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# Polymorphism of 16SrRNA Gene and Its Association with Pathogenecity and Antimicrobial Resistance of Free Ranged Chicken

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## Abstract

Molecular markers have over the years permitted the rapid identification, phylogenetic classification and antimicrobial resistance profile of microbial taxa as compared to the traditional culture method. Through molecular analysis of 16S rRNA gene, this study investigated the molecular and genetic differences that exist between *E. coli* isolated from local scavenging chicken in relation to their pathogenicity and antimicrobial resistance. Our analysis revealed that *E. coli* samples from the two different housing systems showed significant genetic diversity and we postulated that this is attributable to the pressure created by vaccines and antimicrobial drugs. The different genetic patterns corresponded to different antimicrobial susceptibility patterns and prevalence of virulence genes. Thus, the 16SrRNA gene can also be used as a molecular marker to indicate the antimicrobial resistance and pathogenicity of chicken.

***Keywords:*** Pathogenecity; 16SrRNA; free-ranged chicken

## Introduction

One of the world's major challenges for clinical practice and public health surveillance is rapid and accurate identification of infectious agents. Currently, identification and antimicrobial resistance profiling of microbial species is widely based on culture techniques (Clarridge 2004, Mignard an Flandrois 2006). Although such practices are acceptable, they are time-consuming and frequently fails to produce relevant data within the critical window of opportunity. On the other hand, molecular and genomic based methods allow for highly specific and sensitive identification of clinical isolates and clinical specimens in a relatively short time (Adekambi et al., 2009) Molecular markers have over the years permitted the identification, phylogenetic classification and antimicrobial resistance profile of microbial taxa (Bartual et al., 2005 2000, Slack et al., 2006) and one of genes of references used is the 16S rRNA gene. The 16S rRNA is a 401bp base pair gene that codes for a catalytic RNA and is part of the 30S ribosomal subunit. It has desirable properties that allows it to be used for the assignment of close relationships at the genus and in many cases at the species level (Conlan et al., 2012, Newton and Roeselers 2012).

Scavenging local chicken, *Gallus domesticus* are predominant among other poultry in African villages, owing to their relatively low input requirement in their rearing (Pousga, 2007). They thus serve as means of converting low-quality feed into high protein. They also have short life cycles and quick turnover (Carvalho et al., 2015). All these characteristics influence the spread of virulence genes and antimicrobial resistance pattern of pathogens that are harbored by these birds (Schwaiger et al., 2010). Few researches focused on the career rate of pathogens and their antimicrobial resistance, and even fewer highlight the genetic basis for these differences (Kaufmann-Bart and Hoop, 2009). This study intends to investigate the molecular and genetic differences that exists between *E. coli* isolated from local scavenging chicken based on their different housing systems in relation to their carriage of virulence genes and antimicrobial resistance.

With the current population of about 45 million and a land area of 945000km$^2$, about 80% of Tanzanians live in rural areas where scavenging local chicken is mostly distributed (Minga et al., 2001). Among the east African community, Tanzania is relatively rich is wildlife and livestock production. Livestock accounts for 18% of the country's gross domestic product (GDP) and 30% of agricultural GDP. Thus an investigation into the welfare of these indigenous chicken would help boost the agricultural sector of the economy.

## Methodology

### *Study Design and Sample Collection*

A total of 400 swabs were collected from six different households within the Morogoro municipality. Specifically, oral-pharyngeal and cloacae swabs were collected from each of 200 scavenging local chicken. Half of these birds were kept intensively whiles the remaining were on extensively kept. The swabs were collected and kept in transport media and transferred into the laboratory on ice. Table one represents the characteristics of the various production systems of local chicken in Tanzania.

| ***Production System*** | | |
|---|---|---|
| ***Variable*** | ***Intensive*** | ***Extensive*** |
| Biosecurity | High | Low |
| Location | Near towns/cities | Dominant in rural areas |
| Housing type | Permanent chicken house | Coups, kitchen, habitat house |
| Input | High | Low |
| Veterinary service | Pays for veterinary service | Depends on veterinary service |
| Feeding regime | Full ration | Free range |

***Table 1:*** Characteristics of different production systems of indigenous chicken.

### *Isolation of Escherichia coli.*

Based on procedures described in the Bacterial Analytical Manual (BAM 2007), *E.coli* were isolated from all the samples using MacConkey and Blood Agar media (OXOID, Hampshire, England). The following biochemical tests were performed to confirm the suspected *E.coli*: Indole, Methyl Red, Voges-Paskeur, Citrate, Triple Sugar Iron and Motility test.

