Chromosome Mediated Fluoroquinolone (*qnrS*) and Extended Spectrum Beta-lactamase (*blaCMY*) Resistant Genes in *E. coli* of Poultry Origin in Ido-Ekiti

ABSTRACT

Background: The phenomenon of one health approach linking the health of humans, animals and the environment has contributed to the classification of antibiotics used in both human and animal health based on expert opinions.

Aims: The aim of this research work is to determine the predominance of *qnrS* and *blaCMY* resistant genes in *E. coli* of poultry and waste origin and the phylogenetic relationship of the isolates obtained from waste in Ido Ekiti.

Place and Duration of Study: Department on Microbiology, Ekiti State University, Ado-Ekiti, from February, 2017 to December, 2019.

Methodology: *E. coli* was isolated from fresh poultry droppings, feed and water from twelve farms as well as waste disposal sites using eosine methylene blue agar. The antibiogram profile of the isolates was determined using the modified Kirby Bauer disc diffusion method. High levels of phenotypic expression of fluoroquinolone and beta-lactamase resistant traits were further investigated by amplification of the chromosomal DNA and detection of the *qnrS* and *blaCMY* resistant genes through Polymerase Chain Reaction and Agarose Gel Electrophoresis. The 16S rRNA gene sequencing was carried out followed by sequence alignment of *E. coli* genes with those from GenBank. Spearman's correlation coefficient (r_s) was run to determine the relationship between 3 tested antibiotics consumed by birds and resistant profile of isolates. The phylogenetic relationship of the isolates was determined using Bio edit and Mega 6 software.

Results: The percentage occurrence of *E. coli* from fresh poultry droppings (44.1 %) and samples recovered from waste disposal sites (33.3 %) were relatively low. Higher antibiotic percentage resistance to ciprofloxacin (87 %), ofloxacin (83 %), sulfonamide and tetracycline (78 %) were found in the isolates. In addition, 46 multiple antibiotics resistant patterns were recorded and two of these patterns (AMC, CIP, OFX, CN, SXT, TET and CRO, CAZ, AMC, CIP, OFX, CN, MEM, SXT) were spread across 6 out of the 12 farms. There was a strong, positive correlation between antibiotic treatment and resistant profile of isolates to antibiotics which was statistically significant (r_s (3) = 0.866, p = .333). The percentage similarity of gene sequence with those from Gene Data Bank (≥99.29 %) validates the identity of the isolates as *E. coli*. From the Polymerase Chain Reaction, 40 % of the sampled isolates possessed *blaCMY* (the extended spectrum beta-lactamase resistant gene) with a band size of approximately 460 base pair. In addition, 60 % of the sampled population had the *qnrS* (fluoroquinolone resistant) gene with a band size of approximately 322 bp. Both genes co-existed in the chromosome of 15 % of the isolates. Evidence from phylogenetic analysis showed that variant strains of

multiple antibiotic resistant *E. coli* from poultry with antibiotic treatment were more diverse compared to those raised without antibiotics.

Conclusion: The *blaCMY* and *qnrS* 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. Wrong policies on antibiotic formulation and crude treatment regime in poultry promote the expression of these genes haboured in the genome of variant strains of *E. coli*.

Keywords: [Poultry, antibiotic resistant genes]
Abbreviations: [M. A. R. B. - multiple antibiotic resistant bacteria]

1. INTRODUCTION

The phenomenon of one health approach linking the health of humans, animals and the environment has contributed to the classification of antibiotics used in both human and animal health based on expert opinions. Critically important antibiotics include the aminoglycosides, third and fourth generation cephalosporins, tetracyclines, sulfonamides, fluoroquinolones, macrolides and penicillins); veterinary highly important antibiotics (rifampicin), first generation cephalosporin, lincomycins, bacitracin, colistin, first generation quinolones and 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 concentrations can promote genetic changes through multiple pathways involving various stress responses [2]. Resistant bacterial strains, or genes encoding resistance, may also be transferred to the normal gut flora of the consumer without causing an infection. The resistant bacteria can potentially cause infections and spread to other people [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 transfer of genetic material to another 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 strains of *E. coli* can have plasmids with an intact promoter and genes that encode for resistance to streptomycin ß-lactamase, sulfonamides and tetracycline, but not all expressed these genes, presumably due to a chromosomal transcriptional control that silenced the expression of plasmid genes. However, plasmid borne cells are still liable to elimination in a population [10]. Besides, natural plasmids are usually large, maintained at low copy number and prone to loss [11]. Apparently, the

bacterial chromosome is considered a more stable reservoir and natural repository of antibiotic resistant genes than any other known source(s).

