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Antimicrobial Resistance of Pathogenic *Escherichia coli* isolated from Broiler Production Systems

Bajrang Kagane[1*], Rupesh Waghamare[1], Vivek Deshmukh[1], Sanjay Londhe[2], Kakasaheb Khose[3] and Pandit Nandedkar[4]

[1]*Department of Veterinary Public Health and Epidemiology, College of Veterinary and Animal Sciences, Parbhani, INDIA*
[2]*Department of Livestock Product Technology, College of Veterinary and Animal Sciences, Parbhani, INDIA*
[3]*Department of Poultry Science, College of Veterinary and Animal Sciences, Parbhani, INDIA*
[4]*Department of Animal Genetics and Breeding, College of Veterinary and Animal Sciences, Parbhani, INDIA*

**Corresponding author: BR Kagane; E-mail: bajrang.kagane@gmail.com*

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ABSTRACT

The present study was carried out with an objective to evaluate antimicrobial resistant pathogenic *E. coli* from selected broiler production systems in and around Parbhani city. A total of 216 samples comprising 36 various contamination sources from each farm were collected from six broiler units. The initial isolation of *E. coli* was done on EMB and MacConkey agar. On morphological and biochemical tests, 105(48.61%) isolates were preliminary confirmed as *E. coli*. Out of 105 preliminary confirmed isolates, randomly selected 55 isolates were further confirmed as *E. coli* by PCR targeting 16S rRNA gene and phenotypically marked as pathogenic by Congo Red Binding Assay. Out of 55 confirmed isolates, 20 randomly selected isolates were tested against commonly used 15 antimicrobials. antimicrobial susceptibility testing revealed higher resistance against Erythromycin (100%), Cephalothin (95%), Tetracycline (90%), Nalidixic acid (90%) Ceftazidime (85%), Cefotaxime (85%), Amikacin (80%), Gentamicin (75%), Amoxiclav (75%), Ciprofloxacin (70%), Enrofloxacin (70%), Levofloxacin (60%) and sensitivity observed against ampicillin/Sulbactam (85%), Amoxicillin/sulbactam (75%) and Chloramphenicol (50%). The average multiple antimicrobial resistance index of 20 isolates was 0.7. These 20 isolates subjected to genotypic antimicrobial resistance by PCR and found that 85 and 80% isolates expressed *tet A* and *blaTEM* genes which indicates resistance against Tetracycline and β-lactam antimicrobials. The study concludes that higher occurrence of multiple antimicrobial resistant *E. coli* with genotypic characteristics in broiler farms may pose high risk to human and animal health, therefore regional surveillance on use of antimicrobials and studies on antimicrobial resistant bacteria in broiler needs to be conducted regularly.

HIGHLIGHTS

- All the *E. coli* isolates of poultry found pathogenic with Congo red binding assay.
- *tet A* and *blaTEM* genes were observed in *E. coli* isolated from broiler poultry farming system.
- The average MAR index of *E. coli* isolates was 0.7 which indicates that isolates were originated from high-risk sources and resistant against multiple antibiotics.

Keywords: Antimicrobial, Resistant, *E. coli*, Tetracycline, β-lactam

India ranks 4th in the world for its broiler production and India's poultry industry has developed from backyard activity into a major commercial activity (Livestock census, 2019). Contribution of poultry sector to meat production in India accounts for 45 % and growth rate of broiler production is 11 percent (APEDA, 2019). Meat consumption was increasing in India and poultry sector is one of the major contributors to economic development.

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Chicken meat demand is rising due to its versatility, relatively low cost in comparison to other meat and it is accepted by all religions (Devi *et al.,* 2014). Indian Council of Medical Research (ICMR) recommends 10.8 Kg meat consumption/capita/annum but the current consumption of meat is 5.6 Kg.

E. coli is the natural gut microflora of poultry birds which is spread in litter via droppings of birds. It may find its way to the broiler production chain through various sources (Islam *et al.,* 2014). *E. coli* is the opportunistic pathogen capable of causing common infectious diseases in poultry birds like colibacillosis, omphalitis, yolk sac infection, respiratory tract infection, septicemia, enteritis etc and leads to morbidity and mortality, associated with heavy economic losses to the poultry industry (Chowdhuri *et al.,* 2011).