### *Antibiogram of E. coli*

### *Virulence factor profiling*

The positive *E. coli* strains were investigated for various virulence genes by multiplex PCR, with protocol based on Ewers et al., (2007). The procedures were performed in 25µl reaction mixture. This includes: 12.5 µl of Taq polymerase (Dream Tag PCR Master mix, InqabaBiotec East Africa Ltd), 0.5 µl of each 100Mm dNTP, 0.1µl (100pmol) oligonucleotide primer pair, 6.9 µl of nuclease-free water and 4µl of template DNA. Primer concerntration is 0.4 M. Conditions of the reaction mixtures include: 5mins at 95°C initial denaturation, 94°C of denaturation for 30s, annealing at 56ºC for 30s, elongation at72°C for 3minutes at 25 cycles, a final elongation at 72°C for 10 minutes and a hold at 4°C.

### *Antimicrobial Susceptibility: Kirby-Bauer disc diffusion test*

The Kirby-Bauer antimicrobial sensitivity test method was used to determine the antibiogram profile of isolates. Seven antimicrobial drugs were used. These include CRO: ceftriaxone, CTX: cefotaxime, CAZ: ceftazadime,, STX: Trimethroprim-Sulfamethoxazole, AUG: augmentin, NA: nalidixic acid, KF: cephalothin.

Mueller-Hinton agar was prepared according to the manufacturer's protocol. The organisms were cultured on nutrient agar. Between 4 and 5 isolated colonies of the organisms were suspended into about 2ml of sterile saline by use of inoculating loop. The saline tube was vortexed to create smooth suspension. The turbidity of the suspension was adjusted to a 0.5 McFarland standard. 200ml of the suspension was introduced unto the Mueller-Hinton plate. Sterile glass spreader was used to spread the organisms on the plate. The surface of the plate was allowed to dry for 5minutes before the antibiotic discs was placed on them.

A pair of sterile forceps was used to remove the antibiotic discs from the dispensers. After placing the discs on the agar, each disc was gently touched with the inoculating loop to ensure their contact with the agar surface. The plates were then incubated upside down for 24hours at 37°C.

### *16SrRNA sequencing*

The 16S rRNA genes in the Escherichia coli isolates were amplified by Polymerase Chain Reaction (PCR) and sequenced. The protocol for the PCR was as follows: The procedures were performed in 20µl reaction mixture. This includes: 10 µl of Taq polymerase (Dream Tag PCR Master mix, (InqabaBiotec East Africa Ltd), 1 of each 100Mm dNTP, 0.1µl (100pmol) oligonucleotide primer pair, 5 µl of nuclease-free water and 3µl of template DNA. Primer concentration is 0.5 M. The cycling Conditions of the reaction include: 5mins at 95°C initial denaturation, 95°C of denaturation for 30s, annealing at 56°C for 30s, elongation at72°C for 45 seconds at 25 cycles, a final elongation at 72°C for 10 minutes and a hold at 4°C. The primer sequence for the amplification of the gene of interest as well as its size is listed in table 2. The PCR products were then sequenced by the Sanger method of sequencing with the BigDye terminator v3.1 sequencing kit.

| *Target gene* | *Primer sequence* | *size* | *Location within gene* |
|---|---|---|---|
| 16SrRNA | CCCCCTGGACGAAGACTGAC<br>ACCGCTGGCAACAAAGGATA | 401bp | 1682-1701 |

***Table 2:*** Primer for 16SrRNA sequencing.

### *Molecular phylogenetics analysis*

ClustalW (Thompson et al., 1994) were used to align the nucleotide sequences. The best nucleotide substitutional method was determined by computing the minimum theoretical Akaike information criterion (AIC), corrected minimum theoretical Akaikei information criterion (AICc), Bayesian information criterion (BIC), and using the Hierarchical likelihood ratio test with a confidence level of 0.01 (Schwarz, 1978; Sakamoto et al., 1986; Frati et al., 1997; Huelsenbeck et al., 1997; Posada and Crandall, 1998).

