RESEARCH ARTICLE

Determination of Antibiotic Susceptibility of Avian Pathogenic *Escherichia coli* by Phenotypic and Genotypic Methods

Bhargav B. Limbachiya, Rafiyuddin A. Mathakiya, Krishna J. Patel*, Harshrajsinh B. Joddha

Abstract

Escherichia coli infection is one of the most important infections, which causes heavy economic losses in the poultry industry. It is caused by avian pathogenic *E.coli* (APEC), which belongs to the group Extra-intestinal Pathogenic *E. coli* (ExPEC). In this study, a total of 60 APEC isolates from various poultry farms located in and around Anand were evaluated for antibiotics susceptibility by disc diffusion (phenotypic) method for 21 different antibiotics. The *E. coli* isolates showed 100% resistance against pefloxacin, moxifloxacin and tetracycline followed by ampicillin (86.67%), levofloxacin (81.67%), amoxiclav and ciprofloxacin (71.67% each), co-trimoxazole (56.67%), sulphadiazine (53.34%) and erythromycin (33.33%). The study also revealed that *E. coli* isolates were highly susceptible to colistin (100.00%), followed by ceftriaxone and spectinomycin (85.00% each), cefixime (81.67%), amikacin (80.00%) and gentamicin (76.67%). All these isolates were also screened for the presence of 20 different antibiotic-resistant genes (ARGs) by genotypic method, *i.e.*, polymerase chain reaction. PCR revealed presence of *cmlA* gene responsible for chloramphenicol resistance in cent percent isolates. The distribution of other ARGs in the *E. coli* isolates were *qnrS* (85.00%), *aac(3)-IV* (56.66%), *strB* (53.33%), *aadA1* (51.66%), *dhfrI* (50.00%), *tetB* (41.66%) *sulI* and *tetA* (33.33% each), bla_{OXA} (31.66%), *cat1* and bla_{CMY} (21.66% each), *strA* (6.67%), bla_{SHV} (3.33%) and *dhfrV* (1.66%), while all the isolates were negative for $bla_{TEM,}$ *ere(A), qnrA, qnrB* and *mcr-1* genes.

Keywords: Antibiotic-resistant genes (ARGs), Avian Pathogenic *E. coli,* Genotypic method, Phenotypic method, Polymerase Chain Reaction, Poultry industry.

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Introduction

Escherichia coli is one of the most important pathogen causing secondary bacterial infection in poultry. *E. coli* can be divided into two main groups: commensals and pathogenic. The commensals are located in the intestinal tract of both humans and animals. At the same time, the pathogenic group is divided into two other subgroups, known as Extra-intestinal Pathogenic *E. coli* (ExPEC) and Diarrheagenic *E. coli* (DEC), which has been implicated in gastrointestinal diseases (Saif, 2003).

Avian Pathogenic *E. coli* (APEC), the etiological agent of extra-intestinal infections in birds, is a pathotype that belongs to the ExPEC group. APEC infections also lead to reduced yield, quality and hatching of eggs (Kemmett *et al*., 2014). Zoonotic transmission must be considered since poultry serves as the main host for APEC. The consumption of undercooked poultry may infect humans, which can serve as a reservoir of this pathotype (Markland *et al*., 2015). The present study was aimed to determine antibiotic susceptibility patterns and detection of ARGs in *E. coli* isolates.

Materials and Methods

Revival of Avian Pathogenic *E. coli* Isolates

For the present study, preserved isolates were used. Sixty APEC isolates obtained from various poultry farms located in and around Anand were used for the study. These isolates were initially stored in glycerol at -40°C. They were inoculated on MacConkey agar for the revival and incubated overnight at 37°C. From each plate of MacConkey agar, isolated lactose fermenting colony was inoculated on Eosin methylene blue (EMB) agar and incubated overnight at 37°C for preliminary identification. The colonies on EMB agar showing greenish metallic sheen were presumptively considered *E. coli*. The pure cultures of *E. coli* isolates were stored in Brain heart infusion agar (BHI) slants for further study.