The heavy production of flocks may pose a strong source for proliferation of *E. coli* from environmental sources such as water, feed, litter and workers have been proved by various researchers (Mamun *et al.,* 2016). The chicken meat is contaminated by the intestinal content at the time of slaughter (Jakaria *et al.,* 2012). *E. coli* enters the human body through the consumption of imperfectly cooked meat or handling of raw meat and causes food poisoning (Gormley *et al.,* 2011).

The developing antimicrobial resistance in *E. coli* is great concern to public health around the world (Rahman *et al.,* 2020). Emerging strains of pathogenic *E. coli* equipped with genotypic resistance to clinically common antimicrobials rise a red flag in the area of research (Smith *et al.,* 2007). Antimicrobials can be used in poultry birds for therapeutic purpose, prophylactic purpose and as growth promoters. The common antimicrobials used in poultry birds are Tetracyclines, Aminoglycosides, beta-lactams (Penicillins and Cephalosporins), Sulphonamides, and fluoroquinolones. The indiscriminate use or abuse of antimicrobials may lead to antimicrobial resistance (Ibrahim *et al.,* 2019).

The development of antimicrobial resistance is usually associated with genetic changes encoded by chromosomal and plasmid genes. The multidrug resistance *E. coli* carry resistance genes on plasmids and it can be transferred to other species of *E. coli* (Amer *et al.,* 2018). The transfer of resistance genes between bacterial generations may be inherent or acquired through vertical or horizontal transfer (transformation, conjugation, and transduction) mechanisms (Ibrahim *et al.,* 2019). The multidrug resistant *E. coli* and resistance genes can be transferred from food animals to humans via food or environment which is a public health concern (Ibrahim *et al.,* 2019).

Considering all above facts, in order to attempt systemic study on antimicrobial resistant *E. coli* in poultry farm this piece of study was proposed to undertake. The data on antimicrobial resistant *E. coli* in the area may provide epidemiological information for medical and veterinary field to take the problem of food borne *E. coli*. The present study was designed to study the pathogenicity, phenotypic and genotypic antimicrobial resistance of *E. coli* isolates.

MATERIALS AND METHODS

Collection of samples

The samples were collected from six different broiler poultry units in and around Parbhani city. A total of 216 farm origin samples (comprising of 36 samples each farm) were randomly collected from 6 different contamination sources from each broiler poultry farm used for sample collection. The sources of sample include cloacal swab, litter, feed, water, utensil and worker hand swabs. A total of 6 lots from each of 6 sources of each broiler poultry farm were collected. All the samples were collected aseptically, using sterilized polythene bags and containers. Soon after collection the swab samples were placed in the Carry Blair transport medium and brought to laboratory under low temperature (≤10° C) as per the standard methods (Han *et al.,* 2020).

Isolation and identification of *E. coli*

Isolation and identification of *E. coli* from collected samples as per Han *et al.* (2020) Which were further confirmed by conventional method based on Gram's staining, biochemical test and 16S rRNA PCR assay. The *E. coli* isolates were identified by different biochemical tests as per Cheesbrough (2006).

Characterization of *E. coli* isolates by PCR targeting 16S rRNA gene

Conformation of *E. coli* was done with PCR targeting 16S

rRNA gene as per the method described by Ibrahim *et al.* (2019). The primer details and amplicon size are given in Table1.

In-vitro pathogenicity tests

The *E. coli* isolates confirmed by 16S rRNA PCR assay were subjected to congo red binding assay for *in vitro* pathogenicity testing. Congo red binding assay was carried out as per the method described by Berkhoff and Vinal, (1985). Trypticase soya agar was added with 0.3 percent congo red dye and plates were prepared aseptically. These plates were streaked by all *E. coli* isolates and incubated for 3 days at 37°C. The colour change of the colonies were examined daily and appearance of brick red colour was considered as positive or invasive *E. coli*. The greyish white colour colonies were considered as negative or noninvasive.