The model that had the lowest BIC, AIC, or AICc value was chosen as the best-fit. This best- fit model was used to compute the final phylogenetic tree. Phylogenetic tree construction was by Maximum Likelihood method and Tamura 3-parameter model. (Ronquist et al., 2012) by the MegaX software (Kumar et al., 2018). Multiple phylogenetic trees that indicated similar ancestral relationships were critically examined and a representative tree with the overall best bootstrap values chosen.

### *Haplotypic analysis*

Relationships between individual genotypes at the population level were further examined by haplotype networks with Popart 1.7 (Bandelt et al., 1999). Same software was also used to compute the genetic distances, diversity indices and molecular variance. Tajima's D tests of statistical analysis, as well as haplotype diversity, nucleotide diversity and the S segregating sites was computed using

DnaSP ver. 5.10.00 (Librado and Rozas, 2009).

## Results

### *Molecular diversity*

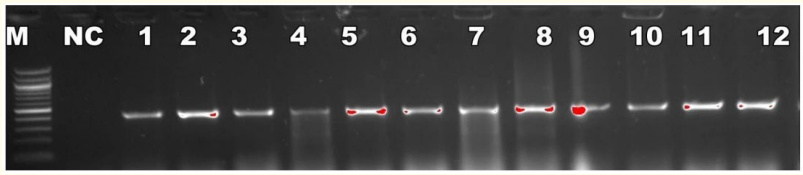


***Figure 1:*** PCR detection of 16SrRNA gene(401bp). PCR products visualized under gel documentation. NC is negative control. M is marker (100bp).

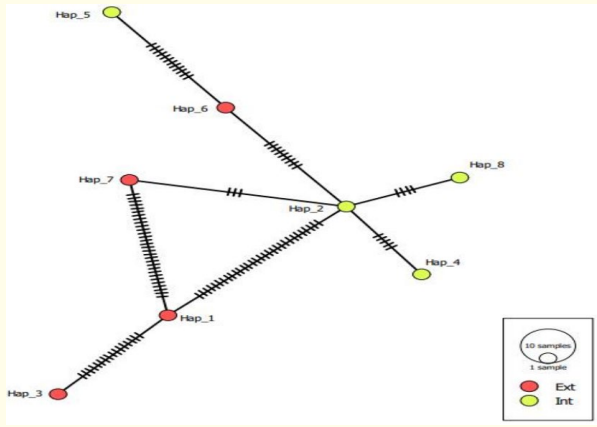


***Figure 2:*** Haplotype network of 16SrRNA gene sequences from *E.coli*. Each circle in the haplotype network corresponds to one haplotype, and the size is proportional to its frequency among the samples. Colours of the circles correspond to housing system from which *E.coli* were isolated, with colour red = Extensive (Ext), yellow = Intensive (Int)The haplotype network was produced using minimum spanning network and Epsilon of 0 in Popart 1.7 (Bandelt et al., 1999).

The haplotype network revealed a total of 8 distinct haplotypes. Extensive (Ext) and Intensive (Int) populations each produced four haplotypes. Haps 1,3, 6 and 7 was each shared by isolates from Extensive (Ext) housing system(red) whiles the other four haplotypes; haps 2,4,5 and 8 were by samples from Intensive (Int) housing system(yellow). Each haplotype was shared by one population; thus all the haplotypes were of same size (fig 2). From Hap 1, three haplotypes evolved; Haps 3, 7 and 2 with mutations base pair changes of 16, 30 and 30 respectively. Five haplotypes evolved from Hap2: haps 8, 6, 7,1 and 4 with mutation base pair changes of 4, 9, 2, 30 and 5 respectively. Hap 5 evolved from hap 6 with 12 base pair distance.

### *Genetic structure*

The analysis of variance results revealed that there is a higher percentage of variation within the two populations (88.92%) than amongst populations (11.08%). The FST value was significantly different from zero (FST = 0.312; P \0.00001), indicating a significant genetic structure among the 2 populations analysed (Table 3).

| *Source of variation* | *Df* | *Sum of squares* | *Variance components* | *Percentage of variation* | *F statistic* |
|---|---|---|---|---|---|
| Among population | 1 | 477.375 | 29.792 | 11.08184 | 0.312 |
| Within population | 6 | 1434.250 | 239.042 | 88.91816 | |
| Total | 7 | 1911.625 | 268.833 | 100 | |

***Table 3:*** Analysis of molecular variance (AMOVA) for all populations of Extensive and Intensively housed local scavenging chicken.

