Indian Journal of Animal Sciences **93** (10): 946–950, October 2023/Article
https://doi.org/10.56093/ijans.v93i10.111002

Prevalence of antimicrobial resistance genes among *Escherichia coli* isolated from poultry

ARPIT[1], M CHANDRA[1]✉, G KAUR[1], D NARANG[1] and A K ARORA[1]

Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab 141 004 India

Received: 3 March 2021; Accepted: 6 September 2023

ABSTRACT

Antimicrobial resistance has become a global threat. In the poultry industry, antibiotic usage has been widespread and been used for multiple purposes, viz. growth promoters, therapeutic agent and prophylaxis. This usage has probably led to accumulation of antimicrobial resistant genes. A study on presence of antibiotic resistant genes in poultry farms of Ferozepur and Ludhiana, Punjab were undertaken. A total of 50 faecal samples were collected from eight farms. The samples were processed for isolation of *E. coli* by using selective media, were identified using various biochemical tests and confirmed with the help of PCR. A total of 35 *E. coli* isolates were obtained and all were subjected to antibiotic sensitivity test against 10 antibiotics. Also, these isolates were subjected to amplification of antibiotic resistance genes, viz. *blaTEM*, *blaSHV*, *DHAM*, *MOXM*, *sul1*, *dhfrV*, *aadA*, *tetA* and *tetB* using published primers. The isolates revealed resistance to penicillin (100%), ampicillin/sulbactum (100%), erythromycin (94.28%), streptomycin (91.4%), tetracycline (60%), chloramphenicol (60%), trimethoprim (51.4%), co-trimoxazole (48.57%), gentamicin (8.5%) and colistin (8.5%). Seven isolates were found to be positive for *blaTEM*, nine for *sulI*, four for *dhfrV*, 11 for *aadA* and *cmlA*, respectively, while none of the isolate showed the *blaSHV, DHAM, MOXM, tetA* and *tetB.* The present study revealed that the multiple AMR genes may be prevalent among *E. coli* isolates of poultry origin which needs urgent attention.

Keywords: Antibiotic resistance, Antibiotic resistance genes, *E. coli*, Poultry, Prevalence

Antimicrobial resistance (AMR) is a global problem. As per one estimate, drug resistant infections are likely to cause 10 million deaths annually by the year 2050 (O'Neill 2016). Antibiotics have been used widely in poultry production as growth promoters, prophylactic and therapeutic agents that could lead to development of antimicrobial resistance (Plata *et al.* 2022). Antimicrobial resistant pathogens results in treatment failure which leads to high economic loss. Antimicrobial resistant bacteria also pose a risk to human health (Nhung *et al.* 2017).

In poultry, *E. coli* plays a pivotal role in maintaining homeostasis of intestinal morphology. Though, most strains of *E. coli* are commensal, some strains are pathogenic too. Both virulent and avirulent *E. coli* have been shown to colonize and persist efficiently in the intestinal tract of poultry. But, whenever, there is imbalance in the presence of stressors (Dominick and Jensen 1984), extra intestinal movement of *E. coli* occurs, leading to colibacillosis. Avian strains of *E. coli* also raise public health concern as these strains share serotypes as well as virulence factors with human pathogens (Dhamal *et al*. 2013). The faecal *E. coli* from poultry that harbour antibiotic resistant genes could transmit to the humans and lead to accumulation of resistant genes. Further, these bacteria could transfer the AMR genes to the normal endogenous bacterial population too (Bogaard *et al.* 2001). In view of this, the present study was aimed to know the presence of antibiotic resistance and associated genes in the *E. coli* of poultry origin.

Present address: [1]Department of Veterinary Microbiology, College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab. ✉Corresponding author email: drmuditchandra@rediffmail.com

MATERIALS AND METHODS

Collection of samples: Poultry faecal samples (n=50) were collected from two poultry farms of Ferozepur and six poultry farms of Ludhiana from August 2019-February 2020. The samples with cold chain were transported to the Department of Veterinary Microbiology, College of Veterinary Science, GADVASU, Ludhiana, Punjab for further processing.