Antimicrobial susceptibility test

The *E. coli* isolates confirmed by biochemical test, 16S rRNA PCR assay and *in-vitro* pathogenicity test are subjected to antimicrobial sensitivity test. It was carried out by disc diffusion technique of Bauer *et al.* (1966) against the panel of 15 antimicrobials. The Antimicrobials used are Levofloxacin, Tetracycline, Chloramphenicol, Erythromycin, Ciprofloxacin, Ampicillin/Sulbactam, Gentamicin, Amoxicillin/Sulbactam, Enrofloxacin, Nalidixic Acid, Ceftazidime, Amikacin, Amoxyclav, Cephalothin and Cefotaxime. Mueller Hinton agar plates were inoculated with purely grown bacterial culture equal to 0.5 McFarland standard by streaking with swab. Antimicrobial discs were distinctly placed over the inoculated agar plates and incubated at 37° C for 16-18 hrs. The size of zones of inhibition was interpreted by zone diameter of standard *E. coli* and Enterobacteriaceae as per chart given in HiMedia manual; Mumbai based on CLSI guidelines, 2017.

Multiple antimicrobial resistance (MAR) index

The MAR index of individual isolates and poultry broiler farms were calculated according to the method of Krumperman, (1983).

Molecular characterization of *E. coli* by conventional polymerase chain reaction assay of antimicrobial resistance genes

E. coli isolates confirmed by PCR targeting 16S rRNA gene was further randomly selected 20 isolates for presence of antimicrobial resistance genes (*tet A* and *blaTEM*).

Extraction of genomic DNA from *E. coli* colonies by standard heat lysis protocol

The DNA of *E. coli* was extracted by boiling method described by Rawool *et al.* (2007) with slight modification. Approximately loopful of *E. coli* culture was inoculated in 2 ml of Nutrient broth and incubated for overnight. The incubated broth 1.5 ml transferred in Eppendorf tube and centrifuged at 10,000 rpm for 10 min to form the pellet of bacteria. The supernatant was discarded and the pellet of *E. coli* was mixed with 1000 µl of normal saline solution and vortexed. The mixture was again centrifuged as above, and the resultant pellet was dissolved in 200 µl of nuclease free water. This mixture was vortexed and further boiled at 100ºC for 8 min. The centrifuge tube was subjected to rapid cooling on ice which was followed by centrifugation at 4,000 rpm for 3 min. The 50 µl of upper aqueous phase containing DNA was transferred to sterile micro-centrifuge tube. These extracted DNAs were stored at -20º C till further use.

Oligonucleotide primers

Oligonucleotide primers used for genotypic characterization of *E. coli* are mentioned in Table 1.

Reaction mixture

PCR for 16S rRNA gene was performed in 25 µl reaction volume containing 12.5 µl of 2 × PCR master mix, 1 µl of each primer (10 pmol/ µl), 2 µl of genomic DNA and 8.5 nuclease free water, used to make the desired volume.

PCR for *blaTEM* and tetA was performed in 25 µl volumes containing 12.5 µl of 2 × PCR master mix, 2 µl of each primer (10 pmol/ µl), 2 µl of genomic DNA and 6.5 µl nuclease free water used to make desired volume (Fonseca *et al.,* 2006 and Bhattacharjee *et al.,* 2007).

Table 1: Primers used for genotypic characterization of *E. coli*

Sl. No.	Primer	Target gene	Primer Sequence (5' — 3')	Product Size (bp)	Reference
1	*16S rRNA*	Conserved and specific gene sequence	F: GACCTCGGTTTAGTTCACAGA R: CACACGCTGACGCTGACCA	485	Ibrahim *et al.,* 2019
2	*bla TEM*	Broad spectrum β-lactamases	F: ATGAGTATTCAACATTTCCG R: CTGACAGTTACCAATGCTTA	867	Bhattacharjee *et al.,* 2007
3	*tet A*	Tetracycline	F: GCTACATCCTGCTTGCCTTC R: CATAGATCGCCGTGAAGAGG	210	Fonseca *et al.,* 2006

PCR amplification

The PCR assay for the detection of *E. coli* by 16S rRNA and antimicrobial resistance genes (*blaTEM* and *tet A*) of *E. coli* was standardized as per the method described by respective references (Table 1). PCR was performed using a gradient thermal cycler.

The cycling conditions for *16S rRNA* gene consisted of initial denaturation at 95 °C for 5 min., followed by 30 cycles of 94 °C for 1 min., 55 °C for 45 seconds and 72 °C for 1 min. with final extension at 72 °C for 7 min. The cycling conditions for *tetA* gene consisted of initial denaturation at 95 °C for 5 min., followed by 40 cycles of 95 °C for 1 min., 64 °C for 30 seconds and 72 °C for 30 seconds with final extension at 72 °C for 10 min. The cycling conditions for *blaTEM* gene consisted of initial denaturation at 95 °C for 5 min., followed by 35 cycles of 95 °C for 1 min., 55 °C for 1 min. and 72 °C for 1 min. with final extension at 72 °C for 7 min.