Table 4 shows parameters of genetic diversity and neutrality diversity. Both populations recorded a genetic diversity of 1.000. The extensive population and intensive population had nucleotide diversities of 0.05827 and 0.04071 respectively and 26 and 50 segregating sites respectively. The Tajima's D value was 0.00797 and -0.01102 for Extensive and Intensive populations respectively (Table 4).

| *Population* | *Gene diversity* | | | | *Neutrality diversity* |
|---|---|---|---|---|---|
| | ***N*** | ***Hd*** | 𝕀𝕀 | ***S*** | |
| Extensive | 13 | 1.00 | 0.05827 | 26 | 0.00797 |
| Intensive | 4 | 1.00 | 0.04071 | 50 | -0.01102 |

*N sample size, h number of haplotypes, Hd haplotype diversity, 𝕀𝕀 nucleotide diversity, S segregating sites.*

***Table 4:*** Genetic diversity and test of neutrality among and within populations.

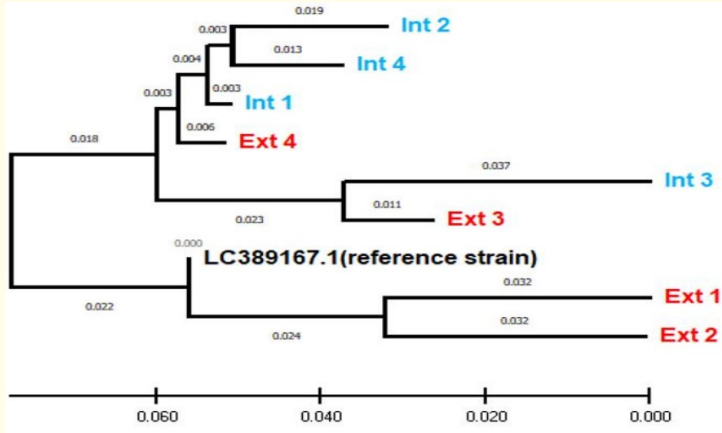


***Figure 3:*** The evolutionary history was inferred by using the Maximum Likelihood method and Tamura 3-parameter model. The tree with the highest log likelihood (-2175.63) is shown. 4 sequences each from Intensive (Int) and Extensive (Ext) and a reference strain (LC389167.1 strain).

The evolutionary history was inferred by using the Maximum Likelihood method using MEGA software. 4 sequences each from Intensive (Int) and Extensive (Ext) and a reference strain (LC389167.1 strain).

The phylogeny consisted on two main clusters. One cluster was shared by the reference strain, Ex1 and Ex 2 with a branch length of 0.022 from the root. Ext 1 and Ex 2 was found on the same node with a branch length of 0.0032 each, separated from the reference stain with a branch length of 0.024. The other cluster, which had a branch length of 0.018 from the main root, had two subclusters. One subcluster had a branch length of 0.023 and has one node which is shared by Int 3 and Ext 3 with branch lengths of 0.037 and 0.011 respectively. The other subcluster has Int 2 and Int 4 sharing a node with branch lengths of 0.019 and 0.013 respectively. These together shared a node with Int 1 with a branch length of 0.003, and they all intend share a node with Ext 4 with a branch length of

0.006 (figure 3).

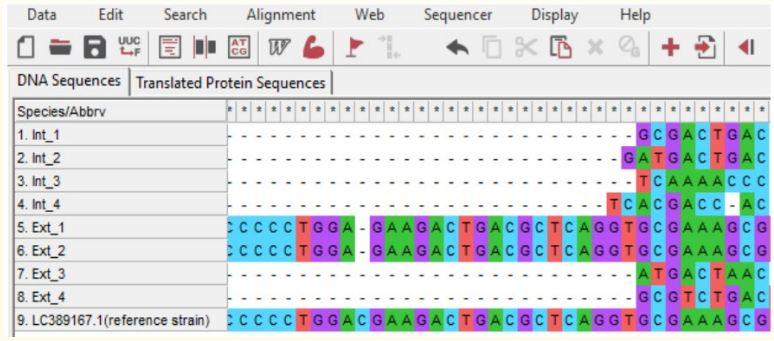


***Figure 4:*** Clustal W method of alignment of 16Sr RNA sequences of *E.coli* isolated from Intensive (Int) and Extensive (Ext) samples.

