TÜBİTAK

Turkish Journal of Veterinary and Animal Sciences

http://journals.tubitak.gov.tr/veterinary/

Research Article

Turk J Vet Anim Sci
(2019) 43: 117-126
© TÜBİTAK
doi:10.3906/vet-1808-47

# Phylogrouping and antimicrobial resistance analysis of extraintestinal pathogenic *Escherichia coli* isolated from poultry species

**Sujata BHAVE[1], Rahul KOLHE[1,*], Ranjan MAHADEVASWAMY[1], Chandrakant BHONG[1], Samir JADHAV[2], Saba NALBAND[1], Dattatray GANDHALE[1], Dushyant MUGLIKAR[3]**

[1]Department of Veterinary Public Health, KNP College of Veterinary Science Shirwal, District Satara, Maharashtra, India
[2]Department of Veterinary Biochemistry, KNP College of Veterinary Science Shirwal, District Satara, Maharashtra, India
[3]Department of Veterinary Microbiology, KNP College of Veterinary Science Shirwal, District Satara, Maharashtra, India

**Received:** 15.08.2018 • **Accepted/Published Online:** 25.01.2019 • **Final Version:** 12.02.2019

**Abstract:** This study was undertaken to determine the prevalence and antimicrobial resistance pattern of extraintestinal pathogenic *Escherichia coli* (ExPEC) in the feces of healthy poultry and retail chicken. All 146 *E. coli* recovered from 351 samples were screened by PCR for detection of ExPEC strains. Nineteen (13.01%) isolates were confirmed as ExPEC. Distribution of ExPEC strains was revealed as follows: broiler (25%), layer (15.87%), and raw chicken (3.12%). Turkey, duck, and water samples were negative for ExPEC strains. ExPEC strains belonged to phylogenetic groups B2 (52.63%), A (36.84%), and D (10.53%). Twenty-three (15.75%) isolates were ESBL-positive, including four ExPEC strains. ESBL-positive *E. coli* were isolated from all the samples except turkey. A high degree of resistance to commonly used antimicrobials, namely nalidixic acid (95.89%), tetracycline (95.89%), trimethoprim (89.04%), colistin (82.88%), and ciprofloxacin (54.11%), including β-lactam antimicrobials ampicillin (84.93%) and amoxicillin/clavulanic acid (81.51%), was expressed by the isolates. Out of fifteen randomly selected ESBL-positive *E. coli* isolates, β-lactam genes, namely *bla*TEM, *bla*CTX-M, and *bla*OXA, were detected in three, six, and one, respectively. Poultry and raw chicken harbor multidrug-resistant, ESBL-type *E. coli* as well as ExPEC and these strains may be transmitted to humans via the food chain.

**Key words:** Extraintestinal pathogenic *E. coli*, ESBL, antimicrobial resistance, poultry

## 1. Introduction

Antimicrobial resistance in foodborne pathogens and their risk of transmission to humans through the food chain has been speculated by several researchers (1,2). Despite the call for limiting antimicrobial use in livestock, its use has been increased several fold. Livestock and poultry harbor different types of commensal, indicator, and pathogenic bacteria of zoonotic importance. *Escherichia coli* is one of the predominant organisms crucial in widespread dissemination of antimicrobial resistance. It is also considered as an indicator organism of antimicrobial resistance in members of Enterobacteriaceae. Pathogenic strains of *E. coli* are classified as diarrheagenic and extraintestinal types and speculations regarding their foodborne origin are in existence (3). Despite the high degree of gene flow, the population structure of this species remains mostly clonal, with clear delineation of phylogroups; however, little is known about the relationships between phylogenetic groups and host specificity (4). Criteria to differentiate extraintestinal pathogenic *E. coli* (ExPEC) strains based on the presence of two or more virulence markers have been documented and referred to widely (5). ExPEC strains are responsible for urinary tract infection, meningitis, and septicemia in humans (6). Intestines and the environment of healthy chickens could act as reservoirs for ExPEC strains with zoonotic potential (7).