Isolation and identification: All the faecal samples were subjected to the isolation of *E. coli* following the standard protocol (Cruickshank *et al.* 1975). About 10 g of each faecal material was inoculated into 100 ml of buffered peptone water and incubated at 37°C for 12-24 h. A loopful of broth culture was streaked onto MacConkey's Lactose Agar (MLA) and Eosine Methylene Blue Agar (EMB) and incubated at 37°C for 12-24 h. After incubation the pink colonies on MLA and colonies with metallic sheen on EMB were confirmed by various biochemical tests, viz. oxidase,

catalase, indole, methyl red, voges proskauer's, citrate and triple sugar iron. After the biochemical confirmation, isolates of *E. coli* were sub-cultured on nutrient agar slants and stored at 4°C.

Antibiotic sensitivity test: Antimicrobial susceptibility testing was performed as per the method of Bauer *et al.* (1965). Overnight grown culture of individual bacteria in LB broth was uniformly spread onto Muller Hinton Agar with the help of a sterilized cotton swab. Antibiotic discs, viz. penicillin (1IU), ampicillin/sulbactum (10/10 mcg), tetracycline (30 mcg), gentamicin (10 mcg), chloramphenicol (30 mcg), erythromycin (15 mcg), streptomycin (10 mcg), co-trimoxazole (25 mcg), colistin (10 mcg) and trimethoprim (5 mcg) were placed at an appropriate distance and incubated for 16-24 h. The zone of inhibition was measured in mm with the help of a ruler and the isolates were classified as sensitive/resistant as per the guidelines of CLSI (2018).

Extraction of DNA from bacteria: The DNA from all the *E. coli* isolates was extracted using NucleoSpin® Microbial DNA kit as per manufacturer's instructions and stored at -20°C till further use. The optical density (OD) at 260 nm and 280 nm of the individual sample was measured by using a Nanodrop (Thermoscientific, USA) and a ration of 1.6-1.8 was considered satisfactory.

Polymerase chain reaction for confirmation of E. coli: All the *E. coli* isolates were subjected to identification by amplification of the specific gene using polymerase chain reaction (PCR) as per Riffon *et al.* (2001) (Table 1). The reaction mixture was prepared by adding 12.5 µl (2×one Taq Master Mix) (New England Bio Labs, USA), 1 µl of 20 pmol/ul of each forward and reverse primers (Cusabio, China), 1 µl of template DNA (100 ng/µl) and the reaction was made up to 25 µl using Nuclease free water. PCR was performed on a thermocycler (Veriti, ABI, USA) with the following conditions: an initial denaturation at 94°C for 5 min and 35 cycles each of denaturation at 94°C for 45 s, annealing at 60°C for 1 min and extension at 72°C for 1 min followed by a final extension at 72°C for 10 min. PCR reaction products were run on agarose gel electrophoresis (1.5%) at 80 V for an hour. Following electrophoresis, DNA bands were visualized under UV trans-illuminator and the images were captured using Gel Documentation System (AlphaImager, Alpha Innotech).

Polymerase chain reaction for antibiotic resistance: All the *E. coli* isolates were subjected to PCR to identify the presence of *blaTEM*, *blaSHV*, *sull*, *dhfrV*, *cmlA*, *aadA*, *DHAM*, *MOXM*, *tetA* and *tetB* antibiotic resistance genes using published primers (Table 1). The reaction mixture was prepared by adding 12.5 µl (2× one Taq Master Mix) (New England Bio Labs, USA), 1 µl each of 20 pmol/ul forward and reverse primers for each of the antibiotic resistant gene (Metabion International AG, Germany), 1 µl of template DNA (100 ng/µl) and 9.5 µl of nuclease free water. The PCR conditions for amplification of *blaTEM, blaSHV, dhfrV, Sul1* genes were: an initial denaturation at 94°C for 5 min followed by 30 cycles of 94°C for 30 sec, annealing at 50°C for 30 s and extension at 72°C for 1.5 min and final extension at 72°C for 10 min.