All the sequences were aligned by Clustal W method of pairwise and multiple alignment. The alignment revealed that the nucleotides sequences of the extensive group were different from those of the intensive group. (fig 4).

***Antimicrobial Resistance***

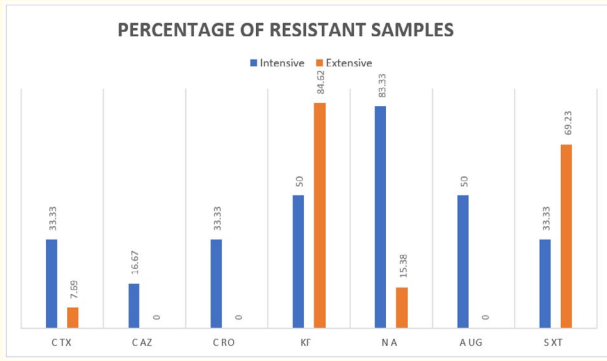


***Figure 5:*** Proportion of antibiotic resistance profile. CRO: ceftriaxone, CTX: cefotaxime, CAZ: ceftazadime, STX: Trimethroprim-Sulfamethoxazole, AUG: augmentin, NA: nalidixic acid, KF: cephalothin.

The antibiogram showed that there were no intensive isolates that are resistant to ceftazidime (CAZ), ceftriaxone (CRO) and augmentinin (AUG), whiles they were 16.67%, 33.33% and 50% of extensive samples were resistant to these drugs respectively. 33.33% and 7.69% of intensive and extensive isolates were resistant to cefotaxime (CTX) respectively. In the extensive isolates, the highest resistance was towards the cephalothin (K); 84.44% of these isolates were resistant to it whiles 50% of intensive isolates were resistant to it. The highest resistance of the intensive isolates was toward the nalidixic acid (NA); 83.33% of the intensive isolates were resistant to this drug whiles only 15.38% of the extensive samples were resistant to it. Regarding the Trimethoprim-Sulfamethoxazole (STX), 33.33% of the intensive isolates were resistant to it whiles 69.23% of the extensive isolates were resistant to it (figure 5).

***Occurrence of Virulence Factors***

| *Virulence factor* | *Extensive* | *Intensive* | *Significance* |
|---|---|---|---|
| Iron Acquisition | 21 | 6 | |
| Serum resistance | 19 | 13 | |
| Adhesins | 2 | 2 | |
| Toxins | 5 | 4 | P<0.05 |
| Invasins | 13 | 4 | |
| Total | 60 | 29 | |

***Table 5:*** Prevalence of virulence genes among extensive and intensive strains.

The most prevalent virulence factor among the extensive samples is the iron acquisition a total of 21 factors whiles the least was adhesins with only 2 factors. Serum resistance, Toxins and invasions were 19, 5 and 13 among the extensive samples. Serum resistance was the most prevalent factor among the intensive group with 13 factors whiles adhesins were the least with 2 factors. Iron acquisition, toxins and invasins were 6, 4 and 4 respectively amongst this group. (table 5).

## Discussion

The genetic variability and adaption of local scavenging chicken to different housing systems is critical to augment the exploration strategies for their improvement. The nutritional requirements of chickens are genetically predetermined and this is affected by the kind of housing system (Khobondo et al., 2015). The different ecotypes of local scavenging chicken are predetermined by their genetic variability, acted on by environmental factors. This study used the 16SrRNA gene nucleotide sequences isolated from two different housing system as indicator to access the genetic variability between birds of these systems. Our initial observation revealed that genetic differences exist between *E. coli* from these different housing system.

Based on the Tamura 3 parameter model, the phylogeny revealed *E. coli* strains from these two different housing systems have different parentage and genetic lineage with significance. Of the two main clusters produced by the phylogenetic tree, all of the samples from the internally kept birds are clustered at one side of the tree whiles fifty percent of the externally kept samples are found on the other cluster of the tree. The other fifty percentage of the *E. coli* from the extensively kept birds are also found amongst the intensive group but with branch lengths that signifies weak similarities. The different mode of feeding and different environments could be the basis for this variation. The genetic variability has been known to be associated with different ecotypes of various types of indigenous chicken in different parts of Africa. In Ethiopia, Mujyambere et al., 2021 showed that differences in sexual maturity among different indigenous ecotypes of may be due to genetic variability. Same results were observed by Mutinda et al., 2013 and Lwelamira et al., 2008 in Tanzania and Kenya respectively. Due to the fact that the nutritional requirements are genetically predetermined, when birds are kept at a system that is in variance with their genetic makeup, these birds have high time trying to adapt and in most times, this leads to morbidity and mortality.

