**CAZ (%)**	**AMC (%)**	**CIP (%)**	**OFX (%)**	**CN (%)**	**MEM (%)**	**SXT (%)**	**TET (%)**	**n (%)**
A	5	0	0	5 (100)	4 (80)	3 (60)	4 (80)	0	3 (60)	4 (80)	4 (80)
B	3	1 (33.3)	1 (33.3)	3 (100)	1 (33.3)	1 (33.3)	1 (33.3)	0	1 (33.3)	3 (100)	3 (100)
C	3	1 (33.3)	1 (33.3)	3 (100)	1 (33.3)	2 (66.7)	3 (100)	0	2 (66.7)	2 (66.7)	3 (100)
D	5	1 (20)	1 (20)	5 (100)	4 (80)	5 (100)	5 (100)	0	2 (40)	3 (60)	5 (100)
E	6	0	0	5 (83.3)	5 (83.3)	5 (83.3)	3 (50)	2 (33.3)	4 (66.7)	5 (83.3)	6 (100)
F	5	1 (20)	1 (20)	5 (100)	5 (100)	5 (100)	1 (20)	3 (60)	4 (80)	4 (80)	5 (100)
G	8	5 (62.5)	5 (62.5)	2 (25)	8 (100)	6 (75)	3 (37.5)	3 (37.5)	6 (75)	7 (87.5)	8 (100)
H	11	9 (81.9)	8 (72.7)	7 (63.6)	11 (100)	9 (81.9)	8 (72.7)	1 (8.7)	11 (100)	11 (100)	11 (100)
I	13	13 (100)	12 (92.3)	9 (69.2)	11 (84.6)	12 (92.3)	13 (100)	9 (69.2)	10 (76.9)	10 (76.9)	12 (92)
J	12	11 (91.7)	10 (83.3)	7 (58.3)	12 (100)	10 (83.3)	7 (58.3)	7 (58.3)	10 (83.3)	11 (91.7)	12 (100)
K	14	9 (64.3)	10 (71.4)	10 (71.4)	12 (85.7)	12 (85.7)	12 (85.7)	7 (50)	11 (78.6)	10 (71.4)	12 (86)
L	5	5 (100)	4 (80)	4 (80)	5 (100)	5 (100)	4 (80)	4 (80)	5 (100)	1 (20)	5 (100)
F/W	-	-	-	-		-	-	-	-	-	-
DS	4	4 (100)	3 (75)	3 (75)	3 (75)	3 (75)	4 (100)	2 (50)	4 (100)	2 (50)	4 (100)
Total	94	60 (63.8)	56 (60)	68 (72.3)	82 (87.2)	78 (83)	68 (72.3)	38 (40.4)	73 (77.7)	73 (77.7)	90 (95.7)

Keys: n=number of isolates, N- Number of isolates showing multiple antibiotic resistance, F/W- isolates from feed and water, DS-number of isolates from disposal site, OFX-Ofloxacin; CIP-Ciprofloxacin, GN-Gentamycin; AMC-Amoxycillin-clavulanate, CRO-Cefriaxone; MEM-Meropenem, CAZ= Ceftaxidime TET= Tetracycline, SXT= Trimethroprim/Sulfamethoxazole. Source A- pullets, B- layers, C- broilers, D- broilers, E-,turkeys, F-,turkey, G-layers, H-layers, I-layers, J-cockerels, K-local birds, L-layer

Table 4. Spearman's statistical correlation of antibiotic consumption and resistant profile