Antibiotic Susceptibility Testing (AST) of *E. coli* Isolates

All 60 isolates of *E. coli* were subjected for determination of AST by disc diffusion method, *i.e.,* phenotypic method

Department of Veterinary Microbiology, College of Veterinary Science & Animal Husbandry, Kamdhenu University, Anand-388 001, Gujarat, India

Corresponding Author: Krishna J. Patel, Department of Veterinary Microbiology, College of Veterinary Science & Animal Husbandry, Kamdhenu University, Anand-388 001, Gujarat, India, e-mail: kj-patelvirpur@gmail.com

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Table 1: Primer sequence used for the detection of *E. coli*

Target Genes	*Name of Primers*	*Sequences (5'→3')*	*Expected Product size (bp)*	*References*
phoA	F	CGATTCTGGAAATGGCAAAAG	720	Hu *et al.* (2011)
	R	CGTGATCAGCGGTGACTATGAC		

for 21 different antibiotics, *viz.*, amikacin (AK, 30 µg), amoxyclav (AMC, 30 µg), ampicillin (AMP, 10 µg), cefixime (CFM, 5 µg), cefoparazone (CPZ, 75 µg), cefotaxime (CTX, 30 µg), ceftriaxone (CTR, 30 µg), cephalothin (CEP, 30 µg), chloramphenicol (C, 30 µg), ciprofloxacin (CIP, 5 µg), colistin (CL, 10 µg), co-trimoxazole (COT, 25 µg), erythromycin (E, 15 µg), gentamicin (GEN, 10 µg), levofloxacin (LE, 5 µg), moxifloxacin (MO, 5 µg), pefloxacin (PF, 5 µg), spectinomycin (SPT, 100 µg), streptomycin (S, 10 µg), sulphadiazine (SZ, 300 µg) and tetracycline (TE, 30 µg). The culture of *E. coli* was inoculated in BHI broth and incubated at 37°C for usually 4-6 h. The turbidity of broth culture was adjusted to 0.5 McFarland standard (~1.5 X 10^8 cfu/mL). Optimally, within 15 min after adjusting the turbidity of the inoculum suspension, a sterile swab dipped into the adjusted bacterial suspension was used to prepare lawn culture on Mueller Hinton Agar (MHA). Then, antibiotic discs were impregnated on an agar surface, plates were incubated at 37°C overnight, and the diameter of the zone of inhibition was measured on the next day (Fig. 1). The zone of inhibition was compared with the zone size interpretative chart supplied by the manufacturer.

Extraction of DNA and Isolation of Plasmid from *E. coli* Isolates

The suspension of organisms was made in 100 µL of Milli-Q water by picking up a typical colony in a 200 µL PCR tube. The suspension was heated at 95°C for 15 min and all cell debris were removed by centrifugation at 7,000 × g for 1 µL and 3 µL of the supernatant was used as a template DNA.

All the 60 Avian Pathogenic *E. coli* isolates were used for plasmid isolation. Isolates were grown overnight at 37°C in 3 to 5 mL Luria broth. Plasmid DNA was extracted by using Qiagen Plasmid Mini Kit, QIAGEN, Germany, according to the manufacturer's instructions. The presence of plasmid DNA was detected by agarose gel electrophoresis.

Molecular Detection of *E. coli* Isolates

To detect *E. coli*, extracted DNAs from isolates were subjected to PCR amplification of *E. coli* specific *phoA* gene. The PCR amplification of *phoA* gene was carried out using primers as in Table 1. The steps and thermal cycling condition was Initial denaturation (94°C for 5 min), denaturation (94°C for 45 s), annealing (56°C for 45 s), extension (72°C for 60 s), cycle was repeated for 35 times, and final extension (72°C for 8 min). The amplified PCR products were analyzed by agarose gel electrophoresis on a 2% agarose gel stained with ethidium bromide (EtBr) and visualized under UV light in gel documentation system.

