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Antimicrobial sensitivity pattern and detection of antimicrobial resistance genes of *E. coli* isolated from respiratory tract infections in poultry

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ABSTRACT

The present study was conducted at College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University with the objective to determine the antimicrobial sensitivity pattern and anti-microbial resistance genes on *E. coli* isolates obtained from respiratory infection samples of poultry. A total of 115 samples were collected from different respiratory disease outbreaks from various poultry farms of Ludhiana district of Punjab. Various bacteria related to respiratory infections were isolated and *E. coli* was found to be in highest number among the isolated bacteria. The isolates of *E. coli* were confirmed by MALDI-TOF and were subjected to Kirby Bauer's disc diffusion method to study the antimicrobial sensitivity pattern phenotypically. The isolates were also screened for the presence of six antimicrobial resistance genes associated with certain antibiotics by Polymerase Chain Reaction (PCR). All the isolates showed 100% resistance towards the antibiotics, viz. tetracycline, chlortetracycline, enrofloxacin, erythromycin, ofloxacin, tylosin, amikacin, and ciprofloxacin. This demonstrates the multidrug-resistance of the isolates. The antimicrobial resistance gene *strA* (60%) was found to be expressed more among the isolates followed by *ere* (50%), *tetA* (47.5%), *aac-(3)-(IV)* (37.5%) and *blaTEM* (32.5%). None of the isolate was found to have *tetC* gene.

Keywords: Antimicrobial resistance, *Escherichia coli*, MALDI-TOF, Poultry, Resistance genes

Escherichia coli is a Gram-negative, facultative anaerobic rod-shaped bacterium that belongs to the family Enterobacteriaceae. They are common inhabitants of the intestinal tract of poultry which rarely affects respiratory tract of poultry thereby causing systemic infection (Mol *et al.* 2019). Infection of the respiratory tract by Avian Pathogenic *E. coli* (APEC) increases mortality in the flocks. The poultry industry has attained a tremendous growth from small backyard business to a commercial industry which has increased the antibiotic usage either therapeutically or as a growth promoter. This has resulted in the evolution of anti-microbial resistance micro-organisms (Hedman *et al.* 2020). The resistance genes are transferred from one generation of bacteria to the other either through horizontal or vertical gene transfer (Amer *et al.* 2018). The resistance transferred to the next generation occurs not only in pathogenic organisms but also in non-pathogenic commensal organisms which may be attributed to the mass administration of antimicrobials in intensive system of rearing in commercial poultry industry (Miles *et al.* 2006). The antimicrobial resistance is mainly associated with the integrons that can actually capture, incorporate and result in the expression of the gene clusters related to antimicrobial resistance (Skurnik *et al.* 2005, Kumar and Gupta 2019). The present study aims at determining the antibiotic sensitivity pattern of the *E. coli* isolates, isolated from respiratory tract infections of poultry by disc diffusion method and to determine the antimicrobial resistant genes by molecular technique (PCR) using specific primers.

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MATERIALS AND METHODS

Sample collection: A total of 115 samples were collected from various respiratory outbreaks of the poultry in and around areas of Punjab. The samples were collected from birds of different age groups and samples like tracheal swabs, tracheal and lung tissues were collected aseptically in a sample collection vial containing Normal saline solution (0.9% NaCl). The samples were collected from dead birds during postmortem examination. The samples were brought immediately to the laboratory in ice and processed. The samples were also processed for the molecular detection of other fastidious organisms involved in respiratory tract infections of poultry.

Isolation and identification of E. coli isolates: The samples were streaked on Brain Heart Infusion (BHI) agar and incubated at 37°C for 16 to 24 h. The colonies were subjected to Gram's staining to differentiate between Gram-positive and Gram-negative. The colonies showing Gram-negative were streaked on MacConkey lactose agar

and incubated for 24 h at 37°C. The lactose fermenters were further streaked on Eosin Methylene Blue (EMB) agar and incubated at 37°C for 24 h and observed for the metallic sheen. All the lactose fermenters were screened by Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI Biotyper Sirius system, using MALDI biotyper 4.1.100 software, Bruker Daltonics, Germany) and *E.coli* isolates were sub-cultured on BHI agar till further use for antimicrobial sensitivity or PCR for resistance genes.

Antimicrobial sensitivity assay: The isolates were studied for antimicrobial sensitivity by Kirby Bauer's disc diffusion method (Bauer *et al.* 1966). Test inoculum were prepared by inoculating the isolates in Brain Heart infusion broth and incubated at 37°C for 4 to 6 h. The turbidity was adjusted according to half the McFarland standard no. 1 (i.e. 1.5×10^8 bacteria/ml of the suspension). The inoculum prepared with each test isolate was streaked on Mueller Hinton agar (MHA) by swabbing it on the whole surface at an angle of 60° to obtain confluent growth. The antibiotic discs were placed on the surface with sterile forceps equidistant from each other as well as away from the edge of the plates and the plates were incubated at 37°C for 24 h.