Antibiotics	Frequency of use (%)	r_s	Resistant profile (%)	r_s	Market survey (%)
Cotrimoxazole	6 (50)		73 (77.7)		1 (100)
Tetracycline	5 (41.7)		73 (77.7)		2 (100)
Gentamycin	3 (25)		68 (72.3)		2 (100)
Enrofloxacin	2 (16.7)		NT		2 (100)
Erythromycin	2 (16.7)		NT		2 (100)
Streptomycin	2 (16.7)		NT		1 (50)
Neomycin	2 (16.7)		NT		2 (100)
Colistin	2 (16.7)		NT		1 (50)
Metronidazole	1 (8.3)	r_s (.866)	NT	r_s (.500)	NS
Chloramphenicol	1 (8.3)		NT		1 (50)
Ceftriazone	NA		60 (63.8)		NS
Ceftazidime	NA		56 (60)		NS
Amoxycillin-clavulanate	NA		68 (72.3)		NS
Ciprofloxacin	NA		82 (87.2)		NS
Meropenem	NA		38 (40.4)		NS
Ofloxacin	NA		78 (83)		NS
Penicillin	NA		NT		1 (50)
Tylosine	1 (8.3)		NT		1 (50)

Keys: NA – not administered, NS not sold, NT- not tested, r_s- Spearman's correlation coefficient

Table 5. Sequence alignment of isolates with strains from Gene data bank

Isolate code	Accession number	Matched *E. coli* strain from Gene data bank	Percentage identity (%)
F 3	SUB5294851 KENECF3MK606083	MH67149.1	99.71
J 11	SUB5294851 KENECJ11MK606084	CP042934.2	99.86
G 3	SUB5294851 KENECG3MK606085	CP044314.1	99.66
I 1	SUB5294851 KENECI1MK606086	MK606086.1	100
L 6	SUB5294851 KENECL6 MK606087	KM198100.1	99.79
H 4	SUB5294851 KENECH4MK606088	JQ781559.1	100
J 12	SUB5294851 KENECJ12 MK606089	EU420950.1	99.89
I 2	SUB5294851 KENECI2 MK606090	MH656755.1	99.69
L 5	SUB5294851 KENECL5 MK606091	KP789331.1	99.65
I 14	SUB5294851 KENECI14MK606092	KJ477001.1	99.44
A 2	SUB5294851 KENECA2 MK606093	KJ477001.1	100
D 1	SUB5294851 KENECD1 MK606094	MG602206.1	99.65
H 8	SUB5294851 KENECH8 MK606095	CP026641.1	99.42
K 7	SUB5294851 KENECK7 MK606096	KY780353.1	99.29
E 3	SUB5294851 KENECE3 MK606097	KY655103.1	100
C 4	SUB5294851 KENECC4 MK606098	CP040269.1	99.79
B 5	SUB5294851 KENECB5 MK606099	CP0462591	99.45
K 19	SUB5294851 KENECK19MK606100	CP044315.1	99.65
K 5	SUB5294851 KENECK5 MK606101	KU870317.1	99.86
G 9	SUB5294851 KENECG9 MK606102	MG602205.1	99.58