Table 2: Quantity and concentration of various components used in PCR

Components	*Volume*	*Concentration*
2 X PCR Master Mix	12.50 µL	2X
Forward Primer (10 pmol/µl)	1.0 µL	10 pmole
Reverse Primer (10 pmol/µl)	1.0 µL	10 pmole
Template DNA	3.0 µL	-
Nuclease Free Water	7.50 µL	-
Total	25.0 µL	-

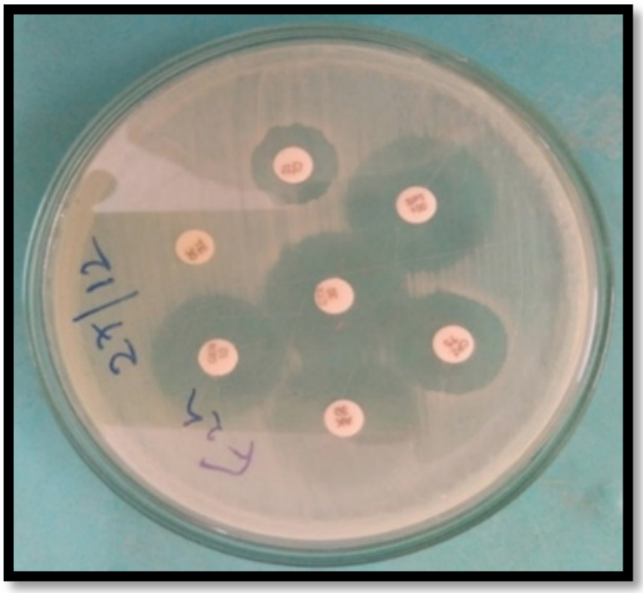

Fig. 1: Petri-dish showing antibiotic susceptibility pattern of *E. coli* by disc diffusion method (phenotypic method)

Detection of Antibiotic Resistance Genes by Polymerase Chain Reaction:

Detection of antibiotic resistance genes responsible for resistance to tetracycline, sulfonamide, chloramphenicol, trimethoprim, erythromycin, colistin, quinolones, aminoglycosides and β-lactamases was done by PCR in total 25 µL reaction mixture (Table 2). Total 60 *E. coli* isolates were screened for the presence of 20 different ARGs, *viz.*, *tetA, tetB, sull, cat1, cmlA, dhfrI, dhfrV, ere(A), mcr-1, qnrA, qnrB, qnrS, aac(3)-IV, aadA1, strA, strB,* bla_{OXA}, bla_{SHV}, bla_{TEM}, and bla_{CMY}. All genes PCR amplification was carried out using primers as detailed in Table 3. and PCR conditions used for the reaction are given in Table 4.

Results and Discussion

Identification of *E. coli* Isolates:

In the following revival of APEC isolates stored in glycerol on MacConkey agar by incubation, the nature of growth and

Table 3: Primer sequence used for the detection of antibiotic resistance *E. coli* isolates