2. MATERIALS AND METHODS

Research tools

Study population and site

The study population comprises 12 poultry farms with their managers. Poultry birds such as broilers, turkeys and free range birds were reared in the selected poultry farms in Ido and Usi Ekiti. The study population comprises layers, broilers, turkeys and free range birds. A total of 204 faecal droppings, 12 feed samples, 12 water samples and 12 samples comprising soil and water from disposal sites were collected for bacteriological analysis.

Collection of samples

Fresh feacal 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 for bacteriological analysis [12]. The samples were cultured within 2 hours of collection.

Isolation techniques and biochemical tests

Swab sticks containing the feacal droppings were suspended in 5 mL of sterile saline water, prepared as 10 % suspension. The suspension was streaked on E.M.B plates with sterile wire loop and incubated at 37 °C for 18-24 hours. Distinct colonies with green metallic sheen and dark centres from the primary culture were preliminarily identified as *E. coli* [13, 12]. One gram of the collected soil sample was weighed and added to test tube containing 9 mL of sterile distilled water. A volume of 1 mL of the stock solution was serially diluted in series of test tubes containing 9 mL of sterile distilled water. About 0.1 mL of the tenth-fold dilution from the eighth tube was inoculated into plates and pour plating was carried out with eosine methylene blue agar. The plates were incubated at 37 °C for 24 hours A loopful of diluents from test tubes with dilution factor of 10^{-5} and 10^{-6} were streaked on EMB agar. The plates were incubated as previous. The identified distinct colonies were sub-cultured on sterile eosin methylene blue agar in order to obtain a pure secondary culture. They were further preserved on nutrient agar slant for biochemical tests [12].

Antibiotic susceptibility testing

This was carried out using the modified Kirby Bauer disc diffusion method. Antibiotic discs (Oxoid) comprising ciprofloxacin (5 µg), Tetracycline (30 µg), ofloxacin (5 µg), Trimethroprim/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 ensure standardization by adjusting the density of the suspension with sterile distilled water. A sterile cotton swab was dipped into the adjusted (standardized) broth culture and excess inoculum was drained by pressing the cotton swab against the test tube above the broth suspension. The swab stick was evenly spread over the entire surface of the 15 mL Muella Hinton agar plate to obtain uniform distribution of inoculums. The inoculated plates were then allowed to dry for 3-5 minutes. Antibiotics impregnated discs were loaded and positioned on the surface of the inoculated plates with a multiple disc dispenser to ensure adequate spacing of the discs. A forceps was used to press the antibiotic discs slightly on the agar to ensure contact. The antibiotics impregnated in the discs were allowed to diffuse into the agar medium for about 15 minutes of application of the discs. The plates were inverted

and incubated at 37 °C for 16-18 hours. They zones of inhibition were recorded and interpreted as susceptible, intermediate and resistant based on procedures of Clinical and Laboratory Standard Institute [14].

DNA extraction and molecular identification of isolates

Single colonies of each isolate were sub-cultured in 3 mL of Luria Bertoni broth and cultured at 37°C for 18 hours. The bacterial DNA samples were extracted and purified from the twenty selected *E. coli* isolates using the phenol chloroform extraction protocol as previously described [15]. The quality of the extracted DNA was estimated in 1.5 % agarose gel electrophoresis. Gels were stained with ethidium bromide and DNA bands 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′-AAGGAGGTGATC CAGCC-3′ [16]. PCR was carried out in a GeneAmp 9700 PCR System Thermalcycler (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′-AAGGAG GTGATCCAGCC-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 consist of an initial denaturation at 94 °C for 5 min; followed by a 30 cycles of 94 °C for 30 s, 50 °C for 60 s and 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 and photographed. The sizes of the PCR products were estimated by comparison with the mobility of a 100 bp molecular weight ladder ran alongside experimental samples in the gel [19]. Prior to DNA sequencing the amplified fragments were purified to remove residual primers, buffers, polymerase enzyme and deoxyribonucleotides from PCR reactions. They were checked on a 1.5 % agarose gel ran on a voltage of 110 V for about 1 hr as previous to confirm the presence of the purified product. It was quantified using a nano drop of model 2000 from Thermo Scientific [15]. The amplified fragments were sequenced using a Genetic Analyzer 3130 XL sequencer from Applied Biosystems using 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].