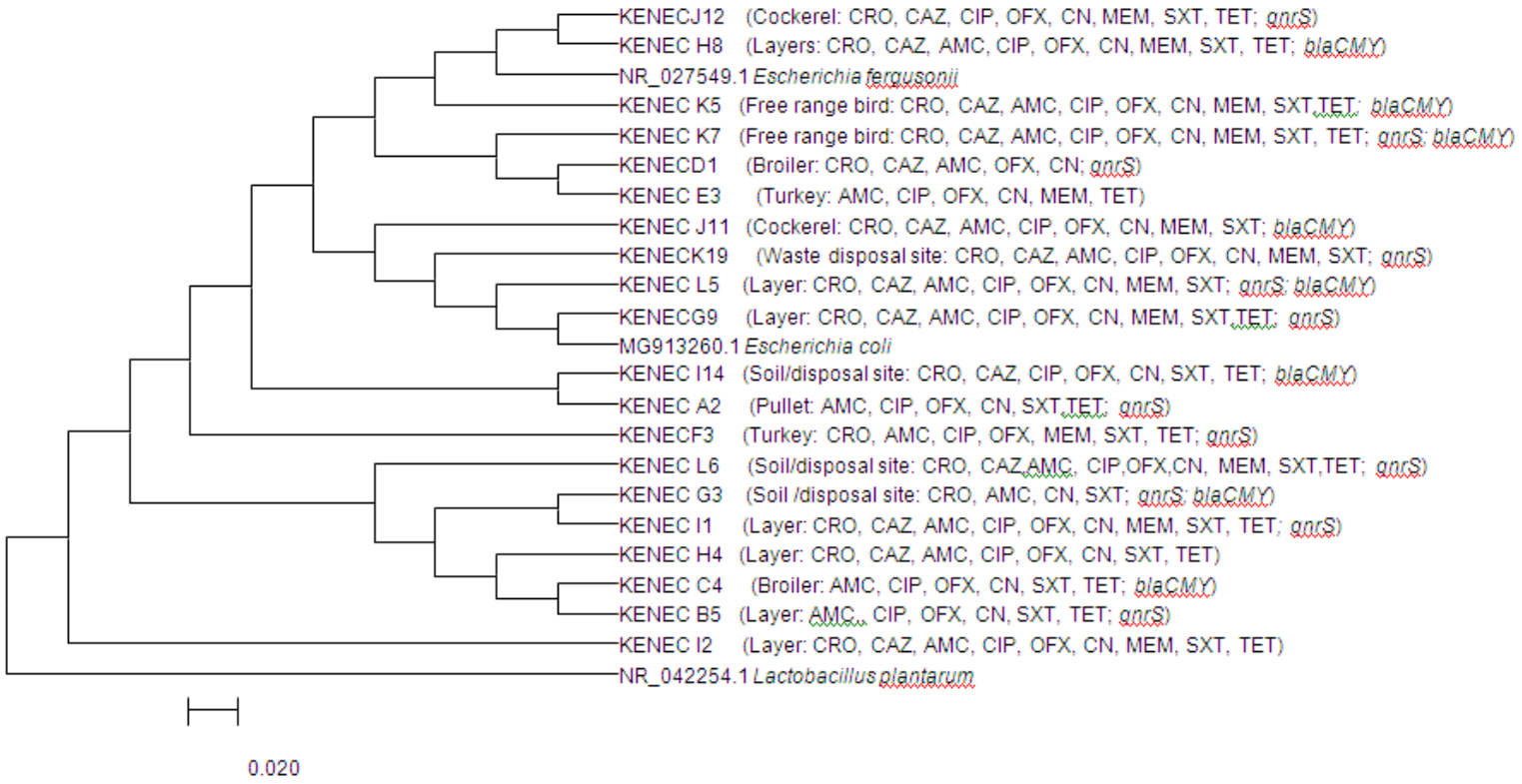

Fig. 1. Phylogenetic analysis of selected *E. coli* isolates

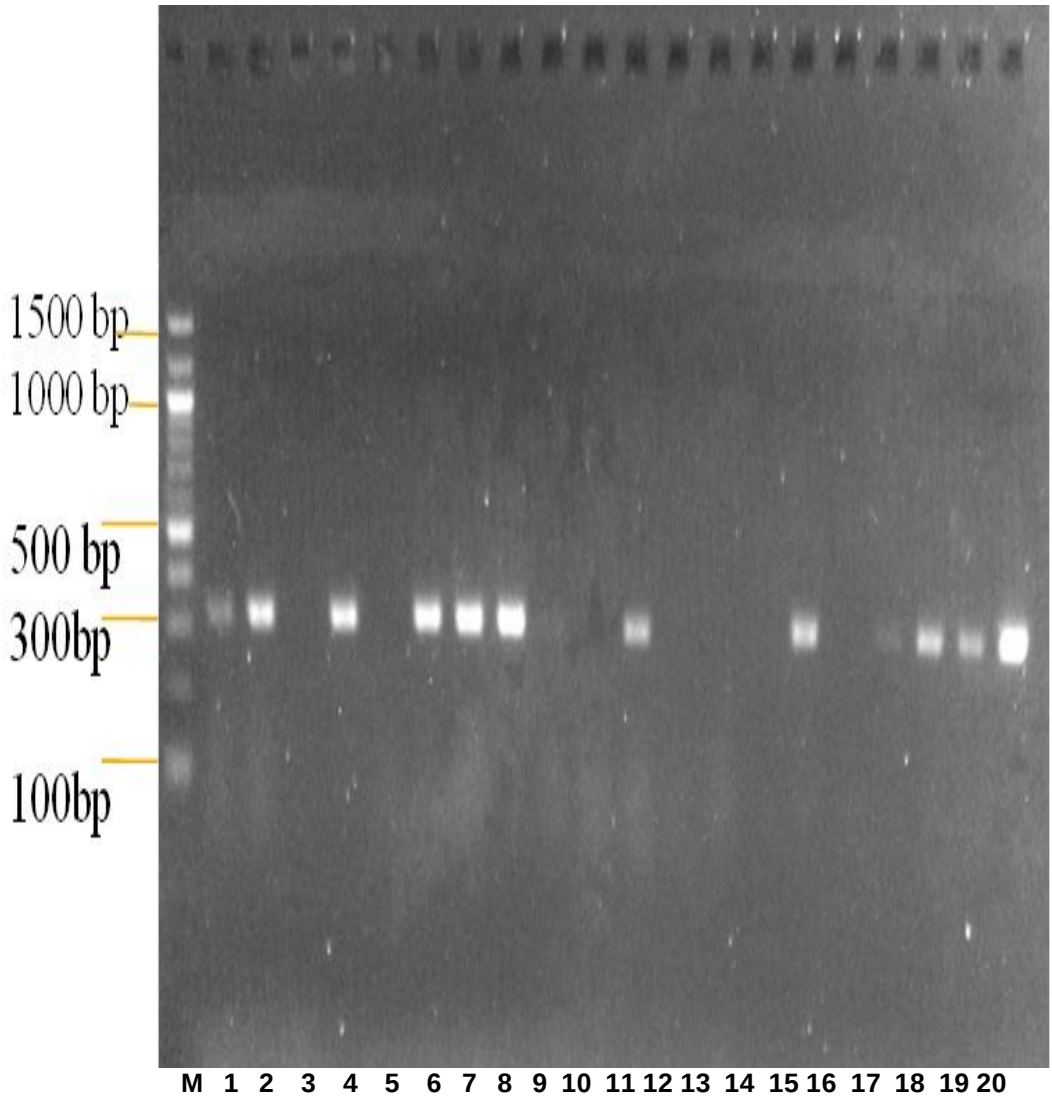

Plate 1. Agarose gel electrophoresis of the PCR products of *qnrS* resistant genes amplified from *E. coli* isolates (Band size approximately 322 bp)

Key: Lane M= Molecular Marker (100-1,500 bp), Lane 1= A2, Lane 2= B5, Lane 3= C4, Lane 4= D1, Lane 5= E3, Lane 6= F3, Lane 7= G3, Lane 8= G9, Lane 9= H4, Lane 10=H8. Lane 11= I1, Lane 12 = I2, Lane 13 = I14, Lane 14 = J11, Lane 15 = J12, Lane 16 =K5, Lane 17 = K7, Lane 18 = K19, Lane 19 = L5, Lane 20 = L6

Results from molecular analysis assert that poultry droppings are the major reservoir of the *qnrS* gene while waste water and soil samples from disposal sites are secondary reservoir. These research findings agree with the assertions of [29] that the frequency of occurrence of *qnrS* gene among poultry and their production sites in Nigeria is high. The *qnrS* gene originated from the chromosome of an organism occupying a human, animal or environmental reservoir [30]. The high level of occurrence of *qnrS* genes in *E. coli* from poultry could be associated with the selection pressure constituted by frequent administration of fluoroquinolone (enrofloxacin) on the farms under study. Resistance to fluoroquinolones in enterobacteriaceae can be chromosomal or plasmid mediated. Chromosome-mediated resistance majorly occurs due to accumulation of mutations primarily in DNA gyrase (GyrA) then in topoisomerase IV [9]. In addition, quinolone resistance can be associated with an over expression of efflux pump systems [31]. High presence of fluoroquinolone resistant gene may constitute a threat to both human and animal health because fluoroquinolones are effective antibiotics in health care delivery.