Target Genes	Name of Primers	Sequences (5'→3')	Expected product size (bp)
sulI	F	TTCGGCATTCTGAATCTCAC	822
	R	ATGATCTAACCCTCGGTCTC	
tetA	F	GTGAAACCCAACATACCCC	887
	R	GAAGGCAAGCAGGATGTAG	
tetB	F	CCTTATCATGCCAGTCTTGC	773
	R	ACTGCCGTTTTTTCGCC	
bla_{OXA}	F	GCAGCGCCAGTGCATCAAC	710
	R	CCGCATCAAATGCCATAAGTG	
bla_{SHV}	F	TCGCCTGTGTATTATCTCCC	768
	R	CGCAGATAAATCACCACAATG	
bla_{TEM}	F	GAGTATTCAACATTTTCGT	698
	R	ACCAATGCTTAATCAGTGA	
dhfrV	F	CTGCAAAAGCGAAAAACGG	432
	R	AGCAATAGTTAATGTTTGAGCTAAAG	
dhfrI	F	AAGAATGGAGTTATCGGGAATG	391
	R	GGGTAAAAACTGGCCTAAAATTG	
cat1	F	AGTTGCTCAATGTACCTATAACC	551
	R	TTGTAATTCATTAAGCATTCTGCC	
cmlA	F	CCGCCACGGTGTTGTTGTTATC	699
	R	CACCTTGCCTGCCCATCATTAG	
aadA1	F	TATCCAGCTAAGCGCGAACT	490
	R	ATTTGCCGACTACCTTGGTC	
aac(3)-IV	F	CTTCAGGATGGCAAGTTGGT	286
	R	TCATCTCGTTCTCCGCTCAT	
ere(A)	F	GCCGGTGCTCATGAACTTGAG	419
	R	CGACTCTATTCGATCAGAGGC	
bla_{CMY}	F	TGGCCAGAACTGACAGGCAAA	462
	R	TTTCTCCTGAACGTGGCTGGC	
qnrA	F	ATTTCTCACGCCAGGATTTG	516
	R	GATCGGCAAAGGTTAGGTCA	
qnrB	F	GATCGTGAAAGCCAGAAAGG	469
	R	ACGATGCCTGGTAGTTGTCC	
qnrS	F	ACGACATTCGTCAACTGCAA	417
	R	TAAATTGGCACCCTGTAGGC	
strA	F	TTGAATCGAACTAATA	806
	R	TCAACCCCAAGTCAGAGG	
strB	F	ATGTTCATGCCGCCTGTTTTT	837
	R	CTAGTATGACGTCTGTCGC	
mcr-1	F	CGGTCAGTCCGTTTGTTC	309
	R	CTTGGTCGGTCTGTAGGG	

cultural characters of colonies were studied. Morphological identification was made based on Gram staining, and further

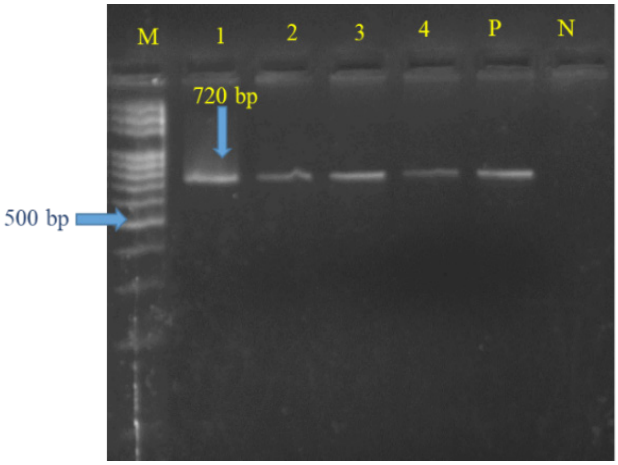

Fig. 2: Agarose gel showing amplified product for *phoA* gene of *E. coli* isolates (approx. 720 bp)

isolates were conformed culturally using EMB. All the isolates produced pink color colonies on MacConkey agar and produced a greenish metallic sheen on EMB agar. Molecular identification was done based on detection of *E. coli* specific *phoA* gene (Fig. 2). PCR further characterized the identified isolates for their detection of antibiotic resistance genes.

Antibiotic Sensitivity of *E. coli* Isolates

In vitro antibiotic sensitivity pattern of all the 60 *E. coli* isolates against 21 different antibiotics revealed that *E. coli* isolates had 100% resistance against pefloxacin, moxifloxacin, and tetracycline followed by ampicillin (86.67%), levofloxacin (81.67%), amoxiclav and ciprofloxacin (71.67% each), co-trimoxazole (56.67%), sulphadiazine (53.34%) and erythromycin (33.33%). The study also revealed that *E. coli* isolates were highly susceptible to colistin (100%), followed by ceftriaxone and spectinomycin (85% each), cefixime (81.67%), amikacin (80%), gentamicin (76.67%), cefotaxime (71.67%), chloramphenicol (70%), streptomycin (61.66%), cefoperazone (58.34%) and cephalothin (33.33%).