The antibiotics used in the study were ampicillin (25 µg), ceftriaxone (30 µg), streptomycin (25 µg), gatifloxacin (5 µg), erythromycin (15 µg), tetracycline (30 µg), enrofloxacin (10 µg), ofloxacin (5 µg), tylosin (15 µg), chlortetracycline (30 µg), amikacin (30 µg), ciprofloxacin (30 µg) and gentamicin (10 µg). The diameters of zone of inhibition (ZOI) were expressed in millimeters (mm) and the results were elucidated according to the CLSI guidelines. The isolates showing resistance to more than two antibiotics tested were considered as multi-drug resistant.

DNA extraction: DNA was extracted from the colonies of the isolates by crude lysate method or hot cold lysis method. The colonies (4-5 colonies) were aseptically picked and mixed with 200 µl of nuclease free water (NFW) in 2.0 ml sterile micro-centrifuge tubes. The samples were kept in dry bath at 100°C for 10 min and then snap cooled at -20°C for 5 min. These were centrifuged at 15,850 × g for 10 min. The supernatant was transferred to fresh centrifuge tubes and stored at -20°C.

Molecular detection of AMR genes by Polymerase Chain Reaction (PCR): The DNA extracted by crude lysate method was used as a template in PCR amplification. All the 40 isolates studied for phenotypic resistance were also evaluated for the presence of genotypic resistance of various antibiotic resistance genes. Specific primers were used as per the references for various genes, viz. *blaTEM, tetA, strA, ere, aac (3)-(IV),* and *tetC.* The details of the primer sequences and the annealing temperature are given in the Table 1.

The amplified PCR products were run in gel electrophoresis with 1.5% of agarose at 80 volts for one hour and the products were visualized in gel documentation system (Syngene, USA).

RESULTS AND DISCUSSION

E. coli infections of the respiratory tract are often explicit with respiratory clinical signs which results in increased mortality among the flock. Samples for the present study were collected from the birds exhibiting clinical signs of respiratory distress and gross lesions like congestion of tracheal mucous membrane, air sacculitis, perihepatitis and pericarditis. Out of 115 samples collected from various outbreaks of respiratory diseases, 52 isolates (45.2%) related to respiratory infections were isolated and out of them 40 isolates (76.9%) were found to be *E. coli*. By conventional tests like Gram's staining, the *E. coli* suspected isolates showed Gram negative coccobacilli. The isolates showed lactose fermentation on MacConkey lactose agar (MLA), metallic sheen on Eosin Methylene Blue agar (EMB). All the suspected *E. coli* colonies after conventional tests were confirmed by molecular means using MALDI-TOF.

Phenotypic antimicrobial sensitivity pattern of E.coli isolates: The results of antimicrobial sensitivity assay was observed and out of 13 antibiotics tested, the isolates were sensitive (22.5%) only for ceftriaxone (Supplementary Table 1). The isolates showed intermediate sensitivity for the antibiotics including, streptomycin (32.5%), followed by gentamicin (30%), ceftriaxone (27.5%) and ampicillin (20%). All the isolates showed complete

Table 1. Primers for antimicrobial resistance genes in *E. coli* isolates

Gene	Oligonucleotide sequence (5'-3')	Amplicon size	Annealing temperature	References
blaTEM	F: ATGAGTATTCAACATTTCCG R:ACCAATGCTTAATCAGTGAG	859bp	50°C	Aarestrup *et al.* (2003)
tetA	F:GTAATTCTGAGCACTGTCGC R:TGCCTGGACAACATTGCTT	954bp	49°C	Frech and Schwarz (2000)
strA	F: CCTGGTGATAACGGCAATTC R: CCAATCGCAGATAGAAGGC	548bp	55°C	Madsen *et al.* (2000)
ere	F:GCCGGTGCTCATGAACTTGAG R:CGACTCTATTCGATCAGAGGC	419bp	52°C	Amer *et al.* (2018)
aac	F:CTTCAGGATGGCAAGTTGGT R:TCATCTCGTTCTCCGCTCAT	286bp	55°C	Amer *et al.* (2018)
tetC	F: GGTTGAAGGCTCTCAAGGGC R: CCTCTTGCGGGAATCGTCC	505bp	50°C	Takaichi *et al.* (2022)

resistance (100%) to 8 antibiotics tested which includes tetracycline, erythromycin, enrofloxacin, ofloxacin, tylosin, chlortetracycline, amikacin and ciprofloxacin. The resistance towards most of the antibiotics may be attributed to the emergence of multi-drug resistant microorganisms. The antimicrobial sensitivity patterns to *E.coli* are given in Fig 1.