The presence of *blaCMY*-2 genes in the chromosomes of isolates from free range birds

and waste sources shows that polluted soil and water are secondary reservoir of antibiotic resistant genes. This is consistent with the works of [32] who reported that *blaCMY*-2 genes may be of chromosomal origin. However, resistance to β-lactams in Enterobacteriaceae is majorly due to the production of β-lactamases and it may be encoded either chromosomally or on plasmids [33]. The presence of this gene without the administration of cephamycin (a beta-lactam antibiotic) might be attributed to selection pressure imposed by other antimicrobial substances with similar or broader mode of action. Though farm records asserted that cephamycin, a beta-lactam antibiotic was not administered; a penicillin based formulation was present in the market for treatment of poultry birds and it could have been administered. The expression of the *blaCMY* gene is traceable to the use of an analogue of penicillin, a beta-lactam antibiotic likely used for antibiotic treatment on the farm.

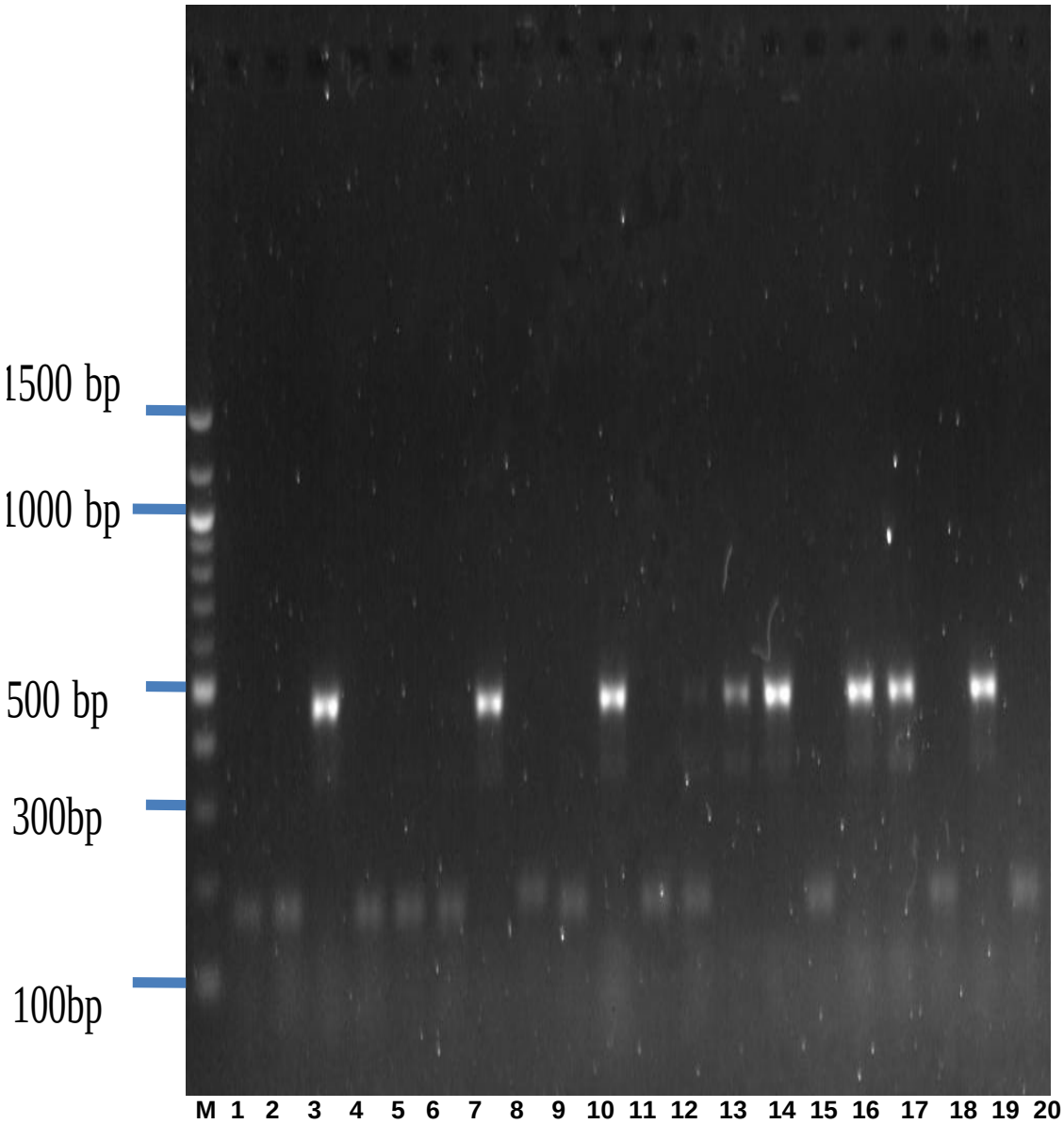

Plate 2. Agarose gel electrophoresis of the PCR products of the *blaCMY*-2 resistant gene amplified from *E. coli* isolates. (Band size approximately 460 bp

Key: Lane M= Molecular Marker (100 -1,500 bp), Lane 1= A2, Lane 2= B5, Lane 3 = C4, Lane 4= D1, Lane 5 = E3, Lane 6= F3, Lane 7 = G3, Lane 8 = G9, Lane 9= H4, Lane 10=H8. Lane 11= I1, Lane 12 = I2, Lane 13 = I14, Lane 14 = J11, Lane 15 = J12, Lane 16 = K5, Lane 17 = K7, Lane 18 = K19, Lane 19 = L5, Lane 20 = L6

Free range birds (animals) have the potential of acquiring antibiotic resistant bacteria from the environment via the ecological food chain. The nomadic life style of free range birds promotes acquisition, release and spread of acquired antibiotic resistant bacteria in droppings to the environment. The fluoroquinolone resistant gene (*qnrS*) is more predominant than the beta lactamase resistant gene (*blaCMY*) in sampled isolates though both genes co-existed in the bacterial chromosome.

