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ORIGINAL RESEARCH ARTICLE

# Antibiotic Resistance and Virulence Gene Patterns Associated with Multi Drug Resistant Avian Pathogenic *Escherichia coli* (APEC) Isolated from Broiler Chickens in India

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**Abstract** In the present study, a total of 102 samples were collected from chickens of different flocks, died due to suspected colibacillosis. Bacteriological and PCR methods were applied to detect avian pathogenic *Escherichia coli* (APEC). Phenotypic antimicrobial resistance (AMR) was determined by disk diffusion method. Extended spectrum beta lactamases (ESBL) detection was carried out via PCR by targeting *bla*TEM, *bla*SHV, *blaOXA,* and *bla*CTX-M groups 1, 2, and 9. Genes of eight virulence factors and class I integrons were also detected by PCR using gene specific primers. Culture, microscopic, biochemical tests and PCR recognised 69/102 (67.64%) samples as *E. coli*. Phenotypic AST revealed higher resistance against fluoroquinolone antibiotics, *i.e*., enrofloxacin (72.46%), levofloxacin (69.56%) & ciprofloxacin (66.66%), followed by amoxyclav (63.77%) and tetracycline (59.42%). Six isolates were found as pan-drug-resistant *E. coli*. A total of 48 (69.56%) and 7 (10.14%) isolates were positive for the presence of *bla*TEM and *bla*CTX-M-G9 genes, respectively, whereas 2 (2.90%) isolates each were found positive for *bla*SHV, *blaOXA,* and *bla*CTX-M-G1 genes. Among APEC associated virulence genes, *iss* (79.71%) was the most predominant, followed by *tsh* (50.72%), *ast* (30.43%), *cvaf* (26.08%), *pap* (23.18%), vat (8.69%) and *stx*-1 (1.44%). Thirty-two isolates harboured class I integrons, either with or without ESBL genes. Conclusively, the isolates under study showed pan and multiple-drug resistance, specifically against fluoroquinolone drugs. ESBL production was mediated principally through $bla_{\text{TEM}}$ and *bla*CTX-M-G9. Multiple virulence factors, toxins, and carriage & spread factor render these as zoonotically potential pathogens for humans.

**Keywords** Antimicrobial resistance (AMR) · Avian pathogenic *E. coli* (APEC) · Extended spectrum beta lactamases (ESBLs) · Fluoroquinolones resistance · Virulence genes

## Introduction

Pathogenic *Escherichia coli* (*E. coli*) produces intestinal and extraintestinal infections in the target hosts [1]. Among these, *E. coli* which causes extra intestinal infections in birds, especially in domestic poultry, is known as avian pathogenic *E. coli* (APEC). APEC can invade multiple organs, causing peritonitis, perihepatitis, air sacculitis, omphalitis, pericarditis etc. in chickens and the infection is collectively termed colibacillosis' [2]. These *E. coli* strains primarily colonise the respiratory and intestinal tracts of birds. The

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colonisation, tissue invasion, and systematic survival of APEC are determined by several virulence factors. Their genes are encoded by virulence-associated genes (VAGs), viz., *iut*A, *iss*, *pap*C, *iuc*D, *tsh*, *irp*-2, *omp*T, *hly*F, iron, *cva/cvi*, and *ast*A etc. [3]. All the genes are not found in a given strain, but, according to the genetic criteria, the pathogenicity of the APEC strain is determined by the presence of combination of virulence genes, which provides them with an advantage of systematic survival [4] over potentially pathogenic *E. coli* strains residing in the intestinal tract [5].

The principal mode of treatment for APEC infection is the administration of antimicrobials. Despite of government guidelines, antimicrobials are commonly used as feed supplements in the poultry industry. The overuse of antimicrobials is a major driving force for the augmentation of AMR [6].

The emergence of AMR is a natural phenomenon in microorganisms, shared by the use of common antimicrobial agents in both humans and animals. Among the several forms of AMR, extended spectrum β-lactamases (ESBLs) production is a major concern in Gram negative bacteria, specifically in *E. coli*. ESBL*s* are enzymes that mediate resistance to extended-spectrum (third generation) cephalosporins (e.g., ceftazidime, cefotaxime, and ceftriaxone) and monobactams (e.g., aztreonam) but do not affect cephamycins (e.g., cefoxitin and cefotetan) or carbapenems (e.g., meropenem or imipenem). There are more than 200 different types of ESBL enzymes, of which *TEM*, *SHV* and *CTX*-M types are more prevalent among *E. coli* [7, 8]. Ubiquitous presence and wide host range made this bacterial species a suitable candidate organism for the spread of antimicrobial resistance. The drug resistance profile can be transferred from poultry to humans and poses a potential risk to human health. Shared homology in virulence genes in APEC and other extraintestinal *E. coli* (ExIEC) poses risk of the development of severe human diseases such as haemorrhagic colitis and haemolytic uremic syndrome [9]. Both virulence genes and antibiotic resistance genes of these pathogens may be transmitted to humans through several genetic mechanisms, including mobile genetic elements class I integrons [10]. This may increase the severity of the disease and complicate the therapeutic strategy [11].

Keeping the importance of APEC in mind, the present study was carried out to see the AMR status of *E. coli* in chickens reared for consumption in Gujarat, India. In addition to that, molecular detection of antimicrobial resistance genes, virulence genes and class I integrons (as carriage and spreading factors) was also investigated as zoonotic spread potential.

## Material and Methods

### Ethical Approval

As the tissue samples were collected from postmortem of dead chickens, ethical approval for the work was not required.