The study revealed the presence of 8 unique haplotypes, thus there were no shared haplotype between extensive and intensive *E. coli*. The extensively kept birds recorded less resistance to anti-microbials than the intensively kept ones. The former recorded resistance to four out of the seven antimicrobials whiles the later were resistant to all seven antimicrobials used. Due to their higher biosecurity nature, the intensively kept birds are recipients of veterinary services, which include the use of vaccines and antimicrobials. These have been shown to be a major contributor to antimicrobial resistance (Micoli et al., 2021).

With regards to genetic diversity our results revealed that *E. coli* from the intensively kept birds samples are more genetically diverse from those from the extensively kept birds. This was evident in their Tajima's D values. A negative Tajima's D value was recorded by the intensive group; this indicates excess of rare (private alleles) and a fewer haplotypes than the number of segregating sites. The extensive group recorded a positive Tajima's D value, a consequence of scarce rare alleles and an indication of low genetic diversity, as

indicated by Stajich and Hahn (2005). This high genetic diversity is attributable to attributable to the vaccines and drugs that are fed to them. Afsaneh Golkar -Narenji and Mozdziak (2020) indicated that vaccines has a genetic deletion that allows differentiation between vaccinated and unvaccinated animals.

Several studies have indicated the rapid and accurate molecular diagnosis of resistance as opposed to the time-consuming culture based methods. Brown and Paladino, 2010 used molecular tool to distinqiush between methicillin-resistant *S. aureus* from methicillin-susceptible *S. aureus* in less than one hour. They concluded that, rapid PCR testing for MRSA appears to have the potential to reduce mortality rates of MRSA (methicillin-resistant *S. aureus*) infections and less costly than empiric therapy involving culture method. This study recorded a wide difference in resistance pattern between extensively and intensively kept *E. coli* in the following antimicrobials; cefotaxime, ceftazidime, ceftriaxone and augmentinin all recorded resistances in 33.33, 16.67, 33.33 and 50 percentage respectively of samples from intensively kept birds and no resistance at all from extensively kept sample. Since these antibiogram of these samples were critical determinants of the resistance genes they carried, it can be inferred that our results have proved that there is a correlation between the 16S r RNA and resistance genes.

Srinivasan et al., 2015 demonstrated that the 16S rRNA gene can be used as a molecular marker for identification of bacterial species. In their study, the 16S rRNA gene clinical identities revealed a genus-level concordance rate of 96% and a species-level concordance rate of 87.5%. They also pointed to multiple cases of probable clinical misidentification with traditional culture based identification across a wide range of gram-negative rods and gram- positive cocci as well as common gram-negative cocci. This current study went a little further beyond species identification to determine which the antimicrobial resistance of the species, as *E. coli* with identical alignment patterns.

Our results also suggest that the 16S rRNA gene can be used a molecular marker for pathogenicity of chicken marker-assisted selection. *E. coli* from the extensively kept housing birds which had a particular haplotype significantly possessed more virulence genes than *E.coli* from the intensively kept birds. 16SrRNA genes are associated with virulence genes and are found in multiple copies per cell (Vos et al., 2012). In this study there was an overall significant difference in virulence genes between *E. coli* from the two different housing systems; 60 of extensive system as against 29 of the intensive group. Individual groups of virulence factors however varied between these two groups. Wide differences were observed in iron acquisition, serum resistance and invasion groups of virulence factors whiles mild differences were recorded among adhesins and toxins. These three factors are very critical to the pathogenicity of APEC (Avian pathogenic *E.coli*) (Kathayat et al., 2021). Thus our development of 16SrRNA as a molecular marker of pathogenecity is more efficient amongst virulence factors of iron acquisition, serum resistance and invasion.

## Conclusion

Our results showed that genetic differences exist between *E. coli* from extensively and intensively kept poultry; and this can be revealed by genetic analysis of the 16SrRNA gene. This genetic differences corresponds to antimicrobial resistance and pathogenicity of the poultry. *E. coli* from the intensively kept birds are more genetically variable than those form the extensively kept birds and this is attributable to their intake of vaccines and antimicrobials.

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