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RESEARCH ARTICLE

Prevalence and Sequence Analysis of *Escherichia Coli* Harboring Colistin, Gentamicin, Streptomycin, Tetracycline and Quinolones Resistant Genes from Commercial Broilers

Waqas Ahmed Madni, Muhammad Asif Zahoor*, Zeeshan Nawaz and Mohsin Khurshid

Institute of Microbiology, Government College University, Faisalabad-Pakistan
*Corresponding author: drasifzahoor@gcuf.edu.pk

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ABSTRACT

The emergence and spread of multidrug resistant *Escherichia coli* (MDR- *E. coli*) among food producing animals is a challenging public health concern, globally. *E. coli* is an opportunist pathogen having zoonotic potential that causes several infections among animals and humans. Currently, there is limited data about the distribution of antibiotics resistance genes in *E. coli* sequence types from commercial broilers. Hence, in this study, cloacal swab samples (n=200) were collected for the isolation and molecular identification of *E. coli* based on *uidA* gene and multi-locus sequence type analysis followed by determination of antimicrobial resistance (colistin, gentamicin, streptomycin, tetracycline & quinolones) along with identification of antimicrobial resistance genes (ARGs) using specific primers. A total of 153/200 (76.5%) *E. coli* were identified and resistance was observed among 49, 54, 62, 77 and 24% of the *E. coli* isolates against colistin, gentamicin, streptomycin, tetracycline and ciprofloxacin, respectively. Minimum inhibitory concentration data showed 49, 54, 50 and 23% of *E. coli* isolates were resistant to colistin, gentamicin, tetracycline and ciprofloxacin, respectively. Further, ARGs data showed detection of *aac(3)-IV*, *aadA1*, *mcr-1*, *tetA* and *qnrA* as 47, 56, 43, 61 and 12% of the isolates, respectively. Virulence genes amplification data showed that one isolate encodes maximum virulence genes i.e. adhesins (*fimH*, *papC*, and *papG*), tissue invasion (*hlyA* and *KpsMTII*) and immune evasion (*traT* and *capU*). Whereas other isolates were identified to encode few virulent genes. MLST data of *E. coli* harboring multiple ARGs showed the detection of ST1035, ST131, ST1650 as (*mcr-1*, *qnrA*, *tetA*) ST1035 (n=10), (*qnrA*, *aadA1*, *tetA*) ST1035 (n=3), (*qnrA*, *aac(3)-IV*, *aadA1*) ST131 (n=7) and (*aac(3)-IV*, *tetA*) ST1650 (n=3). Altogether, it was concluded that ST131 and ST1035 were predominant MDR- *E. coli* strains (harboring *qnrA* gene) isolated from commercial broilers which can potentially spread multidrug resistant *E. coli* to humans.

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INTRODUCTION

The multidrug resistant *Escherichia coli* (MDR- *E. coli*) has widely been recognized as the primary etiological agent of avian colibacillosis among commercial broilers. Whereas a significant population of *E. coli* has been associated with gastrointestinal tract termed as commensal *E.* coli (Montoro-Dasi *et al*., 2021). However, the traditional molecular identification could poorly differentiate commensal versus avian pathogenic *E. coli* (APEC) strains (Delago *et al*., 2023). Further, the mechanism of horizontal and vertical spread of antimicrobial resistant genes (ARGs) between commensals and APEC strains is not widely described in literature as well as the dissemination of *E. coli* form food producing animals to humans is not widely reported (Ho *et al*., 2010; Chalmers *et al*., 2017; Jamil *et al*., 2022). However, there are reports that the commensal *E. coli* could harbor different ARGs (Diarrassouba *et al*., 2007; Montoro-Dasi *et al*., 2021). This could be explained that antibiotics have been widely used among commercial poultry as prophylactic or growth promoters at sub-therapeutic dose to control sub clinical infections and to promote growth. This could modify the intestinal flora by creating selective pressure and favoring the survival of resistant bacterial strains (Aarestrup *et al*., 2001).