The PCR conditions for amplification of *tetA, tetB, DHAM, MOXM, cmlA, aadA* genes were: an initial denaturation at 95°C for 5 min followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, extension at 72°C for 1 min and final extension at 72°C for

Table 1. Primers used for *E. coli* and antibiotic resistance genes

Organism/Antibiotics	Genes/Target	Primers 5′-3′	Product length	References
E. coli	23 S rRNA	F: ATCAACCGAGATTCCCCCA R: TCACTATCGGTCAGTCAGGAG	232 bp	Riffon *et al.* (2001)
Beta lactam	*blaTEM*	F: GAGTATTCAACATTTTCGT R: ACCAATGCTTAATCAGTGA	857 bp	Maynard *et al.* (2004)
	blaSHV	F: TCGCCTGTGTATTATCTCCC R:CGCAGATAAATCACCACAATG	768 bp	Maynard *et al.* (2004)
Tetracycline	*tetA*	F : CGATCT TCCAAG CGTTTG TT R:CCAGAAGAA CGAAGCCAG TC	105 bp	Faldynova *et al.* (2013)
	tetB	F:TACAGGGATTATTGGTGAGC R:ACATGAAGGTCATCGATAGC	162 bp	Faldynova *et al*. (2013)
Sulphonamide	*sulI*	F:TTCGGCATTCTGAATCTCAC R:ATGATCTAACCCTCGGTCTC	822 bp	Shehata *et al*. (2016)
Trimethoprim	*dhfrV*	F :CTGCAAAAGCGAAAAACGG R:AGCAATAGTTAATGTTTGAGCTAAAG	432 bp	Maynard *et al.* (2004)
Streptomycin	*aadA*	F:TGATTTGCTGGTTACGGTGAC R:CGCTATGTTCTCTTGCTTTTG	284 bp	Van *et al*. (2008)
Ampicillin	*MOXM*	F:GCTGCTCAAGGAGCACAGGAT R:CACATTGACATAGGTGTGGTGC	520 bp	Van *et al.* (2008)
	DHAM	F:AACTTTCACAGGTGTGCTGGGT R:CCGTACGCATACTGGCTTTGC	405 bp	Van *et al*. (2008)
Chloramphenicol	*cmlA*	F:CCGCCACGGTGTTGTTGTTATC R:CACCTTGCCTGCCCATCATTAG	698 bp	Van *et al.* (2008)

10 min.

The PCR products were subjected to agarose gel electrophoresis (1.5%) at 80 V for an hour. Following electrophoresis, DNA bands were visualized under UV trans-illuminator and the images were captured by using Gel Documentation System (AlphaImager, Alpha, Innotech).

RESULTS AND DISCUSSION

Isolation and identification of E. coli species: A total of 50 faecal samples collected from two poultry farms of Ferozepur and six poultry farms of Ludhiana were processed for the isolation of *E. coli*. Out of a total of 50 faecal samples, 35 faecal samples (70%) were positive for *E. coli* which is in agreement with the study conducted by Wani *et al.* (2004), Rahman *et al*. (2008), Wesonga *et al.* (2010) and Bandyopadhyay *et al.* (2011) but in contrast to a study conducted by Muglikar *et al.* (2019) in which recovery rate of *E. coli* was 33.77% only. In poultry, *E. coli* resides in the lower digestive tract and it colonizes in the first 24 h after hatching (Ballou *et al.* 2016) which may be the possible reason behind high recovery rate of *E. coli* in the present study. Another possible reason behind high recovery of *E. coli* is that the samples were collected from the faeces of healthy birds while the others collected tissue samples from birds suspected for colibacillosis.

Antibiotic sensitivity testing: The bacterial isolates revealed resistance to penicillin (100%), ampicillin/ sulbactum (100%), erythromycin (94.28%), streptomycin (91.4%), tetracycline (60%), chloramphenicol (60%), trimethoprim (51.4%), co-trimoxazole (48.57%), gentamicin (8.5%) and colistin (8.5%).

The present study showed the highest rate of resistance among *E. coli* isolates to penicillin, methicillin, ampicillin /sulbactum, erythromycin and streptomycin which was observed to be consistent with the findings of Shehata *et al*. (2016) and Muglikar *et al.* (2019). The bacterial isolates showed a high degree of susceptibility to gentamicin, and colistin as reported by Joshi *et al.* (2012), Magray *et al.* (2018) and Kaushik *et al.* (2018).