Molecular detection of *qnrS* and *blaCMY-2* genes in strains of *E. coli* via PCR

A sample size of 20 extracted DNA templates earlier identified as *E. coli* via sequencing and sequence alignment of the16S rRNA genes were screened for the presence of *qnrS* and *blaCMY* genes. PCR was carried out in a Gene Amp 9700 PCR System Thermalcycler (Applied Biosystem Inc., USA). Molecular analysis was carried out to determine if the previously observed phenotypic expression of fluoroquinolone resistant trait shown by 87 % of the isolates to ciprofloxacin and 83 % to ofloxacin was chromosomal. Molecular identification of *qnrS* coding gene in the 20 isolates of *E. coli* were by simple PCR on the extracted DNA template using *qnrS* coding regions specific primers. Primers were obtained from Promega, USA. Previously reported primers targeting *qnrS* coding genes, *qnrS*-F: 5′-GCAAGTTCATTGAACAGGGT 3′ and *qnrS*-R: 5′- TCTAAA CCGTCGAGTTCGGCG 3′ with an amplicon size of about 428 bp were used [21]. The reaction cocktail at a volume of 25 µL based on the manufacturer's prescription used for PCR 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 include an initial denaturation at 94 ºC for 45s followed by 35 cycles each of denaturation temperature of 94 ºC for 45 s, annealing at 53 ºC for 45 s), extension (72 ºC for 5 min) and final extension at 72 ºC for 5 min [22]. Similarly, molecular analysis was carried out to determine if the previously observed phenotypic expression of cephalosporin resistant trait shown by 64 % of the isolates to cefriazone and 60 % to ceftazidime was chromosomal. Molecular investigations of *blaCMY*-2 coding gene in the 20 isolates of *E. coli* were 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´-GGCGTGTTG GGCGGCGATG-3´ and *blaCMY*-R 5´ CAGCGG AACCGTAATCCA 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 (Promega, Madison, U.S.A). The PCR profile used for the amplification of *blaCMY*-2 coding genes include an initial denaturation at 95 ºC for 2 min followed by 35 cycles each of denaturation temperature of 95ºC for 45 s, annealing at 50 ºC for 45 s, extension at 72 ºC for 45 s and final termination at 72 ºC for 10 min [23]. The PCR products were analyzed in 1.5 % agarose gel [19].

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. Antibiotics administered on the farms were enrofloxacin, erythromycin, tetracycline, gentamycin, streptomycin, neomycin, colistin, cotrimoxazole, metronidazole and chloramphenicol. Ninety two percent of the examined birds were exposed to antibiotics of which 64 % were administered for therapeutic purpose and routine chart was used by 25 % of the farmers for administration of antibiotics. Majority of the birds (46 %) were first exposed to antibiotics at age of 2 weeks. 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. On the basis of the different classes of antibiotics administered on the farm, sulfonamides were administered most (50 %), tetracycline (41.7 %), amino- glycoside (41 %), fluoroquinolone and polymyxin (17 %), macrolide (25 %) while nitroimidazole and amphenicol were the least (8 %) administered. Diaveridine, an anticoccidiostat was administered in many of the farms (50 %). Carbapenem, penicillin and cephalosporin were not administered. The wide use of antibiotics in poultry management can select for resistant strain of multiple antibiotic resistant bacteria.

The rate of occurrence of *E. coli* is shown in Table 2. The isolation rate of *E. coli* from poultry droppings and disposal sites (environmental sources) were 44.1 % and 33 % respectively. This might be attributed to the sample size. This higher level of occurrence of the bacteria in poultry droppings might be associated with a lower die off rate of the bacteria in fresh feacal samples than in stale samples with prolonged exposure to harsh environmental condition. However, these isolation rates are in contrast with that of [24] who recorded an isolation rate of 83 % in feacal droppings from poultry sources. These strains of *E. coli* could have lost some genes that are responsible for certain physiological functions that promote bacterial growth as a result of exposure to abiotic factors in the environment. *E. coli* was not isolated from feed and water samples consumed by the birds thereby limiting feed