Various antimicrobial agents have widely been used to control *E. coli* infections in commercial poultry i.e. aminoglycosides and tetracyclines, whereas fluoroquinolones, β-lactams and colistin are also extensively used and considered as last resort antibiotics (Yamane *et al*., 2005; Jamil *et al*., 2022). These antimicrobials are also critically important in human medicine (Zárate *et al*., 2018). Further, *E. coli* strains could harbor various ARGs that confer multidrug resistance. For example, aminoglycoside-modifying enzymes are primarily responsible for resistance to gentamicin and streptomycin which are categorized into different classes i.e. aminoglycoside acetyltransferase, O-phosphotransferase and O-nucleotidyltransferase. The genes of these enzymes are chromosomal or plasmid mediated or located on mobile genetic elements (MGE) which collectively confer the spread of ARGs within animal-environment-humans interface (Vakulenko and Mobashery, 2003; Shakil *et al*., 2008; Amer *et al*., 2018; Zárate *et al*., 2018). Similarly, tetracyclines are broad spectrum antibiotics which are widely used in veterinary medicine for prophylactic or therapeutic purposes. Tetracycline resistance genes such as *tetA* and *tetB* encode for membrane-associated efflux proteins and are considered as the most prevalent tetracycline resistant types in clinical or commensal *E. coli* isolates (Miller *et al*., 2016; Pezzella *et al*., 2004) Fluoroquinolones are broad spectrum antimicrobial agents which are highly effective against a variety of infections (Hammerum and Heuer, 2009; Seo and Lee, 2021). The genetic basis of quinolone resistance is mediated by plasmid encoded *qnrA, qnrB, qnrC, qnrD* or *qnrS* genes (Jamil *et al*., 2022; Madni *et al*., 2024).

Based on the significance of commensal *E. coli* strains and potential ARGs among commercial poultry, the current study has focused on isolation, genomic identification, antimicrobial susceptibility testing, sequence type analysis and the determination of different ARGs (*aac(3)- IV, aadA1, mcr-1, tetA and qnrA*) of *E. coli* isolated from cloacal swabs of commercial broilers.

MATERIALS AND METHODS

Samples Collection and Initial Processing: The cloacal swab samples (n=200) were collected from commercial broiler farms (between 20-35 days of age) located in Jhang and Faisalabad Districts of Punjab-Pakistan. Farms with ongoing antimicrobial treatment or clinically sick birds were excluded from the current study. The samples were collected using aseptic conditions and were transported using ice-containers. The samples were processed within 24-48 hours and initially inoculated on MacConkey agar (Oxoid™, UK) and individual colonies were inoculated on eosin-methylene blue agar (EMB-agar, Oxoid™, UK), supplemented with colistin (2µg/ml). Afterward, bacterial colonies were analyzed using biochemical tests including Gram Staining, Oxidase test, Catalase test, VP test, Indole Test, Methyl red test (Zhang *et al*., 2018; Jamil *et al*., 2022).

DNA Extraction and Molecular Detection of *E. coli*: Initially, confirmed bacterial isolates were inoculated into brain heart infusion broth (BHI broth, Oxoid™, UK) and incubated overnight at 37º C. DNA was extracted using a DNA extraction and purification kit (ThermoFisher Scientific-USA) according to manufacturer's instructions and as described recently (Jamil *et al*., 2022). The genomic DNA was amplified using species specific primers F= 5´-ATCACCGTGGTGACGCATGTCGC-3´, R=5´-CACCACGATGCCATGTTCATCTGC-3´targeting *uidA* gene as described (Jamil *et al*., 2007).

Antimicrobial Susceptibility Testing: Genetically confirmed *E. coli* isolates were subjected to Kirby-Bauer disc diffusion method to determine the antimicrobial resistance patterns following the Clinical Laboratory Standards Institute (CLSI-2023) guidelines against different antibiotics including gentamicin (CN-10µg), streptomycin (STR-10µg), tetracycline (TET-30µg) and ciprofloxacin (CIP-5µg), commercially available antibiotic disc were used (Oxoid-Uk). *E. coli* strain (ATCC-25922) was used as quality control. Colistin resistance was detected by cultivating *E. coli* isolates on EMB agar, supplemented with 22µg/ml (Jamil *et al*., 2022).