PCR for identification of genus and antibiotic resistant genes: PCR amplification using gene specific primers produced the expected band of 232 bp indicative of *E. coli*. Out of 35 isolates resistant for penicillin, seven (20%) isolates were found to harbour the *blaTEM* gene (Fig. 1), while none of the isolate showed the *blaSHV* gene. The above finding was similar to the findings of various workers (Obeng *et al.* 2012, Dhawde *et al.* 2018 and Hussain *et al.* 2019). Ibrahim *et al.* (2019) in a study observed that avian pathogenic *Escherichia coli* isolates possessed *blaTEM* gene and *bla*SHV genes with a prevalence of 72.9% and 1.8%, respectively, which were in agreement with the findings of the present study, however, they observed a very high prevalence of *bla*TEM gene. In this study none of the isolates showed *DHAM* and *MOXM* genes which was similar to the findings of Farrokhnazar *et al.* (2016), Nhung *et al.* (2017) and Liu *et al.* (2019).

Resistance to tetracycline is mediated by efflux proteins, ribosomal protection, enzymatic inactivation and target modification (Chopra and Roberts 2001). Different types of *tet* genes found in gram negative bacteria may encode for efflux proteins or ribosomal protection or both. Out of 21 tetracycline resistant isolates subjected to amplification of *tetA* and *tetB* genes none showed either *tetA* and or *tetB*. Amplification of *tetA* and/or *tetB* gene is an inconsistent findings; Miles *et al.* (2006), Faldynova *et al.* (2013), Liu *et al.* (2019) reported very low prevalence or absence of this gene in the resistant isolates, while in a study conducted by Ibrahim *et al.* (2019) *tetA* gene was found to be most predominant gene among tetracycline resistant isolates. Lack of *tetA* and *tetB* gene among tetracycline resistant isolates might be due to the fact that in this study only two genes for tetracycline resistance were tested, while the phenotypic resistance might have been conferred to the isolates by other genes like *tetC*, *tetD*, *tetE*, *tetG*, *tetH*, *tetI*, *tetJ*, *tetY*, *tet30* and *tet31*, etc. (Grossman 2016). However, further studies are required to substantiate this finding.

Resistance to sulfonamides and trimethoprim is caused by non allelic and drug resistant variants of the chromosomal target enzymes di-hydropteroate synthase (*sul* genes) and dihydrofolate reductase (*dfr* genes), respectively (Sköld 2001). Out of 17 isolates resistant for sulphonamides, seven (41.17%) isolates harboured *sulI* gene (Fig. 2) which is in agreement with the studies performed by Momtaz *et al.* (2012), Faldynova *et al.* (2013) but in contrast to Shehata *et al.* (2016) in which all the tested isolates showed the presence of *sul1* gene.

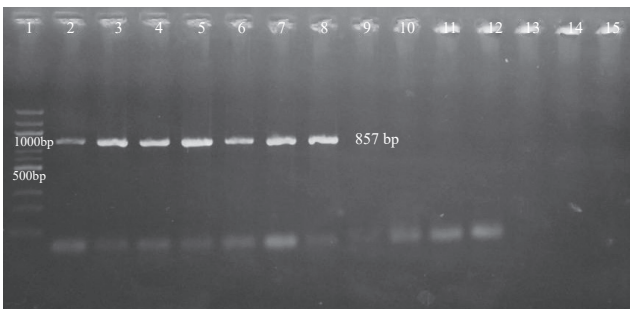

Fig. 1. Amplification of *blaTEM* resistance gene (Lane 1: 100 bp Ladder, Lane 2-8: Positive samples, Lane 9-15: negative samples).

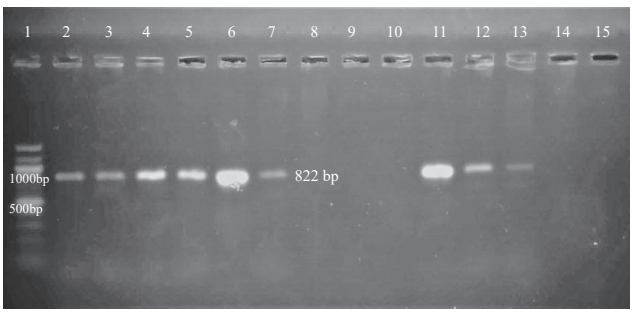

Fig. 2. Amplification of *sul1* gene (Lane 1: 100 bp Ladder, Lane 2-7: 11-13 positive samples, Lane 8-10: 14-15 negative samples).