contamination. 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 resistant profiles on the farms than resistance to single antibiotics. This finding is in agreement with the assertions of [25] that antibiotic use eliminates susceptible bacterial population and selects those unusual strains that continue to grow in its presence through a Darwinian selection process. The resistant variants multiply and become the predominant bacterial population. Their resistant genes are successively transmitted to their offsprings during reproduction. 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 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 comprising both soil and water sources. M.A.R.B. were also isolated from poultry across the 12 farms. The average resistance of the isolates to ciprofloxacin was the highest (87 %), followed by ofloxacin (83 %) and cotrimoxazole. This phenomenon might be associated with frequent administration of enrofloxacin on different farms and the availability of the fluoroquinolone in the market for poultry consumption. In contrast, resistance to meropenem was the lowest and this may be due to non-availability of the antibiotic in tablet form on the counter. This result is higher than that reported by [26] that the percentage resistance of *E. coli* from human origin to carbapenemese in most European countries was less than 1 % with the exception of Belaraus with a higher range of 10 % - 25 %. Also, the antibiotic percentage resistance of *E. coli* from poultry and human sources in Iran to meropenem was reported to be 20 % and 60 % respectively [27]. Besides, resistance to multiple classes of antibiotics was recorded in 95.7 % of the isolates. Resistance was shown to the antibiotics including meropenen, amoxycillin-clavulanate and the cephalosporins that were not administered on the farms. This might be caused by treatment with antibiotics with similar mode of action or class. Fluoroquinolones, cephalosporin, aminoglycoside, sulphonamide, tetracycline and amoxicillin-clavulanate may have less chemotherapeutic value both in human and animal medicine in the area of study because of the high levels of antibiotic resistant profile observed. Meropenem may be recommended for treatment of infection caused by superbugs in the area of study and it should neither be administered indiscriminately as a frontline drug in human medicine nor used in animal medicine without antibiotic susceptibility test to prevent development of possible bacterial resistance to it.

The **s**equence alignment of isolates with strains from Gene data bank is shown in Table 4. The percentage similarity of gene sequence with those from Gene Data Bank falls within the range of 99.29 % - 100 %. This shows 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 SUB5294851 KENECF3 MK606083 to SUB5294851 KENECG9 MK606102. The nucleotide sequences of the isolates were also documented.

The Spearman correlation coefficient (r_s) of variables is shown in Table 5. There was a strong, positive correlation between antibiotics used for treatment and resistant profile of the isolates, which was statistically significant (r_s (3) = .866, p = .333). This indicates a direct relationship between antibiotic treatment on the farm and bacterial resistance to antibiotics. 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) = .500, p = .667). This shows a direct proportional relationship between the two variables. Though both data on antibiotic treatment and that from market survey are positively related to antibiotic resistant profile of isolates, there is an indication that data on antibiotic treatment on the farm are more related to resistance of bacteria than those obtained from market survey. This result is in contrast with the findings of [28] who recorded a negative Spearman correlation coefficient, r_s (8) = -.243

between antibiotic treatment and antibiotic resistant profile of isolates. This may be attributed to the differences in sample size of the antibiotics used for both treatment and antibiotic susceptibility test.

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. Findings from this research show that poultry droppings, soil and water from poultry disposal sites were the major reservoir of the *qnrS* gene. These research findings are in agreement 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]. Presence of quinolone resistant genes in relatively large proportion may constitute a threat to both human and animal health because fluoroquinolones are critically important antibiotics in health care delivery.

Similarly, the agarose gel electrophoresis of the PCR products of extended spectrum beta lactamase gene *blaCMY*-2 amplified from the DNA of *E. coli* 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 *E. coli* isolates from free range birds obtained from the same farm without a record of antibiotic consumption. Free range birds are mobile source of spread of multiple antibiotic resistant bacteria. Natural water bodies are potential sources of human acquisition of multiple antibiotic resistant bacteria via the food chain. Recreational life-style like swimming coupled with religious activity constitutes a predisposing factor to human acquisition of these bacteria.

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 haboured the *blaCMY*-2 gene. Polluted soil and water are secondary reservoir of these bacteria. 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 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.

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). Free range birds (animals) have the potential of acquiring antibiotic resistant bacteria from the environment via the ecological food chain. Frequent mobility of free range birds leads to

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 from the area of study though both genes co-existed on *E. coli* chromosome

The phylogenetic analysis of the sequenced bacterial genes is represented in figure 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 where antibiotics were not administered were restricted to a sub-cluster in one of the major clades while isolates from birds with record of antibiotic consumption and those from disposal sites were distributed across the two major clades.