In the present study, all the *E. coli* isolates were 100% resistant to moxifloxacin, pefloxacin, and tetracycline. Mohamed *et al.* (2014) also found 100 % resistance of *E. coli* isolates to pefloxacin. In contrast, Sharada *et al.* (2008) reported only 23.08% resistance to pefloxacin. Touzain *et al.* (2018) and Awad *et al.* (2020) reported 100% resistance against tetracycline. Various authors (Sharada *et al.*, 2008; Soufi *et al.*, 2009; Wang *et al.*, 2013; Awad *et al.*, 2016; Abbassi *et al.*, 2017) reported variable resistance to tetracycline from 74.20 % to 98.00 %, while Gregova *et al.* (2012) observed 33.00% resistance.

All the isolates (100.00%) in our study were sensitive to colistin. Soufi *et al.* (2009) and Bakhshi *et al.* (2017) reported 96.00% and 100% sensitivity, respectively, which supports the present study. In contrast, Mohamed *et al.* (2014) observed 44.00% sensitivity, and Awad *et al.* (2020) observed 7.69 % sensitivity of *E. coli* to colistin.

Table 4: Steps and conditions of thermal cycling used for different antibiotic-resistant genes in PCR

	Cycling conditions			
Gene	*Initial denaturation*	*Denaturation*	*Annealing*	*Extension*
sulI, tetB, tetA	94°C, 5 min	94°C, 30 s	50°C, 30 s	72°C, 1 min
bla_{OXA}, bla_{TEM}, dhfrI, bla_{SHV}, dhfrV, cat1, cml A	95°C, 15 min	94°C, 30 s	58°C, 30 s	72°C, 1 min
aadA1, aac(3)-IV, ere(A), bla_{CMY}	95°C, 3 min	94°C, 30 s	55°C, 90 s	72°C, 1 min
qnrA, qnrB, qnrS, strA, strB	95°C, 15 min	94°C, 45 s	55°C, 45 s	72°C, 1 min
mcr-1	94°C, 15 min	94°C, 30 s	58°C, 90 s	72°C, 1 min
		Each cycle was repeated 35 times; Final extension at 72°C for 10 min		

In the present study, multiple drug resistance was seen in all isolates. All isolates showed resistance to at least 3 antibiotics, and maximum resistance was seen against 14 antibiotic drugs. The source of the resistance may be assumed from the poultry feeds consumed since antibiotics are used as feed additives to improve feed efficiency and weight gain. Many antibiotics are also used in feed and water to control the diseases. Indiscriminate use of antibiotics has provided selective pressure for the emergence of drug resistance, resulting in a larger proportion of *E. coli* resistance (Atere *et al.*, 2015).

Detection of Antibiotic Resistant Genes by PCR

A total of 60 *E. coli* isolates were screened for the presence of 20 different ARGs, *viz.*, *tetA, tetB, sulI, cat1, cmlA, dhfrI, dhfrV, ere(A), mcr-1, qnrA, qnrB, qnrS, aac(3)-IV, aadA1, strA, strB, bla_{OXA}, bla_{SHV}, bla_{TEM},* and *bla_{CMY}*.

Gene responsible for tetracycline resistance was targeted using gene-specific primer sets *tetA* and *tetB* (Fig. 3). 20 (33.33%) and 25 (41.66%) isolates possessed *tetA* and *tetB*, respectively. Gene responsible for sulfonamide resistance was targeted using gene-specific primer *sulI*. Twenty isolates (33.33%) harbored *sulI* gene.

Gene responsible for chloramphenicol resistance was targeted using gene-specific primer set *cat1* and *cmlA*. 13 (21.66%), and 60 (100%) isolates possessed *cat1* and *cmlA* (Fig. 4) gene, respectively, while the gene responsible for trimethoprim resistance targeted using gene-specific primer set *dhfrI* and *dhfrV* (Fig. 5), revealed 30 (50.00%) and 1 (1.66%) isolates harboring *dhfrI* and *dhfrV* gene, respectively.