### Study Location and Sample Collection

The present study was conducted in Banaskantha (24.085560° N, 72.144234° E) district, Gujarat, India, during 2019–21. Necropsy was conducted on the chickens, which died due to suspected *E. coli* infection. Clinical picture and necropsy findings, viz., exhibiting pericarditis, perihepatitis, egg peritonitis, omphalitis, tracheitis, intestinal

**Table 1** Details of sampling and culture and biochemical tests used for identification of *E. coli*

| S. No | Condition | No of Sample | LF colonies on MacConkey agar | Greenish Metallic Growth on EMB | biochemical test | | | | TSI (Y/Y/G/H2S-) |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | Indole (+) | MR (+) | VP(−) | Citrate (−) | |
| 1 | Perihepatitis | 29 | 26 | 23 | 23 | 23 | 23 | 23 | 23 |
| 2 | Pericarditis | 12 | 9 | 9 | 9 | 9 | 9 | 9 | 9 |
| 3 | Tracheitis | 8 | 4 | 3 | 3 | 3 | 3 | 3 | 3 |
| 4 | Gall Bladder Infection | 2 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 5 | Peritonitis | 13 | 10 | 9 | 9 | 9 | 9 | 9 | 9 |
| 6 | Intestinal Infection/hemorrhage | 13 | 13 | 11 | 11 | 11 | 11 | 11 | 11 |
| 7 | Spleen infection | 5 | 00 | 00 | 00 | 00 | 00 | 00 | 00 |
| 8 | Omphalitis | 7 | 4 | 3 | 3 | 3 | 3 | 3 | 3 |
| 9 | Air sacculitis | 10 | 7 | 7 | 7 | 7 | 7 | 7 | 7 |
| 10 | Diarrhea | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| | Total | 102 | 77 | 69 | 69 | 69 | 69 | 69 | 69 |

haemorrhage, etc. were taken into account. A total of 102 tissue samples were collected aseptically in MacConkey broth and transported to laboratory under standard conditions. The details of the samples are shown in Table 1.

### Isolation and Identification of Avian Pathogenic E. coli (APEC) Strains

Primary isolation and identification of *E. coli* were carried out as per Edwards & Ewing [12]. Briefly, samples were inoculated on MacConkey agar medium and incubated aerobically at 37 °C overnight. Lactose-fermenting colonies were purified and sub-cultured on Eosin methylene blue (EMB) agar to detect metallic sheen. Biochemical tests, viz., oxidase, catalase, oxidation-fermentation, indole test, methyl red test, Vogus Proskauer test, citrate utilisation test, and sugar utilisation pattern on TSI slants, were employed for the identification of *E. coli* [12] (Table 1). Genomic DNA was extracted from freshly grown cultures by the boiling method. In brief, 3 to 5 bacterial colonies were picked up and suspended in 200 µl of deionized water, followed by boiling at 95 °C for 15 min in a thermal cycler and centrifugation at 4000 rpm for 5 min. The supernatant was then used as the DNA template for further molecular characterization**.** The isolates were confirmed by amplification of the *E. coli* specific genomic region of 16srRNA using primers described by Lee et al. [13] (Supplementary Table 1).

### Phenotypic Antibiotic Susceptibility Testing

Mueller–Hinton agar (Hi Media, India) was used to perform antibiotic susceptibility test (AST) of *E. coli* isolates. A total of 12 different types of antibiotic discs (Hi Media, India) (Table 2), which represent different antibiotic classes and are commonly used in the poultry sector, were tested following the standard Kirby-Bauer disk diffusion method, 1966 and interpretation of results was made according to the recommendations of the CLSI (CLSI VET -08, 2018)/ manufacturer's guidelines [14].

### Molecular Detection of Antimicrobial Resistance (ESBL Genes) and Virulence Genes

Genotypic antimicrobial resistance of *E. coli* isolates was carried out by amplifying β-Lactamase producing genes (*bla*TEM, *bla*SHV, *blaOXA, and bla*CTX-M groups 1, 2, and 9) by PCR using various primers listed in supplementary table 1 [15, 16]. Similarly, APEC-associated virulence genes like *pap*, *ast*, *tsh*, *iss*, *vat*, *cva* [17], *stx1 and stx2 genes* [18] were detected by PCR using respective primers, including the *int*1 gene, which was detected as per Kar et al. [11] (supplementary table 2).

The PCR reaction mixture was prepared in a total reaction volume of 25 µl for each gene, containing 12.5 µl of the 2 × PCR master mixture, 10 pmol of forward and reverse primers, 2 µl template DNA and nuclease free water to make a volume of 25 µl. The cardinal PCR temperatures and cyclic conditions for each PCR have been depicted in supplementary table 3. The amplified products were electrophoresed in a 1.5%–2% agarose gel stained with ethidium bromide (0.5 µg/ml), and the image was documented by the gel documentation system.