Determination of Minimum Inhibitory Concentration (MIC): Broth microdilution method was used to determine the minimum inhibitory concentrations (MICs) of isolated *E. coli* strains against ciprofloxacin, colistin, gentamicin and tetracycline and results were noted according to CLSI-2023 guidelines, *E. coli* strain (ATCC-25922) was used as quality control (Jamil *et al*., 2022).

Genotypic Identification of Antimicrobial Resistance Genes (ARGs): The extracted DNA was also subjected to PCR by targeting the specific genes for identification of different ARGs i.e. *aac(3)-IV* (F=5-CTTCAGGATGGCAAGTTGGT-3, R=5-TCATCTCGTTCTCCGCTCAT-3), *aadA1* (F=5-TATCCAGCTAAGCGCGAACT-3, R=5-ATTTGCCGACTACCTTGGTC-3), *mcr-1 (*F=5-*AGTCCGTTTGTTCTTGTGGC*-3, R=5-*AGATCCTTGGTCTCGGCTTG*-3*)*, *tet*A (F=5-GGGTATGGATATTATTGATAAAG-3, R=5-CTAATCCGGCAGCACTATTTA-3) and *qnr*A (F=5-GTGAAACCCAACATACCCC-3, R=5-GAAGGCAAGCAGGATGTAG-3) as described (Momtaz *et al* ., 2012; Zhang *et al*., 2018; Nawaz *et al*., 2021; Jamil *et al*., 2022).

Determination of Virulence Genes: The DNA of *E. coli* isolates carrying multiple ARGs was also amplified to identify the virulent genes encoding adhesins (*fimH, papC,* and *papG*), tissue invasion (*hlyA* and *KpsMTII*) and immune evasion (*traT* and *capU*) using specific primers as described (Mujahid *et al*., 2024).

Multilocus Sequence Typing (MLST): The genomic DNA of *E. coli* harboring multiple ARGs was further subjected to multilocus sequence typing (MLST) by targeting the amplification of housekeeping genes i.e. *adK, fumC, gyrB, icd, mdh, purA,* and *recA* according to protocol described recently (Jamil *et al*., 2022). Briefly, the amplified products were sequenced at Macrogen (South Korea), a commercial sequencing facility. Following the initial editing from the ChromasPro

(Technelysium, Australia), the sequences were aligned from the ClustalW Algorithm (MEGA software), whereas allelic numbers were assigned, and the Entero-based database (https://pubmlst.org/organisms/escherichia-spp) was accessed to find the allelic profiles of isolates to determine sequence types (STs).

RESULTS

Prevalence of E. coli: A total of (192/200) cloacal swabs samples were found positive for bacterial colonies on MacConkey agar (Oxoid^{TM}, UK). However, (153/200, 76.5%) isolates were identified as E. coli using EMB agar (Fig. 1) and based on biochemical and molecular identification of uidA gene (Fig. 2). Thus, only these bacterial isolates were processed in the current study.

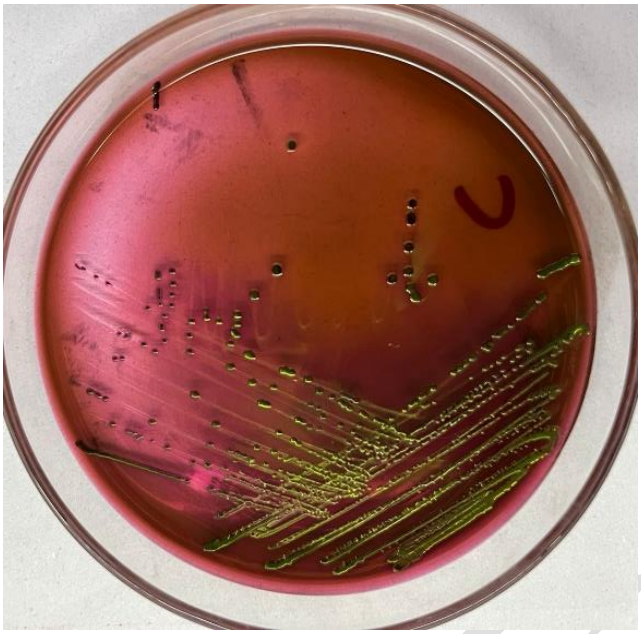

Fig 1: Characteristic metallic sheen color colonies on Methylene Blue (EMB) Agar.