Genes responsible for erythromycin resistance and colistin resistance were targeted using gene-specific primer set *ere(A)* and *mcr-1*, respectively. Still, none of the isolates could be found positive for both genes. Gene responsible for quinolone resistance was targeted using gene-specific primer set *qnrA, qnrB,* and *qnrS*. All the isolates were found negative for the quinolone resistance genes *qnrA* and *qnrB*. Only quinolone resistance gene *qnrS* (Fig. 6) was found in 51 (85.00%) isolates.

Gene responsible for aminoglycoside resistance was targeted using gene-specific primer set *aadA1* and *aac(3)-IV*. 31 (51.66%) and 34 (56.66%) isolates possessed *aadA1* and *aac(3)-IV* gene, respectively. Gene responsible for streptomycin resistance was targeted using gene-specific primer set *strA* and *strB*. 4 (6.67%) and 32 (53.33%) isolates harbored *strA* and *strB* gene, respectively. Further, the gene

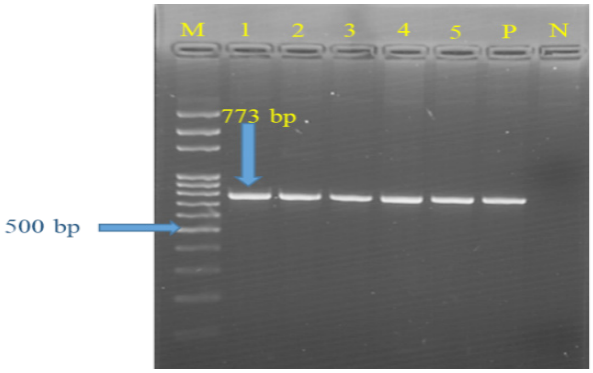

Fig. 3: Agarose gel showing amplified product for *tetB* gene of *E. coli* isolates (approx. 773bp)

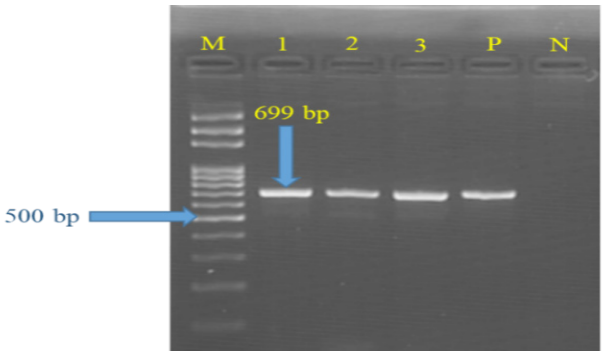

Fig. 4: Agarose gel showing amplified product for *cmlA* gene of *E. coli* isolates (approx. 699bp)

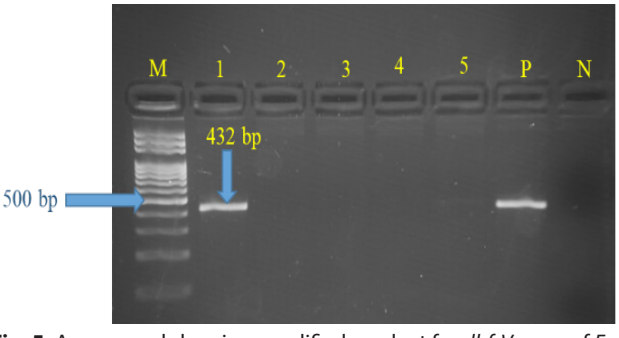

Fig. 5: Agarose gel showing amplified product for *dhfrV* gene of *E. coli* isolates (approx. 432bp)z