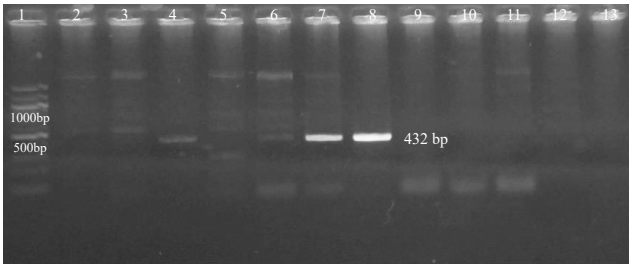

Fig. 3. Amplification of *dhfrV* resistance gene (Lane 1: Ladder, Lane 4, 6-8: positive samples, Lane 2-3, 5, 9-13: negative samples).

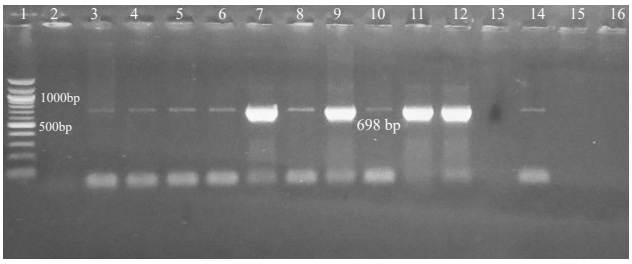

Fig. 4. Amplification of *cmlA* gene (Lane 1: 100bp Ladder, Lane 3-12: 14 positive samples, Lane 2, 13, 15, 16: negative samples).

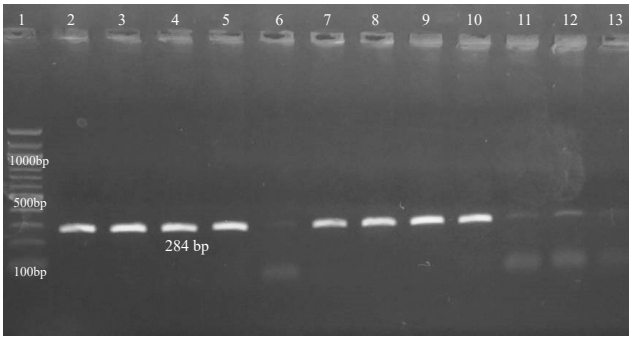

Fig. 5. Amplification of *aadA* gene (Lane 1: 100 bp Ladder, Lane 2-5: 7-12 positive samples, Lane 6: 13 negative samples).

Out of 18 isolates resistant for trimethoprim, four isolates (22%) were found to be positive for *dhfrV* gene (Fig. 3). These results agreed with the findings of Obeng *et al*. (2012), Shehata *et al*. (2016) but were in contrast with the findings of Dehdashti *et al*. (2019) who reported all the isolates negative for *dhfrI* and *dhfrV* genes. The reason for this variation might be the difference in isolation source/ host species, since this study used poultry isolates whereas they worked on buffaloes isolates.

All 21 isolates which showed the resistance to chloramphenicol were screened for *cmlA* gene and 11 (52.38%) isolates had *cmlA* gene (Fig. 4) which was in agreement with the findings of Momtaz *et al.* (2012), Shehata *et al.* (2016) and Poirel *et al.* (2018).

Out of 32 isolates resistant for streptomycin, 11 (34.37%) isolates harboured *aadA* gene (Fig. 5) which was in accordance with the findings of Obeng *et al.* (2012) and Younis *et al.* (2017).

The high percentage of the isolates exhibiting AMR phenotype compared to the specific AMR gene might be due to the presence of some other AMR genes that confer the similar AMR phenotype. Moreover, the target gene might be located on plasmids, while in this study the genomic DNA has been used for testing of the AMR genes among bacterial isolates.

ACKNOWLEDGEMENTS

The authors are grateful to the ICAR Niche area of Excellence on Antibiotic Resistance: Animal Human Interface for providing necessary consumables required during the research work. The authors are also thankful to the Director Research, GADVASU for providing the necessary laboratory facilities.

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