## Results and Discussion

On necropsy, egg peritonitis, perihepatitis, pericarditis, tracheitis, air sacculitis, hepatomegaly, and hemorrhagic intestine were the visible findings. On the basis of microscopic, cultural, and biochemical tests, 69 (67.64%) isolates were

**Table 2** Details of antibiotic discs used to study phenotypic antibiotic susceptibility pattern of *E. coli* isolates

| S. no | Antibiotic class | Antibiotic used | Drug concentration in µg/disc | Sensitivity Criteria (Zone Diameter in mm) | Resistance Criteria (Zone Diameter in mm) |
|---|---|---|---|---|---|
| 1 | Penicillin with beta lactamase inhibitor | Ampicillin + sulbactam | 10 | ≥ 17 | ≤ 16 |
| 2 | | Amoxyclav | 30 | ≥ 18 | ≤ 13 |
| 3 | Sulphonamide | Sulpha + Trimethoprim | 25 | ≥ 18 | ≤ 10 |
| 4 | Fluroquinolone | Ciprofloxacin | 5 | ≥ 21 | ≤ 15 |
| 5 | | Levofloxacin | 5 | ≥ 21 | ≤ 16 |
| 6 | | Enrofloxacin | 10 | ≥ 26 | ≤ 21 |
| 7 | Aminoglycoside | Gentamicin | 10 | ≥ 15 | ≤ 12 |
| 8 | Tetracycline | Tetracycline | 30 | ≥ 11 | ≤ 15 |
| 9 | Amphenicol | Chloramphenicol | 30 | ≥ 12 | ≤ 18 |
| 10 | Polymixin | Colistin | 10 | ≥ 11 | ≤ 10 |
| 11 | Third Gen Cephalosporin | Ceftriaxone + Sulbactam | 30 | ≥ 23 | ≤ 19 |
| 12 | | Cefotaxime (30 µg), | 30 | ≥ 20 | ≤ 17 |

identified as *E. coli*. Further, all 69 isolates were confirmed by PCR employing universal eubacterial primers (SRV3) targeting the 16S rRNA gene. For the in vitro antibiotic susceptibility test, out of 69 isolates, a very high degree of resistance was shown against fluoroquinolone antibiotics, *i.e*., enrofloxacin (72.46%), levofloxacin (69.56%), & ciprofloxacin (66.66%), followed by amoxyclav (63.77%) and tetracycline (59.42%). whereas lower resistance to ampicillin + sulbactam (34.78%) and ceftriaxone + sulbactam (36.23%) was observed (Fig. 1). Out of them, 6 (8.69%), 5 (7.25%) and 3 (4.34%) were found resistant to 8, 9, and 10 drugs, respectively. Six isolates were found sensitive to none of the tested antibiotics and showed resistance to 11–12 antibiotics, therefore being declared pan-drug resistant isolates (Table 3).

In addition to this, these isolates were subjected to detection of ESBL enzymes by targeting *bla*TEM, *bla*SHV, *blaOXA, and bla*CTX-M group 1, 2, and 9 genes using target gene specific primer pairs (Fig. 2). It revealed that a total of 48 (69.56%) and 7 (10.14%) isolates were positive for the presence of *bla*TEM and *bla*CTX-M-G9 genes, respectively, whereas only 2 (2.90%) isolates were found positive for *bla*SHV, *blaOXA,* and *bla*CTX-M-G1 genes out of 69 isolates, and none of the isolates were found positive for *bla*CTX-M-G2 (Fig. 2; Table 3).

The frequency of various virulence genes in 69 *E. coli* isolates revealed that 55 (79.71%), 35 (50.72%), 21 (30.43%), 18 (26.08%), 16 (23.18%), 6 (8.69%), and 1 (1.38%) isolates were found positive for *iss*, *tsh*, *ast*, *cvaf*, *pap*, *vat, and stx*1 genes, respectively (Figs. 3 and 4). But, 8 isolated were negative for any virulence gene. The different combinations of virulence genes have been shown in Table 4. Simultaneous detection of *iss, tsh, cva* (N = 8); *iss, tsh, pap (N = 8); iss, ast, tsh* (N = 7) showed that these were the most prevalent virulence factor combinations in these isolates. Out of six virulence factors tested, the gene *iss* was found in the highest number of isolates (N = 54), followed by *tsh* (N = 35). Whereas *ast, pap,* and *cva* genes were detected in 19, 16, and 12 isolates, respectively. Genes of *cva*, *vat (*N = 06 each), and *stx*1 were the least prevalent genes among the isolates. The total of 9, 27, 10, and 15 isolates showed positive for 4, 3, 2, and 1 virulence gene(s), respectively. A total of 32 isolates were detected with the *int*1 gene (Fig. 4), out of which five were associated with non ESBL producers, but in the others, it had the co-occurrence of one or more ESBL genes. Mainly, the *bla*TEM, and *bla*CTX-M g9 ESBL genes were found to be associated with the *int*1 gene (Table 3).

In the recent past, there has been increased concern about one health approach to tackle anti-microbial resistance [6], and the poultry sector constitutes an important component of the human-animal interface that can transmit antibiotic-resistant bacteria in an interdependent manner. The presence of resistance against newer classes of antibiotics has been increasingly documented in *E. coli* isolated from diseased [19] and healthy [20] poultry birds from India and neighbouring countries [4, 7]. The present study was carried out in the dry and hot region of Gujarat State, India, and the prevalence of *E. coli* was recorded at 67.64% (69/102). The prevalence rate approximated the results obtained in Jordan [21] and India [22]. Though almost 100% positive samples have also been reported for *E. coli* [4, 19], all these reports indicate that this is among the most prevalent pathogens among young poultry birds, and veterinarians are bound to use antibiotics as an obvious choice of treatment. The emergence of AMR is inevitable. AMR is a dynamic phenomenon and therefore, patterns of AMR must be constantly monitored. In recent reports from several parts of the world, like