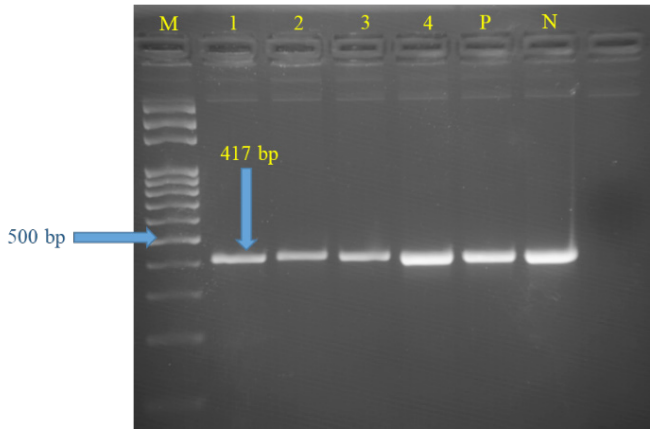

Fig. 6: Agarose gel showing amplified product for *qnrS* gene of *E. coli* isolates (approx. 417bp)

responsible for β-lactamase resistance was targeted using gene-specific primer set bla_{OXA}, bla_{TEM}, bla_{SHV}, and bla_{CMY}.19 (31.66%), 2 (3.33%) and 13 (21.66%) isolates were found positive for bla_{OXA}, bla_{SHV} and bla_{CMY}, respectively. None of the isolates could be found positive for gene bla_{TEM}.

The finding of present study for *catA1* and *cmlA* genes concurred with the observations of Momtaz *et al.* (2012), who detected *catA1* and *cmlA* 36.84% each, Wang *et al.* (2013) found *cat1* and *cmlA* 31.79% and 23.46%, while Shehata *et al.* (2016) observed *cat1* and *cmlA* 0% and 40%, respectively.

The present result of *qnrA* was in agreement with Samanta *et al.* (2013), Xie *et al.* (2014), and Li *et al.* (2015). They also found all isolates negative for *qnrA* gene. Ahmed *et al.* (2013) and Awad *et al.* (2016) found a lower prevalence of this gene at the rate of 8.62 to 9.58%, while Momtaz *et al.* (2012) and Ponce-Rivas *et al.* (2012) detected a high prevalence of this gene upto 36.84% and 52.63%, respectively. Like our findings, Ponce-Rivas *et al.* (2012) and Li *et al.* (2015) found all *E. coli* isolates negative for *qnrB* gene, while Xie *et al.* (2014) and Awad *et al.* (2016) detected a lower prevalence of this gene at the rate of 0.90 and 3.44%.

The absence of bla_{TEM} gene agrees with Samanta *et al.* (2013) and Shehata *et al.* (2016) as they detected all negative isolates for this gene. In contrast Parvez *et al.* (2016) detected all isolates positive for this gene. Ahmed *et al.* (2013), Li *et al.* (2015), and Awad *et al.* (2016) also found this gene ranging from 16.10 to 85.00% in their studies. Barbieri *et al.* (2017) found a lower prevalence of *mcr-1* gene (1.22%). They also found all isolates phenotypically resistant to colistin.

Conclusions

The present study involving 60 Avian Pathogenic *E. coli* (APEC) isolates from various poultry farms revealed that *E. coli* isolates were 100.00% resistant to pefloxacin, moxifloxacin, and tetracycline, followed by ampicillin (86.67%), levofloxacin (81.67%), amoxiclav and ciprofloxacin (71.67% each), co-trimoxazole (56.67%) and sulphadiazine (53.34%). In contrast, the isolates were 100.00% sensitive to colistin followed by ceftriaxone and spectinomycin (85.00%), cefixime (81.67%), amikacin (80.00%), gentamicin (76.67%), cefotaxime (71.67%), and chloramphenicol (70.00%). Phenotypically all the isolates were found to be multidrug resistant as they showed resistance to at least three antibiotics and maximum resistance to fourteen antibiotics. The antibiotic-resistant genes, *viz*, *cmlA*, *qnrS*, *aac(3)-IV*, *strB*, *aadA1*, and *dhfrI* were distributed in more than 50% of the isolates. The ARGs viz. bla_{TEM}, *ere*(*A*), *qnrA*, *qnrB*, and *mcr-1* were absent among all the isolates.

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