Antimicrobial Susceptible Testing and Antimicrobial Resistance Genes: Antimicrobial susceptibility testing data showed that 54, 62, 77 and 24% of total isolates were resistant to gentamicin, streptomycin, tetracycline and ciprofloxacin as shown in Fig. 3. However, the ARGs data showed the presence of *aac(3)-IV*, *aadA1*, *mcr-1*, *tetA* and *qnrA* as 47, 56, 43, 61 and 12%, respectively.

Determination of Minimum Inhibitory Concentration (MIC): MICs of four antimicrobial agents including colistin, gentamicin, tetracycline, and ciprofloxacin were tested against 153 *Escherichia coli* isolates. Resistance breakpoints were defined as follows: colistin ≥4 µg/mL, gentamicin ≥8 µg/mL, tetracycline ≥16 µg/mL, and ciprofloxacin ≥1 µg/mL. For colistin, 75 isolates (49%) were resistant, with notable counts at 8 µg/mL (42 isolates) and 16 µg/mL (26 isolates). Gentamicin resistance was observed in 83 isolates (54.2%), predominantly at 16 µg/mL (35 isolates) and 32 µg/mL (29 isolates). Tetracycline resistance was identified in 77 isolates (50.3%), with most isolates at 16 µg/mL (30 isolates) and 32 µg/mL (31 isolates). Ciprofloxacin resistance was noted in 35 isolates (22.9%). Comparative distribution of MICs of *E. coli* isolates is summarized in Fig. 4.

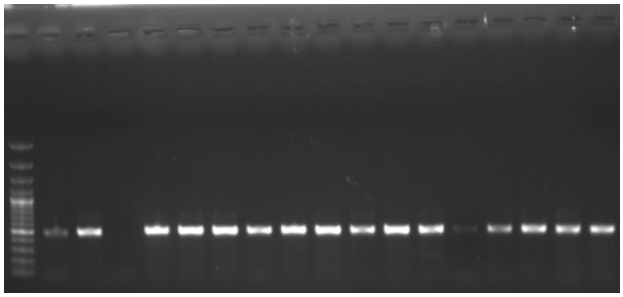

Fig. 2: Amplification of *uid-A* gene of commensal *E. coli* isolates, Lane 1= 100 bp Marker, Lane 2-17= 485bp *uid-A* gene

Determination of Virulence Genes: Virulence genes amplification data showed that EC-20-181 isolate encodes maximum virulence genes i.e. adhesins (*fimH*, *papC*, and *papG*), tissue invasion (*hlyA* and *KpsMTII*) and immune evasion (*traT* and *capU*) followed by EC-20-20 isolate. Whereas other isolates were identified to encode few virulent genes as shown in Fig. 5.

Multilocus Sequence Typing (MLST) and Multiple Occurrence of ARGs: A total of 23 *E. coli* isolates were found to harbor multiple ARGs (*mcr-1*, *qnrA*, *tetA*) belonged to ST1035 (n=10), (*qnrA*, *aadA1*, *tetA*) belonged to ST1035 (n=3), (*qnrA*, *aac(3)-IV*, *aadA1*) ST131 (n=7) and (*aac(3)-IV*, *tetA*) ST1650 (n=3) as described in Table 1.

DISCUSSION

Commercial poultry farming has made significant contributions in the economy of developing as well as developed countries. However, several constraints contributed to increased economic costs, reduced production and spread of various bacteria to humans i.e. antimicrobial resistant bacteria and associated antimicrobial resistant genes (ARGs). These are responsible for several infections in commercial birds as well as food safety concern for humans via the food chain. For example, multidrug resistant *Escherichia coli* (MDR-*E. coli*) has widely been recognized in the horizontal and vertical spread of ARGs among different isolates (Ho *et al*., 2010; Jamil *et al*., 2022). MDR-*E. coli* is responsible for several infections in commercial poultry and could serve as source of ARGs (Al Azad *et al*., 2019; Jamil *et al*., 2022). Hence, in this study multilocus sequence type analysis of commensal MDR- *E. coli* along with prevalence of different ARGs was described from commercial broilers.