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 genomic DNA from certain strains of *E. coli* across the clades is traceable to various responses of genomic DNA to selective pressure imposed by antibiotic treatment. This phenomenon tends to link antibiotic treatment in poultry as the major source of these variant strains of multiple antibiotic resistant *E. coli*. Related strains could have emerged and spread via a common genetic mechanism. Wrong antibiotic policies and crude use of antibiotics 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 links the origin of multiple antibiotic resistant *E. coli* from waste disposal sites to poultry production sites and similarly links the evolution of genomic responses of 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 husbandry to increased environmental burden of multiple antibiotic resistant bacteria.

Natural water bodies used for domestic, agricultural, recreational and religious activities are potential sources of human acquisition of these strains of bacteria. This is in agreement with [34] that evidences such as phylogenetic traits obtained from gene sequencing and direct epidemiologic evidence from locations with poor sanitation level and poor drinking water quality show that human pathogens have acquired resistant genes from environmental bacteria.

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)
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

Poultry site	No of Poultry droppings examined	No positive (%)	Poultry feed examined	No positive(%)	Poultry water examined	No positive(%)	Waste sources/samples examined			Total samples examined
							Waste water samples examined	Soil samples examined	No. positive (%)	
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		Aminoglycoside	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)	r_s (.866)	NT	r_s (.500)	1 (50)
Neomycin	2 (16.7)		NT		2 (100)
Colistin	2 (16.7)		NT		1 (50)
Metronidazole	1 (8.3)		NT		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, *rs*- 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