To observe the amplified band, 15 µl of PCR product from each tube was mixed with 4 µl(6x) gel loading dye. This mixture was electrophorized on 1.8% and 1.4% agarose gel added with 10 µl ethidium bromide for 16S rRNA and antimicrobial resistance genes, respectively. Along with PCR product 100 bp DNA ladder was also electrophorized. The electrophoresis was carried out at constant voltage of 100 V for 55 min and 75 V for 45 min to detect 16S rRNA gene and antimicrobial resistance genes, respectively. Further, the gel was removed carefully and observed the band size under UV transilluminator.

RESULTS AND DISCUSSION

Occurrence of *E. coli* in broiler poultry farms

The presumptive positive *E. coli* isolates were confirmed by growth characteristics of different selective media, Gram staining and biochemical tests. Out of 216 samples collected from broiler poultry farms 105 (48.61%) samples were found positive for *E. coli*. Amongst the various samples ascending percentage occurrence rate observed was 77.77, 36.11, 33.33, 77.77, 41.66 and 25.00 percent for cloacal swab, feed, water, litter, utensils and workers hand swabs, respectively. Cloacal swabs and litter revealed highest occurrence while workers hand swab revealed low occurrence compared to other remaining sources. Statistically significant difference at 1% and 5% was observed between various sources of samples the results indicates that the cloacal swabs and litter have considerable effect on occurrence of *E. coli* in broiler poultry farms. The enteric nature of the organisms itself justifies the highest occurrence in cloacal swab and litter sample. Overall occurrence of *E. coli* 48.61% are in agreement with previous studies of Taralkar, (2002) and Farooq *et al.* (2009) who reported occurrence of 48.38% and 40.32% in apparently healthy broiler poultry.

Characterization of *E. coli* isolates by PCR targeting 16S rRNA gene

Randomly selected 55 isolates from biochemically confirmed isolates were subjected to PCR targeting 16S rRNA gene for final confirmation and all 55 isolates were confirmed as *E. coli.* The 16S rRNA PCR method for the confirmation of *E. coli* is a sensitive, rapid and

Table 2: Details of various sources of samples collected from broiler farms

Sl. No.	Various sources of Contamination	Number of Samples analysed	Total positive samples	Occurrence of *E. coli*
1	Cloacal Swab	36	28.00[a]	77.77%
2	Feed	36	13.00[b]	36.11%
3	Water	36	12.00[b]	33.33%
4	Litter	36	28.00[a]	77.77%
5	Utensils	36	15.00[b]	41.66%
6	Workers Hand Swab	36	09.00[b]	25.00%
	Overall occurrence	216	105	48.61%

accurate method (Hossain *et al.,* 2013). The key points of this molecular target by universal primer have served for determining phylogenetic relationship between closely related bacteria.

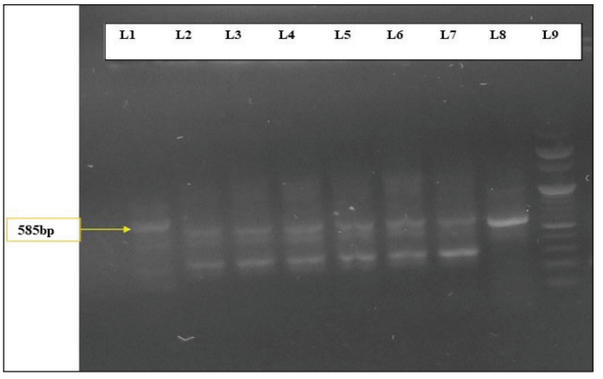

Fig. 1: Agarose gel showing PCR amplified product of 16S rRNA gene of *E. coli.* L1-18: Samples; L9: 100 bp DNA lader. *In-vitro* pathogenicity of *E. coli*

Congo red dye binding in *E. coli* is associated with pathogenicity (Styles and Flammer, 1991). The direct correlation of between congo red dye binding and ability to cause septicaemic infection by *E. coli* was reported by (Berkhoff and Vinal, 1986). Several workers successfully used Congo red binding assay for identification of pathogenic *E. coli* (Berkhoff and Vinal, 1986 and Shekh *et al.,* 2013).