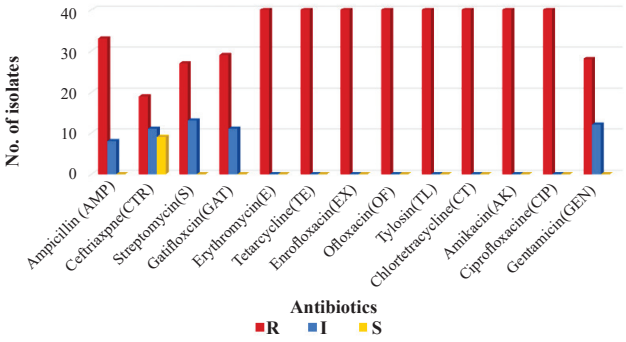

Fig. 1. Antimicrobial sensitivity pattern of *E. coli.*

The isolate can be regarded as multi-drug resistant when it is resistant to three or more antibiotics tested and Momtaz *et al.* (2012) reported 15.78% resistance to a single antibiotic followed by 19.29% resistance to two antibiotics and 64.91% resistance to three or more antibiotics tested for *E.coli* strains isolated from commercial broilers. Abbassi *et al.* (2017) reported that isolates of *E.coli* obtained from avian samples manifested high resistance for the antibiotic tetracycline that was 74.7% out of 174 *E. coli* isolates tested. A similar result was obtained from a study conducted by Halfaoui *et al.* (2017) involving 156 isolates of *E. coli* which exhibited a high level of resistance to tetracycline (94.12%) followed by enrofloxacin (86.27%), ampicillin (83.01%) and few other antibiotics not included in our study. Their study also reported multi-drug resistance with the percentage of 66.66% which were resistant for seven antibiotics tested. The percentage of multi-drug resistance in the present study was 100% among the forty (40) isolates for 8 antibiotics.

In a study conducted by Miles *et al.* (2006), tetracycline showed the highest resistance among the poultry derived *E. coli* isolates that was as high as 82.4% and the resistance in human derived isolates reported to be just 43.8% resistant which was comparatively lower. Multiple-drug resistance was reported from both the poultry and human isolates especially involving tetracycline resistance. The results observed in our present study are similar to the results observed by Amer *et al.* (2018) with 85% resistance towards oxytetracycline, 80% to ampicillin and streptomycin, 75% to enrofloxacin, 55% to gentamicin and 30% to erythromycin and also reported the presence of multi-drug resistant isolates of *E. coli*. Bhardwaj *et al.* (2021) reported high resistance of *E.coli* isolates to the fluoroquinolones group of antibiotics that was 94% and *qnrB* was the most commonly present antimicrobial resistance gene. Similar pattern of resistance was observed by Hassan *et al.* (2014) to tetracycline, enrofloxacin and ciprofloxacin (100%) followed by amoxicillin (84.62%) and the study also reported 100% sensitive pattern to gentamicin which is contrast to the present study where the *E. coli* isolates showed 30% of intermediate sensitivity pattern to gentamicin. Chaudhari *et al.* (2017) reported 93.33% sensitivity of *E. coli* isolates towards ceftriaxone and 80% towards gentamicin which are in contrast to the present study. Ievy *et al.* (2020) reported 100% resistance to ampicillin and tetracycline, 97.2% to erythromycin which are similar to the results of the present study. The resistance pattern of enrofloxacin, ciprofloxacin, streptomycin and gentamicin were 55.5%, 50%, 19.4% and 8.33%, respectively which are different from the results of the present study. Ievy *et al.* (2020) also reported presence of multi-drug resistant isolates.