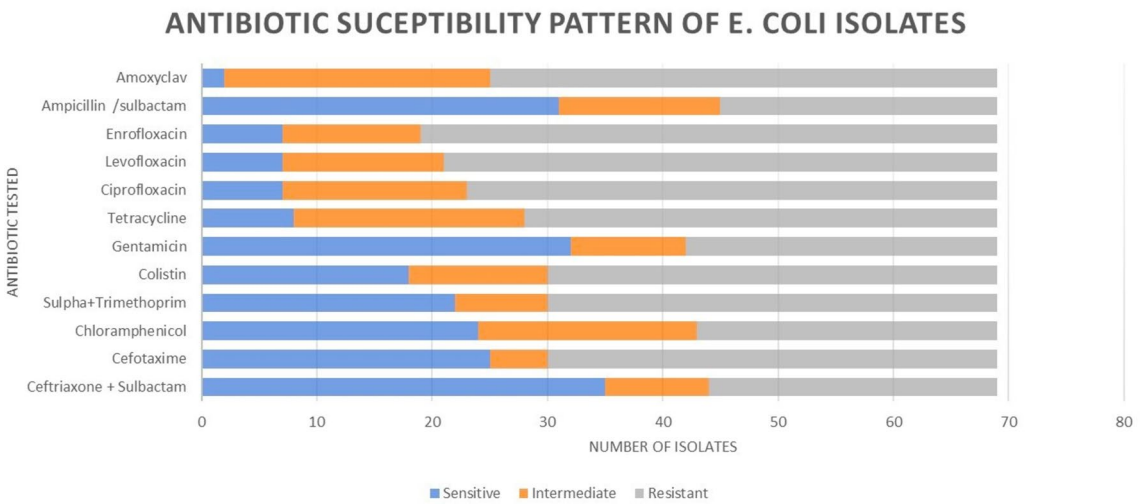


**Fig. 1** Antibiotic susceptibility pattern of avian pathogenic *E. coli* isolates

**Table 3** Distribution of ESBL, APEC virulence determinants and Class I integrons gene among APEC isolates

| Sample ID | Sample source | ESBL genes | Virulence genes | Phenotypic Resistance Pattern[#] | | | Sample ID | Sample source | ESBL genes | Virulence genes | Phenotypic Resistance Pattern[#] | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | S | I | R | | | | | S | I | R |
| 1 | Liver | *blaTEM*, *blaCTX-M -g9* | *iss*, I*nt*1 | 6 | 3 | 3 | 36 | Air sac | *blaTEM* | *iss, tsh, pap,cva,* I*nt*1 | 2 | 1 | 9 |
| 2 | Peritonitis | – | – | 8 | 2 | 2 | 37 | Liver | *blaTEM* | *iss, tsh, cvaf,* I*nt*1 | 2 | 2 | 8 |
| 3 | Liver | – | I*nt*1 | 8 | 0 | 4 | 38 | Liver | – | *tsh,pap* | 2 | 4 | 6 |
| 4 | Liver | *blaTEM* | *iss* | 8 | 2 | 2 | 39 | Liver | – | *iss, tsh* | 2 | 4 | 6 |
| 5 | Heart | *blaTEM* | *iss, pap, cva* | 6 | 2 | 4 | 40 | Air sac | *blaTEM* | *iss, tsh, ast* | 1 | 1 | 10 |
| 6 | Liver | *blaTEM* | *iss, ast* | 6 | 3 | 3 | 41 | Liver | *blaTEM* | *iss* | 2 | 0 | 10 |
| 7 | Heart | *blaTEM* | *iss, ast,tsh* | 3 | 1 | 8 | 42 | Intestinal content | *blaTEM* | *iss, tsh, cva, vat,* I*nt*1 | 5 | 1 | 6 |
| 8 | Liver | *blaTEM* | *iss,ast,tsh* | 4 | 1 | 7 | 43 | Intestinal content | *blaTEM* | *ast* | 8 | 1 | 3 |
| 9 | Peritonitis | *blaTEM*, *blaSHV*, *blaOXA* | *iss, tsh* | 0 | 1 | 11 | 44 | Intestinal content | *blaTEM* | *iss, tsh, cva,* I*nt*1 | 6 | 1 | 5 |
| 10 | Peritonitis | – | – | 4 | 3 | 5 | 45 | Intestinal content | *blaTEM* | *iss, tsh, ast* | 4 | 1 | 7 |
| 11 | Peritonitis` | – | – | 8 | 2 | 2 | 46 | Intestinal content | – | *pap* | 4 | 3 | 5 |
| 12 | Peritonitis | *blaTEM* | *iss* | 6 | 2 | 4 | 47 | Intestinal content | *blaTEM* | *iss, ast, cva, vat,* I*nt*1 | 1 | 5 | 6 |
| 13 | Peritonitis | *blaTEM* | *Iss,* I*nt*1 | 4 | 0 | 8 | 48 | Intestinal content | *blaTEM* | *iss, tsh, ast,* I*nt*1 | 5 | 2 | 5 |
| 14 | Air sac | *blaTEM* | *tsh,pap,* I*nt*1 | 4 | 2 | 6 | 49 | Intestinal content | *blaTEM* | *iss,ast, pap* | 0 | 6 | 6 |
| 15 | Trachea | *blaTEM* | *iss, tsh,* I*nt*1*pap* | 4 | 0 | 8 | 50 | Intestinal content | *blaTEM*, *blaCTX-M -g1* | *iss, tsh, pap,* I*nt*1 | 0 | 0 | 12 |
| 16 | Air sac | *blaTEM* | *iss, tsh, pap,* I*nt*1 | 4 | 4 | 4 | 51 | Trachea | – | *ast, ast, cva, vat* | 5 | 0 | 7 |
| 17 | Trachea | – | *iss, tsh, pap,* I*nt*1 | 4 | 2 | 6 | 52 | Heart | *blaTEM* | *iss, tsh,vat* | 11 | 1 | 0 |
| 18 | Peritonitis` | *blaTEM* | *iss, tsh, pap,* I*nt*1 | 1 | 5 | 6 | 53 | Liver | *blaCTX-M -g1* | *iss,ast,* I*nt*1 | 4 | 1 | 7 |
| 19 | Peritonitis | *blaTEM* | *iss, tsh,* I*nt*1 | 0 | 0 | 12 | 54 | Heart | *blaTEM* | *iss, tsh, cva* | 3 | 0 | 9 |
| 20 | Peritonitis | *blaTEM* | *iss, tsh, ast, cva,* I*nt*1 | 0 | 2 | 10 | 55 | Peritonitis | *blaTEM* | *iss, tsh, ast,* I*nt*1 | 7 | 0 | 5 |
| 21 | Liver | *blaTEM* | *iss, ast,cvaf, vat,* I*nt*1 | 6 | 2 | 4 | 56 | Heart | *blaTEM*, *blaSHV*, *blaOXA* | *iss, tsh, cvaf,* I*nt*1 | 0 | 0 | 12 |
| 22 | Liver | *blaTEM* | *iss, tsh, pap, ast,* I*nt*1 | 0 | 0 | 12 | 57 | Liver | *blaTEM* | *iss, tsh* | 0 | 8 | 4 |
| 23 | Intestinal contents | – | -, I*nt*1 | 3 | 4 | 5 | 58 | Air sac | *blaTEM* | *iss, tsh, pap* | 2 | 4 | 6 |
| 24 | Intestinal contents | – | *iss* | 3 | 3 | 6 | 59 | Heart | *blaCTX-M -g9* | *iss, ast, cva, vat,* I*nt*1 | 0 | 4 | 8 |
| 25 | Gall bladder | – | -, I*nt*1 | 1 | 6 | 5 | 60 | Liver | *blaTEM, blaCTX-M -g9* | *iss, tsh, cvaf* | 0 | 1 | 11 |
| 26 | Liver | – | -, I*nt*1 | 1 | 2 | 9 | 61 | Cloacal swab | *blaTEM* | *iss,* | 2 | 6 | 4 |
| 27 | Liver | *blaTEM* | *iss* | 3 | 5 | 4 | 62 | Air sac | *blaTEM* | *iss, tsh, pap,* I*nt*1 | 2 | 4 | 6 |
| 28 | Liver | – | -, I*nt*1 | 2 | 4 | 6 | 63 | Cloacal swab | *blaTEM* | *iss* | 0 | 7 | 5 |
| 29 | Liver | – | *Pap,* I*nt*1 | 1 | 6 | 5 | 64 | Yolk sac | | *iss, tsh, pap* | 1 | 5 | 6 |
| 30 | Yolk sac | – | *Iss, stx*1 | 5 | 1 | 6 | 65 | Yolk sac | *blaTEM* | *iss* | 1 | 4 | 7 |
| 31 | Liver | *blaCTX-M -g9* | *iss, tsh, ast, cva,* I*nt*1 | 3 | 2 | 7 | 66 | Cloacal swab | *blaTEM* | *iss, tsh, cva* | 2 | 3 | 7 |