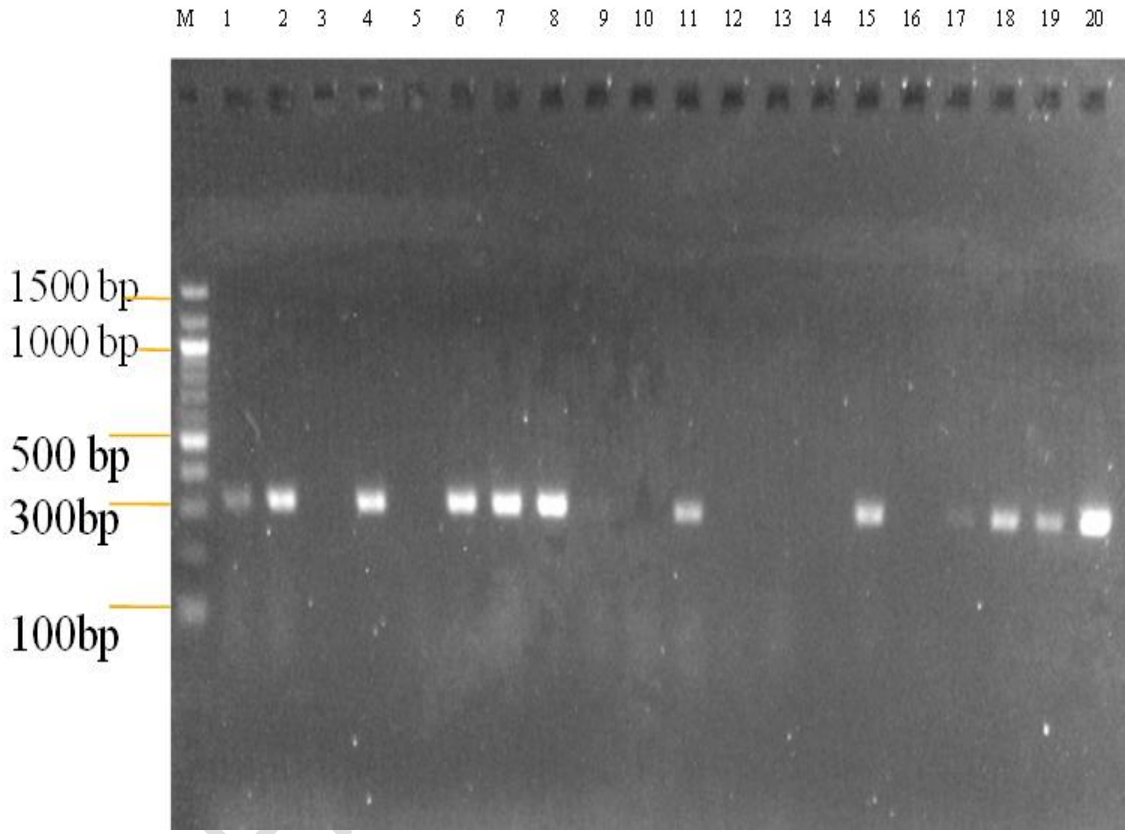

Plate 1: Agarose gel electrophoresis of the PCR products of *qnrS* resistant genes amplified from *E. coli* isolates (Band size approximately 322 bp).

Key: Lane M= Molecular Marker, 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

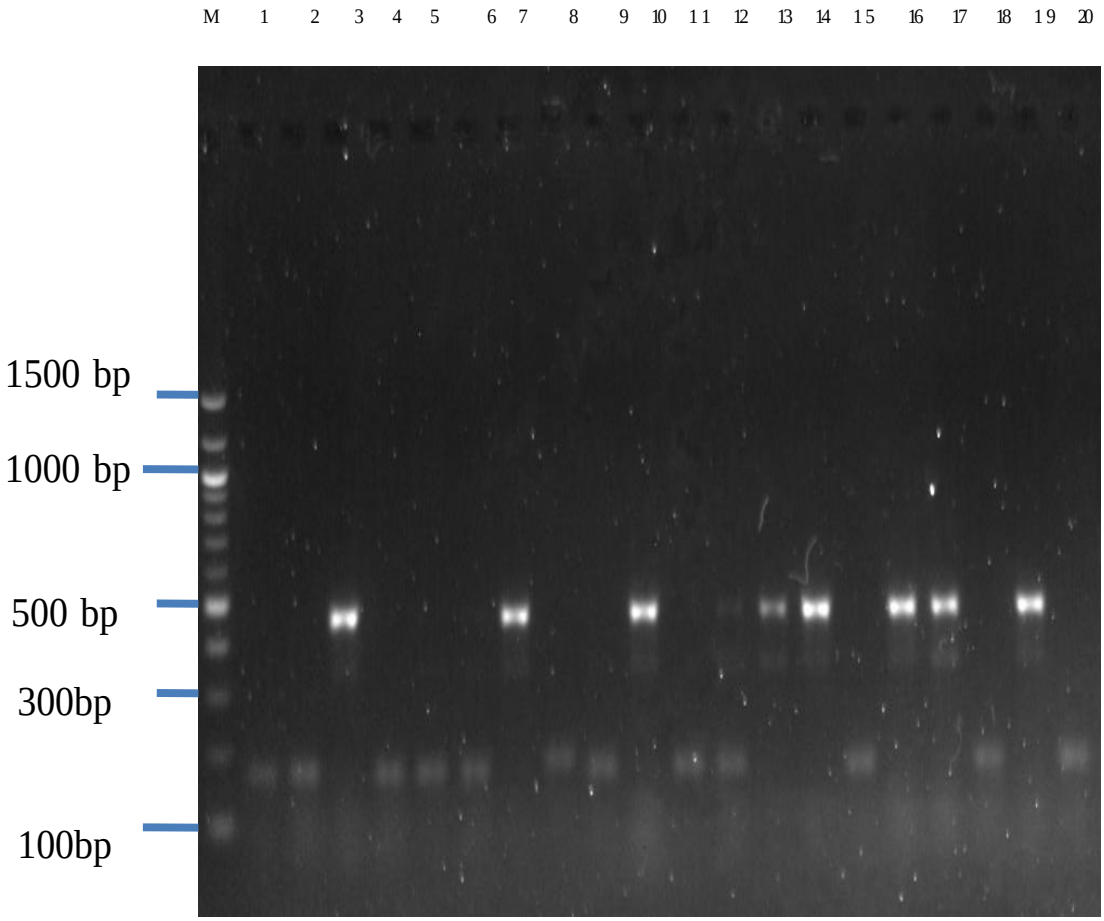

Plate 2: Agarose gel electrophoresis of the PCR products of the *blaCMY*-2 resistant gene amplified from *E. coli* isolates. (Band size approximately 460 bp

Key: Lane M= Molecular Marker, 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