In present study Congo red binding assay was performed using Trypticase Soya Agar. A total of 55 isolates confirmed by 16S rRNA PCR were subjected for Congo red binding assay. Surprisingly the percent positivity of Congo red binding assay observed was 100%.

The results are in agreement with Roy *et al.* (2006) and Berkhoff and Vinal, (1986). They reported 100 % Congo red binding isolates under their study and further suggested use of Congo red binding assay as phenotypic marker to differentiate between invasive and non-invasive isolates.

Phenotypic antimicrobial resistance (antibiogram) of *E. coli*

The PCR assay (targeting 16S rRNA gene) positive randomly selected 20 *E. coli* isolates were tested against 15 commonly used antimicrobials. Higher resistance was recorded against Erythromycin (100%), followed by Cephalothin (95%), Tetracycline (90%), Nalidixic acid (90%), Ceftazidime (85%), Cefotaxime (85%), Amikacin (80%), Gentamicin (75%), Amoxyclav (75%), Ciprofloxacin (70%), Enrofloxacin (70%), Levofloxacin (60%), Chloramphenicol (50%), Amoxicillin /sulbactam (25%) and Ampicillin/sulbactam (15%).

The use of antimicrobials in animal feed plays a crucial load in development (Wegener *et al.,* 1999). Exposure to Antimicrobials enables the selection of resistance strains of *E. coli* exposed to the Antimicrobials in the intestinal flora of birds (Chika *et al.,* 2017). Uncontrolled use of antimicrobials in food animal production systems for prevention and treatment of diseases increases the risk of emergence of resistant bacterial strains (Rahman *et al.,* 2020).

The present study is in agreement with findings of Dandachi *et al.* (2020) wherein Erythromycin, Tetracycline, Chloramphenicol, Amikacin, Gentamicin and Cefotaxime resistance were comparable. Ibrahim *et al.* (2019) observed 70.60, 63.90 and 84.4 % resistance to

Amoxyclav, Ciprofloxacin and Enrofloxacin, respectively. The results pertaining to Levofloxacin, Nalidixic acid and Ceftazidime are similar with Brower *et al.* (2017) and variation in resistance of Cephalothin, Amoxicillin/sulbactam, and Ampicillin/sulbactam was observed by Brower *et al.* (2017).

In the present study *E. coli* isolates found resistance to Erythromycin, Cephalothin, Tetracycline, Nalidixic acid, Ceftazidime, Cefotaxime, Amikacin, Gentamicin, Amoxyclav, Ciprofloxacin, Enrofloxacin, Levofloxacin and Chloramphenicol which is a great concern. In the current era antimicrobial resistance is a common issue and setting a challenge in community infections, therefore Antimicrobial needs to be individualize according to patients risk of acquiring drug resistance pattern (Lin *et al.,* 2019).

Multiple antimicrobial resistance (MAR) index of *E. coli* isolates

MAR index of each isolate was carried out by dividing the number of antimicrobials to which the isolate was resistant by the total number of antimicrobials to which the isolate was exposed. The MAR index greater than 0.2 considered to originating from high-risk source of contamination (Chandran *et al.,* 2008). The incidence of multiple antimicrobial resistance microorganisms in the present study was 100%. All the isolates were resistant to more than 5 Antimicrobials. The MAR index varies from 0.4 to 1 with an average of 0.7. A total of 13 isolates out of 20 were resistant to more than 10 Antimicrobials. The results of present study found are in agreement with the study conducted by Chandran *et al.* (2008) who reported 95% isolates as MAR. The increased concentration of multidrug resistance bacteria in environment creates selective pressure in natural bacterial strains (Alpay-Karoglu *et al.,* 2007).

In the present study MAR index of isolates recovered from area under study exceeds the arbitrary level which indicates isolates are originated from high-risk source. These resistant strains spreading to different ecological areas which could lead to further increase in the number of drug-resistant bacteria (Chandran *et al.,* 2008).

Genotypic antimicrobial resistance (*tet A* and *blaTEM* genes) of *E. coli* isolates

The unprecedented increase of antimicrobial resistant organisms in nature is linked to the extensive and non-judicial use of antimicrobial agents for disease control and prevention (Levy, 2014). The development of antimicrobial resistance is complex process arises due to presence of resistance encoding genes which are found inside plasmids of chromosomal genetic material.