**Table 3** (continued)

| Sample ID | Sample source | ESBL genes | Virulence genes | Phenotypic Resistance Pattern# | | | Sample ID | Sample source | ESBL genes | Virulence genes | Phenotypic Resistance Pattern# | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | S | I | R | | | | | S | I | R |
| 32 | Heart | *blaTEM* | *iss, ast, cvaf* | 4 | 0 | 8 | 67 | Liver | *blaTEM, blaCTX-M -g9* | *iss, tsh, ast* | 2 | 7 | 3 |
| 33 | Liver | *blaCTX-M -g9* | *iss,* | 2 | 1 | 9 | 68 | Heart | | *ast* | 1 | 6 | 5 |
| 34 | Air sac | *blaTEM, bla CTX-M -g9* | *iss, tsh, cvaf, Int1* | 2 | 1 | 9 | 69 | Heart | *blaTEM* | *iss, tsh, cva, Int1* | 3 | 3 | 6 |
| 35 | Liver | *blaTEM* | *iss* | 6 | 3 | 3 | | | | | | | |

Isolates no- 9,19,22,50,56 and 60 were the pan drug resistant isolates and # = number of antibiotic discs showing result as sensitive (S), resistant (R) and intermediate sensitive (I)

Nepal [4], Bangladesh [7, 23], and Italy [24], either ampicillin or tetracycline were found to be the most resistant drugs against APEC. But our work revealed that enrofloxacin was the most resistant antibiotic, and the fluoroquinolone group was the most resistant group of antibiotics. Similar findings have been revealed by several workers, and 81.94–87.0% fluroquinolone resistance was reported [21, 25–27], and one report stated as high as 97% of ciprofloxacin resistance [28]. This change in resistance pattern is conspicuous and clearly indicates widespread use of this antibiotic class for prophylaxis and treatment in the area. Further, the frequency of other resistant classes of antibiotics is also indicative of the proportion of their use in the poultry sector. Earlier, there were reports of colistin resistant [28] and multidrug resistant avian pathogenic *E. coli,* which were isolated from broiler chickens in Bangladesh [29], Iran [27], Qatar [28], and even India [19, 20]. Most importantly, the present quantum of resistant strains against last resort antibiotics like colistin and pan drug resistance is of great concern to human health. Though there are guidelines for restricted use of last resort drugs from different agencies of the government, but an official ban has been notified against colistin only [30].