In the current study, cloacal swab samples (n=200) were processed and a total of 153 *E. coli* were identified based on biochemical and genomic amplification of *uidA* gene. Antimicrobial susceptibility testing data showed 49, 54, 62, 77 and 24% of the isolates were resistant to colistin, gentamicin, streptomycin, tetracycline and ciprofloxacin, respectively. These findings are consistent with one of the previous studies indicating high resistance to tetracycline and aminoglycosides among *E. coli* isolates from duck farms in China (Luo *et al*., 2023). In addition, MICs against these antimicrobials were calculated that

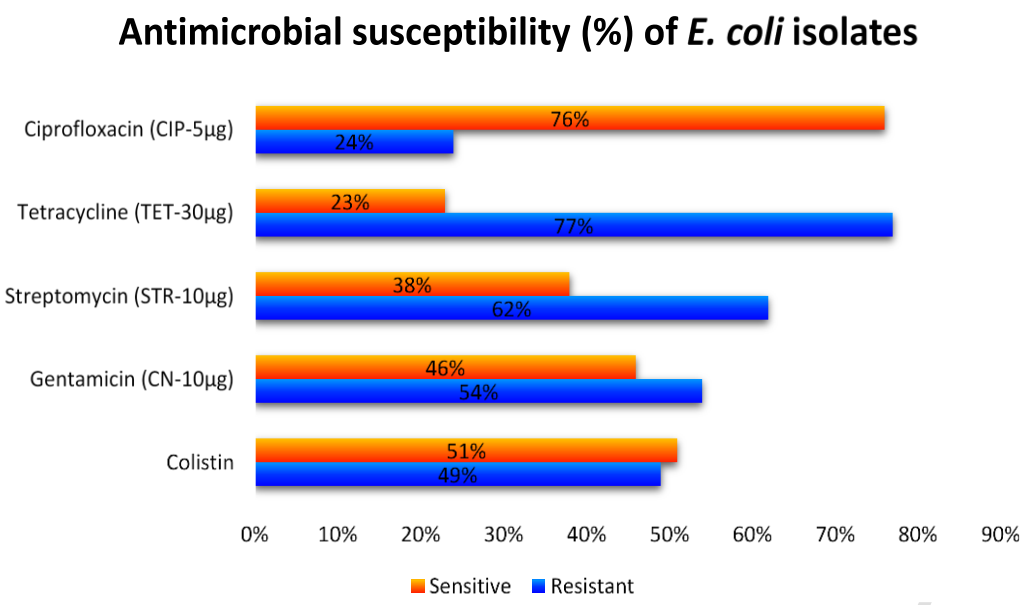

Fig. 3: Antimicrobial susceptibility testing of *E. coli* isolated from cloacal swabs.