Genotypic antimicrobial sensitivity assay of E.coli by PCR: All the 40 isolates of *E. coli* were evaluated genotypically for antimicrobial resistance genes (Supplementary Table 2) and the highest prevalence (60%) of antibiotic resistance gene *strA* was exhibited among the isolates, followed by *ere* (50%), *tetA* (47.5%), *aac-(3)-(IV)* (37.5%) and *blaTEM* (32.5%). None of the isolates of *E. coli* exhibited the presence of *tetC* gene which endorsed the less prevalence of the *tetC* gene among *E. coli* isolates. The gel electrophoresis pictures of the respective genes are shown in Supplementary Figs 1-5. Guerra *et al.* (2003) reported notable higher resistance in *E. coli* isolates obtained from poultry samples and the resistance was observed towards tetracycline, streptomycin, ampicillin, and sulfamethoxazole. The percentage of resistance genes observed included 92% for *blaTEM*, 60% for *aac-(3)-(IV)*, 59% for *strA* and 66% for *tetA* which are almost similar to the present study. Similar pattern of presence of resistance genes was reported by Amer *et al.* (2018) in which the *E. coli* isolates showed presence of *ere* gene (60%), *aac-(3)-(IV)* gene (60%) and *tetA* gene (40%). Mooljuntee *et al.* (2010) reported antimicrobial resistance was high towards the antibiotics, viz. ampicillin, erythromycin and tetracycline and these results are similar to the present study. However, no resistance genes were reported for the antibiotic gentamicin (*aac-(3)-(IV)*) which is contrary to the present study where 37.5% isolates showed resistance. The above study revealed that 90% of the isolates showed presence of *tetA* gene and 73.3% showed *ereA* gene and these results were more comparable to the present study. All the *E. coli* isolates were tested for both antimicrobial sensitivity pattern (phenotypic expression) as well as presence of resistance genes (genotypic expression). A positive relationship was observed between phenotypic and genotypic resistance (Table 2). For erythromycin related resistance gene (*ere*), out of 40 isolates tested, 20 (50%) showed positive relationship, followed by tetracycline resistance gene (*tetA*) that was found in 47.5% isolates. Gene for resistance to streptomycin (*strA*) was found among 45% isolates, gentamicin resistance gene (*aac*) was found among 30% isolates and ampicillin resistance gene (*blaTEM*) was found among 27.5% isolates. The results

Table 2. Relationship between phenotypic and genotypic resistance of the *E. coli* isolates

Sample. No.	AMP	*blaTEM*	TE	*tetA*	*tetC*	S	*strA*	E	*ere*	GEN	*aac*
1	R	+	R	+	-	R	+	R	-	R	+
2	I	+	R	+	-	I	-	R	-	I	-
3	R	+	R	+	-	R	+	R	-	I	-
4	R	-	R	+	-	R	+	R	+	R	-
5	R	-	R	+	-	I	+	R	-	R	+
6	R	-	R	+	-	R	-	R	+	R	+
7	R	-	R	-	-	R	-	R	-	R	+
8	R	-	R	+	-	I	-	R	-	I	-
9	R	-	R	-	-	R	-	R	+	R	-
10	R	-	R	-	-	R	+	R	+	R	-
11	I	-	R	+	-	I	-	R	+	I	-
12	R	-	R	-	-	R	+	R	+	I	-
13	R	+	R	-	-	R	+	R	+	R	-
14	R	-	R	-	-	R	+	R	-	R	-
15	I	-	R	-	-	R	+	R	+	R	-
16	R	-	R	-	-	I	+	R	-	I	-
17	R	-	R	-	-	R	-	R	+	R	+
18	R	-	R	-	-	R	+	R	+	R	-
19	I	-	R	+	-	I	+	R	+	I	+
20	R	-	R	+	-	R	+	R	+	I	-
21	R	+	R	+	-	R	+	R	+	R	+
22	R	+	R	-	-	R	+	R	-	R	+
23	R	-	R	+	-	I	+	R	+	R	-
24	R	+	R	+	-	R	+	R	-	I	-
25	R	+	R	-	-	R	+	R	-	R	+
26	R	-	R	-	-	I	+	R	+	R	+
27	I	-	R	+	-	R	-	R	-	R	-
28	R	+	R	-	-	R	+	R	-	R	+
29	R	-	R	+	-	R	+	R	-	R	-
30	R	+	R	-	-	I	-	R	+	I	+
31	I	-	R	-	-	R	+	R	-	R	-
32	R	+	R	+	-	R	-	R	-	R	-
33	R	-	R	-	-	I	-	R	+	R	+
34	R	-	R	+	-	R	-	R	-	R	-
35	I	-	R	-	-	I	+	R	+	I	-
36	R	+	R	-	-	R	-	R	-	R	+
37	I	+	R	-	-	R	-	R	+	R	-
38	R	-	R	+	-	I	-	R	-	I	+
39	R	-	R	-	-	R	+	R	-	R	-
40	R	-	R	+	-	I	-	R	+	I	-
TOTAL	11 (27.5%)		19 (47.5%)			18 (45%)		20 (50%)		12 (30%)	

were in contrast to the results reported by Amer *et al. (*2018). The present study needs further investigations about pathogenic significance of the *E. coli* isolates by Congo red binding assay or detection of virulent genes by PCR.

The present study on *E. coli* isolates of poultry, determines the presence of multi-drug resistant isolates for most of the commonly used antibiotics in the poultry industry. Most of the isolates exhibited the presence of antimicrobial resistant genes which were targeted in the present study and few isolates showing phenotypic resistance to a particular antibiotic did not show resistance gene(s) which may be due to other type of resistance gene(s) which have not been targeted in the present study.

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