When the genes responsible for ESBL production were tested, the occurrence of *bla*TEM and *bla*CTXM as the most significant ESBL synthesizing genes was found to be consistent with the findings of other workers [24, 26, 31]. It has been reported that *bla*CTX-M has become the dominant ESBL gene group [32], but present findings denote that *bla*TEM is still the most widespread ESBL genes group. The frequency of *bla*SHV was in line with findings of several researchers among poultry APEC [8, 21, 24] and APEC isolated from migratory birds [7]. Detection of *bla*OXA seems a rare phenomenon and was reported previously by only a few workers [26], who also detected *bla*OXA at a very low frequency. Genes viz., *blaTEM* and *bla*CTXM-9 were found to be associated with the pan drug resistant and multi drug resistant strains, except for one isolate (Number 26), where none of the tested genes were detected. The major reason behind this is the presence of at least 12 ESBL groups among Gram negative bacteria [32], which may produce at least 200 different types of ESBL enzymes.

The detection of *iss* as most prevalent virulence factor [19] and *tsh* as another significant factor is supported by earlier studies [2, 8, 21, 24]. Corroborating to present findings, *pap* was found among a significant number of APEC [4, 23]. Whereas in other studies, it was found in a lesser number of isolates [24] or in one isolate only [21]. Likewise, *ast* and *vat* were found to be less prevalent factors, was corroborated with earlier findings [21, 24]. As indicated in results, the total of 9, 27, 10, and 15 isolates were found positive for 4, 3, 2, and 1 virulence gene(s), respectively in various combinations. As for ESBL genes,

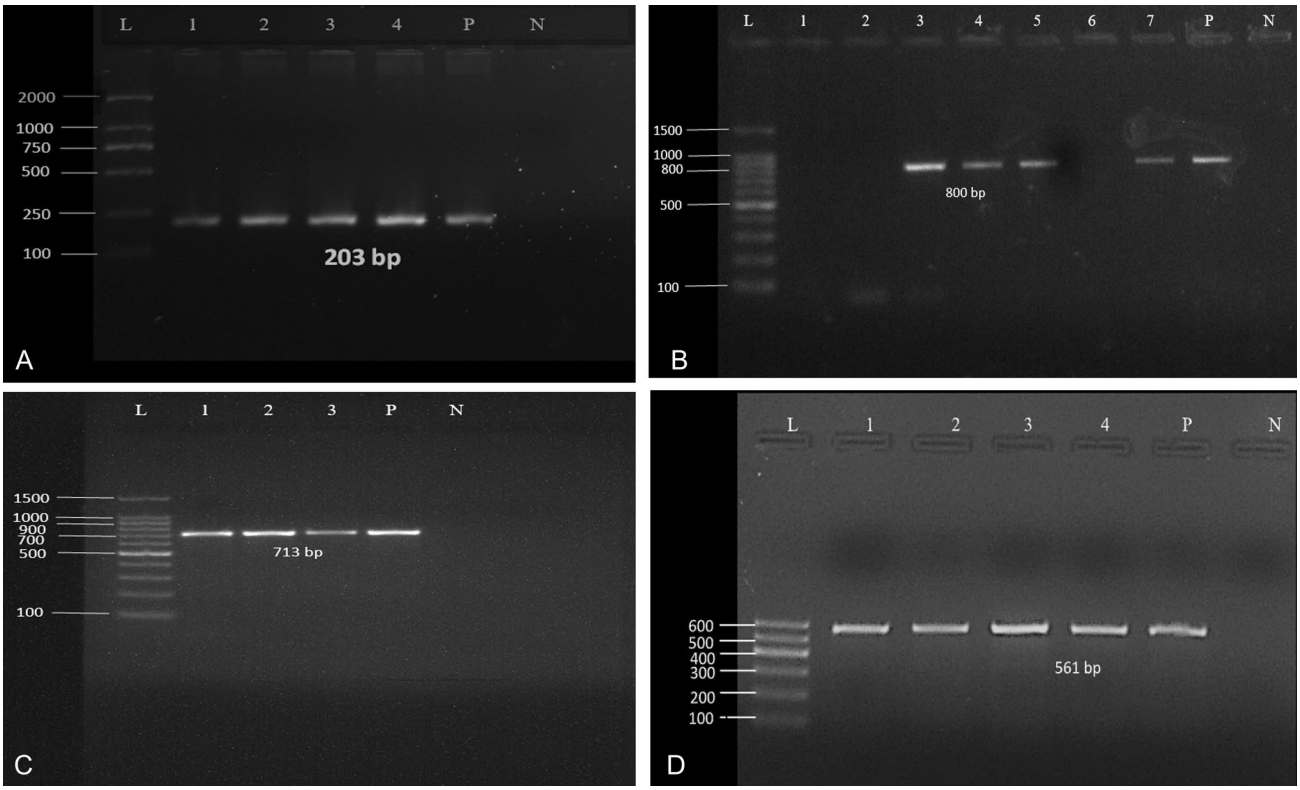


**Fig. 2** Agarose gel electrophoresis of **A** *E. coli* genus specific PCR showing Lane L: Gel pilot mid range ladder (Qiagen); Lane 1,2,3,4: Positive samples with amplicon of the size 203 bp **B** $Bla_{TEM}$ gene for genus specific PCR showing Lane L: 100 bp plus ladder (Qiagen); Lane 3,4,5,7: Positive samples with amplicon of the size 800 bp; Lane 1,2,6: negative sample **C** $Bla_{SHV}$ gene for genus specific PCR showing Lane L: 100 bp plus ladder (Qiagen); Lane 1,2,3: Positive samples with amplicon of the size 713 bp **D** $CTX_{M\text{-}9}$ gene for genus specific PCR showing Lane L: 100 bp ladder (Qiagen); Lane 1,2,3,4: Positive samples with amplicon of the size 561 bp. In all the plates lane P is positive control and lane N is non template control