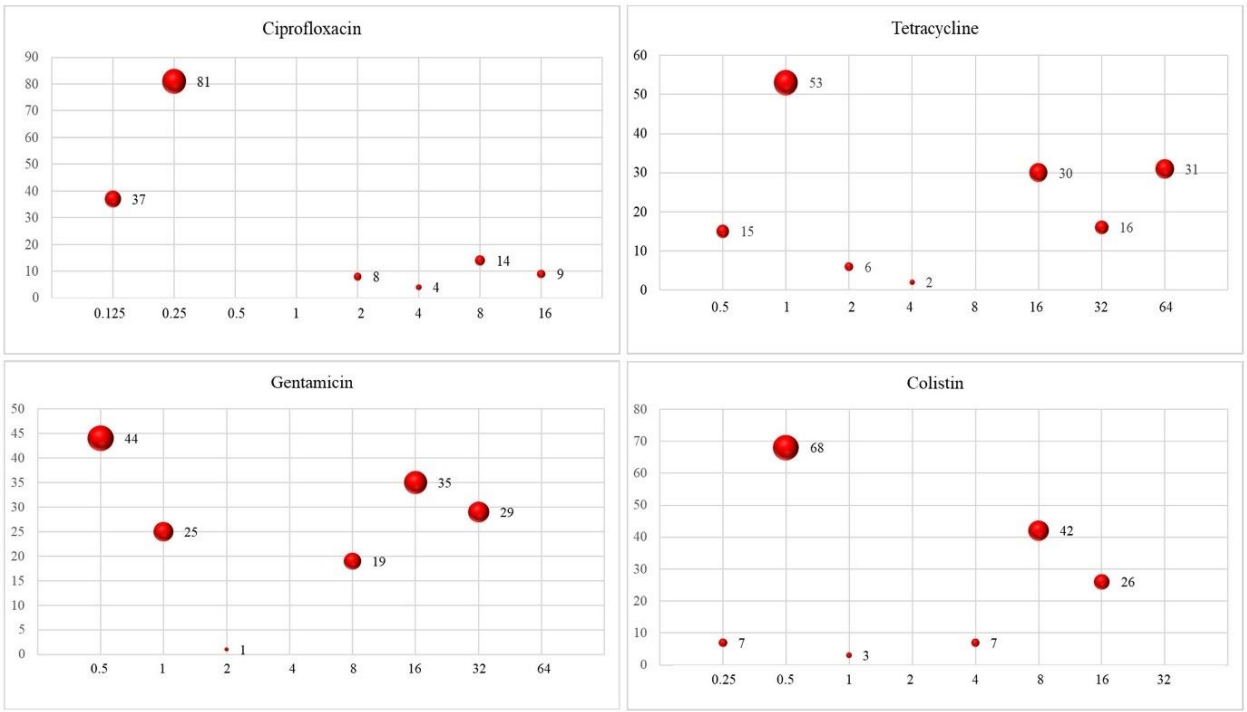

Fig. 4: Comparative distribution of MICs of *E. coli* isolates against Ciprofloxacin, Tetracycline, Gentamicin and Colistin (X-axis= Minimum inhibitory concentrations /mL and Y-axis= Number of *E. coli* isolates).

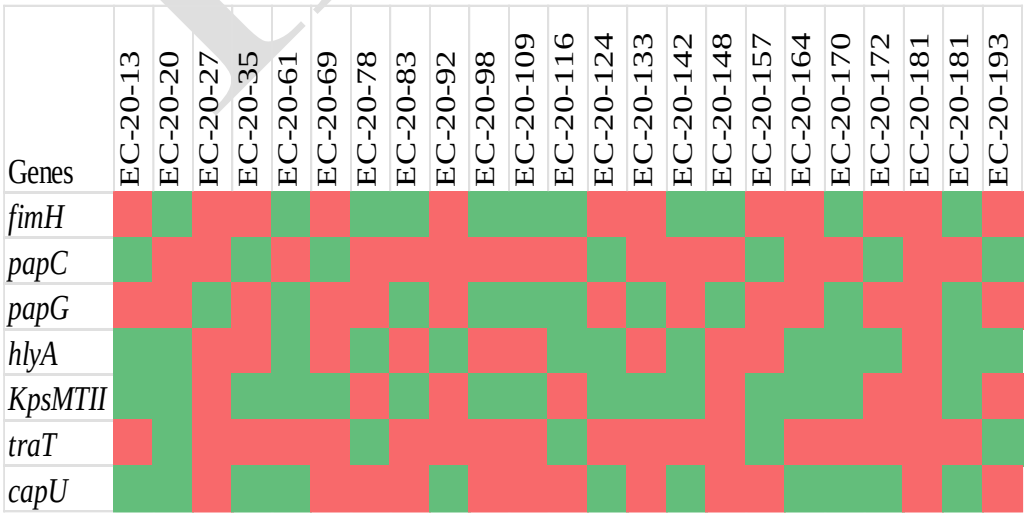

Fig. 5: Distribution of virulence genes among *E. coli* isolates.