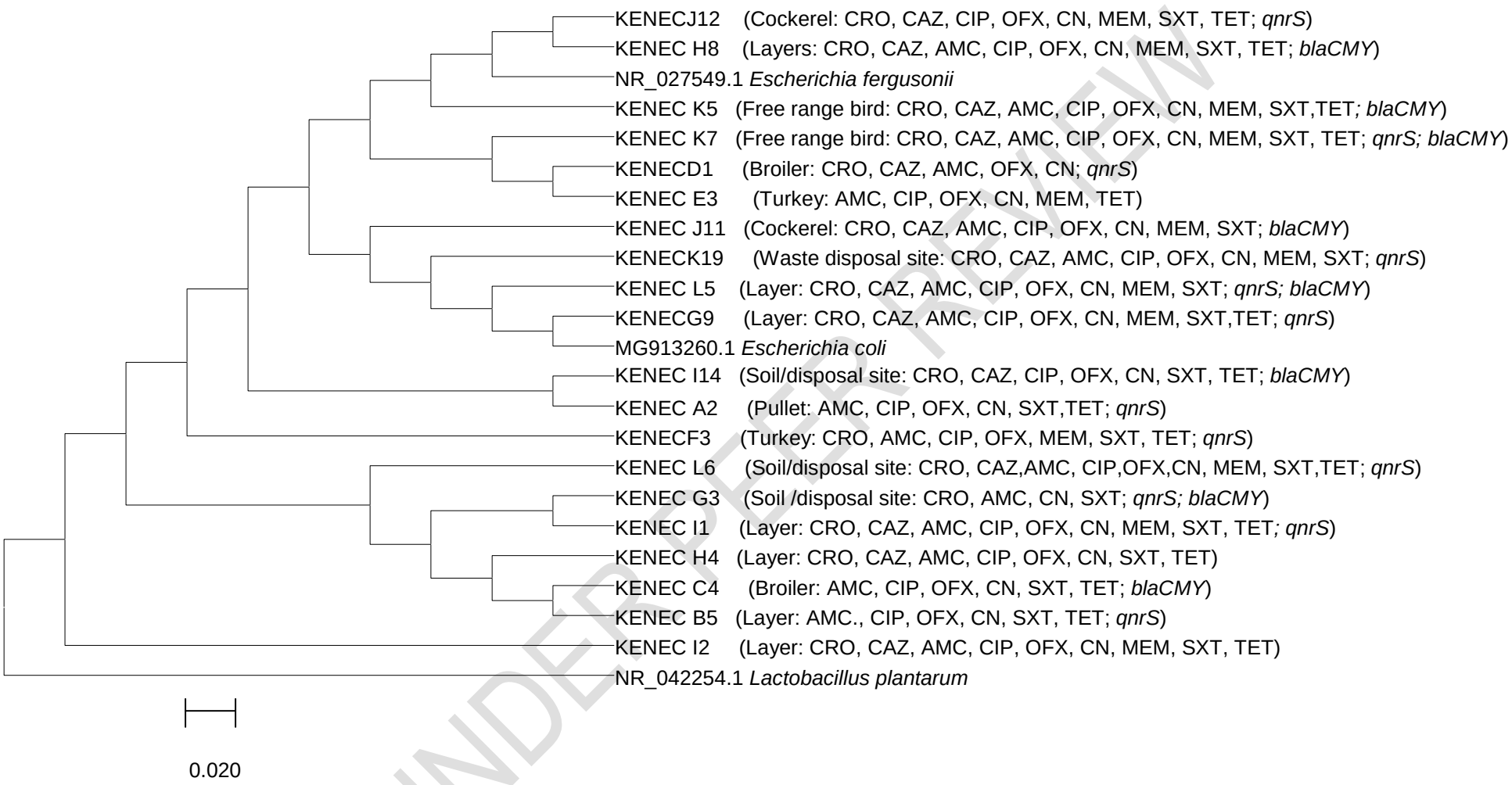

Figure 1: Phylogenetic analysis of selected *E. coli* isolates

4. CONCLUSION

Conclusion: The *blaCMY* and *qnrS* 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. Wrong policies on antibiotic formulation and crude treatment regime in poultry promote the expression of these genes haboured in the genome of variant strains of *E. coli*.

CONSENT (WHERE EVER APPLICABLE)

Not applicable

ETHICAL APPROVAL (WHERE EVER APPLICABLE)

Not applicable

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