there are several varieties of virulence genes but the preponderance of adhesins (*tsh* and *pap*) and protectins (*iss*) makes them essential genes for APEC [2, 3]. Though it has been mentioned that detection of five genes [3, 4] denotes a strain as APEC, the occurrence of these factors in different combinations makes it equivocal to denote any strain conclusively as APEC and differentiate it with faecal *E. coli* [5]. This fact was further substantiated by Tomaz et al. [26], who also found a non-significant association between virulence genes, APEC phenotyping, and AST pattern. But the presence of the *stx*1 gene in one isolate (isolate no. -30) and the *int*1 gene in 46.37% of isolates denotes the increasing zoonotic potential of the APEC. The *stx*1 gene is the Shiga toxin gene, which is frequently isolated in cattle *E. coli* [1, 9] within pathotype Shiga toxigenic *E. coli*, and only seldom reported from poultry birds in India [33] with diarrhoea. Antibiotic resistance, including ESBL production, may be carried and spread by integrons, which are important genetic elements. In our work, class I integrons were detected mostly along with ESBL gene, but their association with strains carrying the lesser number of virulence factors and AMR genes is an important finding which correspond to the previous report that these elements might also be associated with non-pathogenic *E. coli* and antibiotic non exposed strains [34] and considered among the potent factors of antibiotic spread. Apart from this work, class I integrons was detected in the veterinary sector in India by few workers only [11] and their association with human uropathogenic *E. coli* [10] underscore the importance of this study.

## Conclusions

Avian pathogenic *E. coli* is predominantly associated as a causative agent of avian colibacillosis; hence, antibiotics are used in the poultry industry as therapeutic and preventive measures, which leads to the development of different forms of AMR. The current status of antibiotic usage shows higher resistance to fluoroquinolone antibiotics. The report also showed an abundance of ESBL producing APEC,