The extended spectrum beta lactamase producing *E. coli* is a global antimicrobial multidrug resistance problem affecting human and animals. The bacteria which are resistant to Cephalosporins and Penicillins are encoded by bla_{CTX-M}, bla_{SHV} and *blaTEM* genes, these bacteria produce beta lactamase. The *tet A* gene associated with Tetracycline influx pump was predominantly found in *E. coli* isolates from livestock and food animals (Waghamare *et al.,* 2017).

The 20 randomly selected *E. coli* isolates were subjected for screening of Tetracycline resistance and broad-spectrum beta lactamase resistance by targeting *tet A* and *blaTEM* genes, respectively. An amplified product 210 bp and 867 bp specific of *tet A* and *blaTEM* gene, respectively were screened in *E. coli* isolates. Out of the 20 samples assessed, 85 and 80% samples were positive for *tet A* and *blaTEM* genes, respectively. Results are depicted in Fig. 2 and Fig. 3.

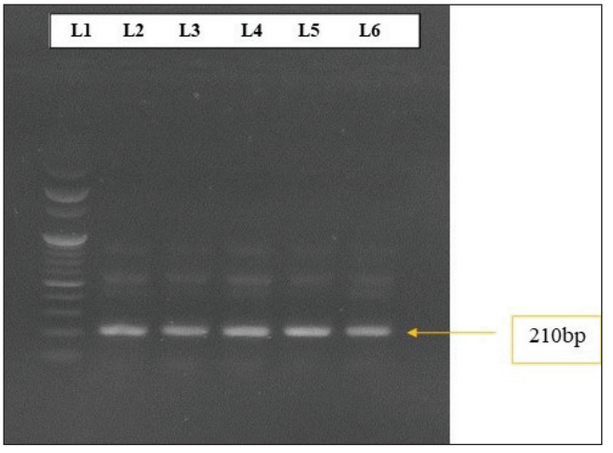

Fig. 2: Agarose gel showing PCR amplified product of *tet A* gene of *E. coli.* L1: 100 bp DNA ladder; L2-L6: Samples

The findings for *tet A* gene were in corroboration with Han *et al.* (2020). The results of *blaTEM* gene are in agreement with results of Younis *et al.* (2017) who reported occurrence of 78%. It is observed that Oxytetracycline, Chlortetracycline and Doxycycline drugs are highly used in poultry farms for therapeutic or prophylactic purposes and could have contributed to the higher resistance these antimicrobial agents (Waghamare *et al.,* 2017).

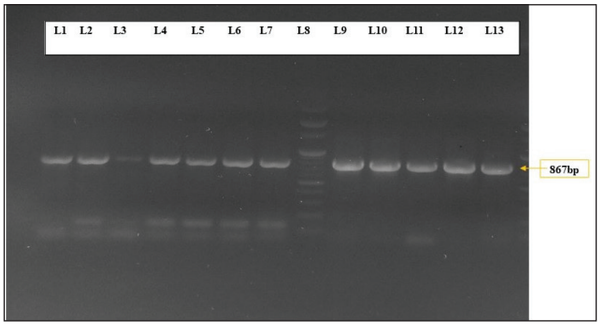

Fig. 3: Agarose gel showing PCR amplified product of blaTEM gene of *E. coli.* L & L2: Samples; L3: negative control; L4-L7: samples; L8: 100 bp DNA ladder; L9-L13: Samples

Results of present study also report presence of *tet A* gene supporting the statement. The isolates were usually expressed phenotypic resistance but failed to express resistance gene in present study might be due to point mutation or other resistance mechanism.

CONCLUSION

E. coli isolates of present study could be pathogenic as all tested isolates were positive for Congo Red Binding assay. High drug resistance was recorded against commonly used antibiotics viz; Erythromycin, Cephalothin, Tetracycline, Nalidixic acid, Ceftazidime and Cefotaxime antibiotics compared to Chloramphenicol, Amoxicillin/sulbactam and Ampicillin/sulbactam. The average MAR index of isolates was 0.7 which indicates that *E. coli* isolates originated from high-risk sources and resistant against multiple antibiotics. Distribution of *tet A* and *blaTEM* in *E. coli* isolates may pose high risk to human and animal health. Regional surveillance on use of antibiotics and studies on antimicrobial resistant bacteria in poultry needs to be conducted regularly.

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