Phylogenetic analysis shows that variant strains of *E. coli* from poultry with record of antibiotic treatment and disposal sites were more diverse in their antibiotic consumption profile and clade distribution compared to those from birds raised without antibiotics. The evolution, divergence and spread of chromosomal DNA from certain strains of *E. coli* across the clades is traceable to genomic responses of the DNA to selective pressure imposed by antibiotic treatment. Related bacterial strains could have emerged and spread via a common genetic mechanism. Crude antibiotic treatment in poultry can lead to emergence of phylogenetically diverse strains of bacteria with potential to evade treatment both in human and veterinary medicine. Polluted soil, water, and waste disposal sites are potential secondary reservoir of emergent strains of multiple antibiotic resistant *E. coli.* Phylogenetically diverse strains of bacteria have the potential to complicate treatment both in poultry and humans during infection. Phylogenetic classification also links the origin of multiple antibiotic resistant *E. coli* from waste disposal sites to poultry production sites. Similarly, it links the origin of chromosomal antibiotic resistant genes in isolates from free range birds to their interaction with the environment. This asserts the contribution of antibiotic treatment in poultry, improper waste disposal and free range system of animal farming to increased presence of multiple antibiotic resistant bacteria in the environment.

4. CONCLUSION

The *qnrS* and *blaCMY* genes found in multiple antibiotic resistant *E. coli* mediated resistance to critically important antibiotics and co-existed in variants strains of *E. coli* occupying different clusters in the phylogenetic analysis. Heavy doses of antibiotics and crude treatment regimen in poultry could have promoted the expression of these genes in the variant strains of *E. coli*. However, the absence of these genes in certain strains may be related to the use of antibiotics at a sub-therapeutic dose. Thus, certain doses of antibiotic may be required to trigger the development and expression of antibiotic resistant genes. Besides, there are phynotypic antibiotic resistant traits that were not captured during molecular analysis. Some antibiotic resistant genes could have been acquired and lost through process(es) not examined in the course of this research work.

CONSENT

As per international standard or university standard, respondents' written consent has been collected and preserved by the author(s).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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