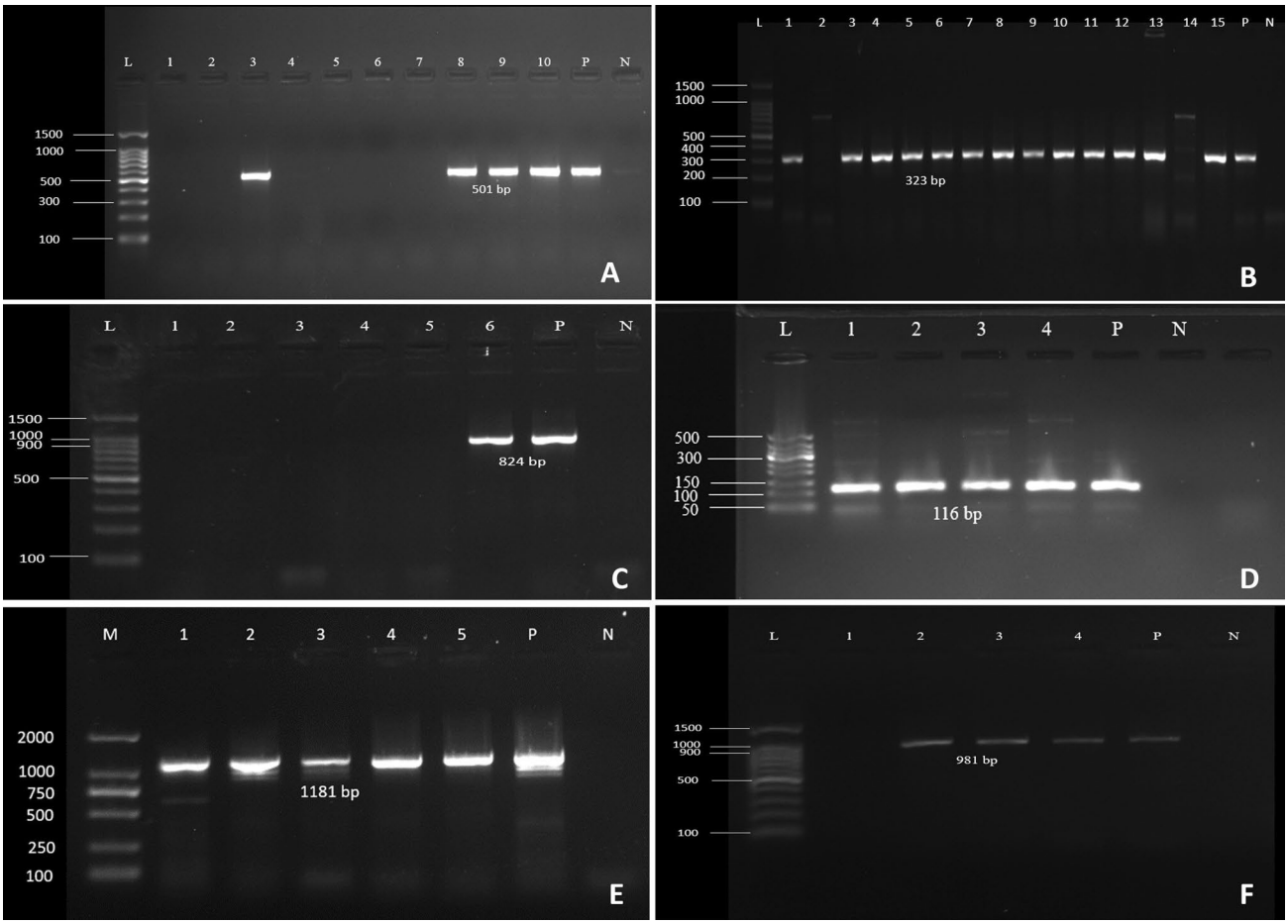


**Fig. 3** Agarose gel electrophoresis of **A** *pap* gene for genus specific PCR showing Lane L: Gel pilot 100 bp plus ladder (Qiagen); Lane 3,8,9,10: Positive samples with amplicon of the size 501 bp **B** *iss* gene for genus specific PCR showing Lane L: Gel pilot 100 bp plus ladder (Qiagen); Lane 1,3,4,5,6,7,8,9,10,11,12,13,15: Positive samples with amplicon of the size 323 bp **C** *tsh* gene for genus specific PCR showing Lane L: Gel pilot 100 bp plus ladder (Qiagen); Lane 6: Positive samples with amplicon of the size 824 bp **D** *ast* gene for genus specific PCR showing Lane L: Gel pilot 50 bp plus ladder (Qiagen); Lane 1,2,3,4: Positive samples with amplicon of the size 116 bp **E** *cvaf* gene for genus specific PCR showing Lane L: Gel pilot mid-range ladder (Qiagen); Lane 1,2,3,4,5: Positive samples with amplicon of the size 1181 bp **F** *vat* gene for genus specific PCR showing Lane L: Gel pilot 100 bp plus ladder (Qiagen); Lane 2,3,4: Positive samples with amplicon of the size 981 bp. In all the plates lane P is positive control and lane N is non template control

**Table 4** Genotypic detection of various APEC related virulence genes by PCR

| S. No | Name of the gene(s) | Total | Isolate number with gene(s) | S. No | Name of the gene | Total | Isolate number with gene(s) |
|---|---|---|---|---|---|---|---|
| 1 | *iss* | 12 | 1,4,12,13,,24,27,30,33,35,41,61,63,65 | 11 | *pap* | 2 | 29,46 |
| 2 | *iss, pap, cvaF* | 1 | 5 | 12 | *iss, ast, cvaF* | 1 | 32 |
| 3 | *iss, ast* | 2 | 6,53 | 13 | *iss, tsh, cvaF* | 8 | 34,37,44,54,56,60,66,69 |
| 4 | *iss, ast, tsh* | 7 | 7,8,40,45, 48,55,67 | 14 | *iss, tsh, pap, cvaF* | 1 | 36 |
| 5 | *iss, tss* | 4 | 9,19,39,57 | 15 | *iss, tsh, cvaF,vat* | 1 | 42 |
| 6 | *tsh, pap* | 2 | 14,38 | 16 | *ast* | 2 | 43,68 |
| 7 | *iss, tsh, pap* | 8 | 15–18,50,58,62,64 | 17 | *iss, ast, pap* | 1 | 49 |
| 8 | *iss,tsh, ast, cvaF* | 2 | 20,31 | 18 | *iss, tsh,vat* | 1 | 52 |
| 9 | *iss,ast, cvaF, vat* | 4 | 21,47,51, 59 | 19 | *iss,stx*1 | 1 | 30 |
| 10 | *iss, tsh, pap,ast* | 1 | 22 | | | | |

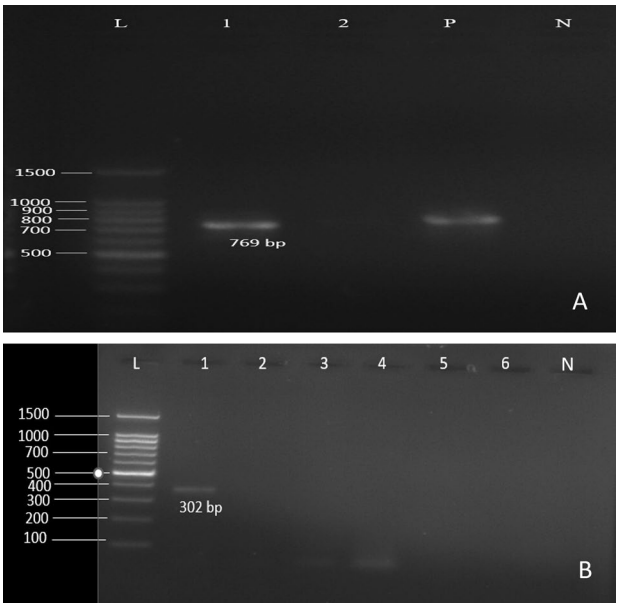


**Fig. 4** Agarose gel electrophoresis of (**A**) int1 gene PCR showing Lane L: Gel pilot 100 bp plus ladder (Qiagen); Lane 1: Positive samples with amplicon of the size 769 bp; Lane 2 negative sample; lane P is positive control and lane N is non template control. (**B**) *stx*I gene PCR showing Lane L: Gel pilot 100 bp plus ladder (Qiagen); Lane 1: Positive samples with amplicon of the size 302 bp; Lane 2–6 negative sample; lane N is non template control; positive control was not used in this PCR. (**C**)

which arose principally through the possession of *bla*TEM and *bla*CTX-M 9 genes, along with *blaSHV* and *bla*OXA. Most isolates carried serum resistance (*iss*) and temperature sensitive haemagglutinin (*tsh*) as the main virulence marker genes. The association of class I integrons with ESBL and virulence markers rendered these isolates potentially zoonotic, and they could easily be spread to humans through the food chain or via the environment, causing serious public health concerns.

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**Declarations**

**Conflict of interest** The authors declare no conflicts of interest related to this article.

**Ethical Approval** No ethical approval was required as samples were obtained from dead birds.

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