allows to determine the quantitative patterns of resistance or susceptibility. The results showed 49, 54, 50 and 23% resistance against colistin, gentamicin, tetracycline and ciprofloxacin, respectively. The breakpoints were defined according to CLSI-2023 guidelines. These results are in line with one of the previous studies (Jamil *et al*., 2022) except resistance to colistin. The data in the current study showed the occurrence of ARGs including *aac(3)-IV), aadA1, mcr-1, tet*A and *qnr*A as 47, 56, 43, 61 and 12%, respectively. Rahman *et al*. (2020) recently reported ARG prevalence rates of 25.8% for *aac-3-IV*, 33.5% for *aadA1*, and 72.58% for *tetA* genes. However, their study was based on *E. coli* isolation from meat samples of broilers. Wu *et al*. (2024) described 100% dissemination of plasmid mediated *mcr-1* gene that was verified by plasmid conjugation transfer analysis. The primary objective in the current study was to demonstrate various ARGs in commensal/ cloacal *E. coli* isolates with the hypothesis that these may serve as a potential source for humans. For this reason, *E. coli* isolates were processed to determine the sequence types based on housekeeping genes along with determination of ARGs encoding for gentamicin, streptomycin, tetracycline and ciprofloxacin resistance. Another study described the prevalence of MDR- *E. coli* isolates based on MALDI-TOF MS from commercial layers and broilers as high as 86.76% along with occurrence of different ARGs (Kitti *et al*., 2021). However, the prevalence of MDR- *E. coli* is lower in the current study. The amplification of virulence genes showed that EC-20-181 isolate (ST1650) encodes maximum virulence genes i.e. adhesins (*fimH, papC*, and *papG*), tissue invasion (*hlyA* and *KpsMTII*) and immune evasion (*traT* and *capU*) followed by EC-20-20 (ST1035). One of the previous studies described the occurrence of AMR or MDR among commensal *E. coli* (Montoro-Dasi *et al*., 2021). However, in the current study we have conducted virulent gene profiles among commensal *E. coli* isolates. Another study described the antimicrobial resistance profiles of the commensal/ cloacal *E. coli* (Kitti *et al*., 2021). In the current study, we found that overall occurrence of virulence genes among *E. coli* isolates is low. This has also been described that virulence genes have strong association with avian pathogenic *E. coli* (Fujimoto *et al*., 2021). A total of 23 *E. coli* isolates were found to have multiple ARGs and the MLST data indicated that these isolates belonged to (*mcr-1, qnrA, tetA*) ST1035 (n=10), (*qnrA, aadA1, tetA*) ST1035 (n=3), (*qnrA, aadA1, aac(3)-IV*) ST131 (n=7) and (*aac(3)-IV, tetA*) ST1650 (n=3). However, previous data showed that MALDI-TOF MS based identification of *E. coli* is also a reliable tool (Kitti *et al*., 2021), further amplification and sequence analysis of 16SrRNA and pulsed field gel electrophoresis (PFGE) could be utilized for molecular identification of *E. coli* isolates (Li *et al*., 2023; Othman *et al*., 2024). Further, MLST has also been reported to determine sequence types (Jamil *et al*., 2022). It has also been described that extra intestinal pathogenic *E. coli* belonging to ST131 has zoonotic potential and is reported to cause millions of infections worldwide, annually. ST131 has also been reported to carry plasmid mediated resistance genes or mobile genetic elements encoding for different ARGs, further ST131 has wide resistance patterns against fluoroquinolones (Pitout and DeVinny, 2017). The data in the current study also demonstrated that all ST131 (n=7) isolates were resistant to ciprofloxacin and were positive for *qnrA* gene. Altogether, the occurrence of different sequence types of MDR- *E. coli* from commercial broilers sufficiently highlights possible zoonotic dissemination among humans via food chain.

Conclusions: In conclusion, the current study highlighted the significant presence of commensal MDR- *E. coli* strains particularly ST131 and ST1035 in cloacal samples of commercial broilers. Further, these strains harboring multiple ARGs have potential to contaminate the broiler meat. The findings underscore the need for monitoring and managing commensal antibiotic resistance *E. coli* isolates among food producing animals.

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