The emergence of extended spectrum β-lactamase (ESBL)-producing *E. coli* in livestock and poultry is a matter of growing concern in the western world (8). In India, ESBL-positive strains of *E. coli* have been isolated from clinical samples of humans (9), but studies on *E. coli* of livestock and poultry origin are limited (10). Chicken is the most preferred nonvegetarian food commodity in India. Thus, there is a need to explore the role of different poultry species in fecal carriage of ESBL-positive *E. coli* and ExPEC strains in the Indian context. In view of these facts, a study was undertaken to determine the prevalence of ExPEC strains, their phylogenetic grouping, ESBL positivity, and antimicrobial resistance profiles of *E. coli* isolates of poultry origin.

* Correspondence: dr_kolherahul@rediffmail.com

## 2. Materials and methods

### 2.1. Sample collection

A total of 351 samples comprising the fecal matter of clinically healthy broilers (176), layers (50), turkeys (17), and ducks (29) together with raw chicken (54) and water (25) were collected aseptically and examined for *E. coli*. Samples were transported under low temperature and processed on the same day for bacterial culture. All sampling was conducted from January to April 2016. Broiler samples were collected from birds reared at the college farm and private farms near the institute. Layer, duck, and turkey samples were taken from birds available at the poultry farm of our institute. Raw chicken samples were collected aseptically from retail chicken markets. Water samples were procured from the poultry farm's premises and water used for carcass washing in retail chicken markets.

### 2.2. Reference strains

Known bacterial strains of *E. coli*, *Salmonella* Enteritis, and *Listeria monocytogenes* maintained in the Department of Veterinary Public Health of this institute were used for the standardization of protocols.

### 2.3. Isolation and identification of *E. coli*

For isolation of pure culture, enrichment followed by selective plating on specific agar was performed. Enrichment was done in Enterobacteriaceae enrichment broth (EEB). A loopful of enriched culture from EEB was first streaked on eosin methylene blue (EMB) agar plates and incubated at 37 °C for 24 h. Colonies with a metallic sheen were further streaked on MUG sorbitol agar plates. Pathogenic *E. coli* were suspected as pale-colored nonfluorescent colonies on MUG sorbitol agar. Cloacal swabs were directly enriched in 10 mL of EEB, while meat samples were enriched in the ratio of 1:10. Presumptive identification of *E. coli* was recorded based on Gram's straining, morphology, catalase, oxidase, and IMViC tests (11). Pure broth and slant cultures of confirmed *E. coli* were maintained for further studies.

### 2.4. Detection of ExPEC strains by PCR

*E. coli* strains were cultured afresh on the EMB agar plates and DNA extraction was carried out by suspending one bacterial colony in 50 µL of sterile water, boiling the suspension for 5 min at 95 °C in a thermal cycler (Applied Biosystems) followed by snap chilling in crushed ice for 10 min. Thereafter, the suspension was centrifuged at 10,000 rpm for 1 min in a refrigerated centrifuge (Hettich Zentrifugen, Universal 320 R). Supernatant (2 µL) was used as the DNA template for the polymerase chain reaction (PCR). Uniplex and multiplex PCR assays were performed to detect virulence markers representing ExPEC strains. Detection of eight virulence genes, namely *astA*, *iss*, *irp2*, *papC*, *iucD*, *tsh*, *vat*, and *cva/cvi*, was done by multiplex PCR (set 1) according to the protocol described earlier (12), whereas uniplex PCR (set 2) was performed for detection of the *kpsMTII* gene (13). All amplifications were carried out in a volume of 25 µL containing 12.5 µL of 2X PCR Master Mix (HiMedia) supplied with Taq DNA polymerase, buffer, $MgCl_2$, and dNTPs; 0.5 µL each of forward and reverse primers; 2 µL of DNA template; and nuclease-free water to make the final volume of 25 µL. For set 1, a cycle of initial denaturation was carried out at 94 °C/3 min followed by 25 cycles of denaturation (94 °C/30 s), annealing (58 °C/30 s), and extension (68 °C/3 min). One cycle of final extension of 10 min at 72 °C followed and amplified products were held at 4 °C, visualized, and stored at –20 °C for further use. For set 2, cycling conditions were as follows: initial denaturation (95 °C/12 min - 1 cycle) followed by denaturation (94 °C/30 s), annealing (63 °C/30 s), and extension (68 °C/3 min), with a final extension (72 °C/10 min) and holding at 4 °C. PCR-amplified products of set 1 (8 µL) and set 2 (5 µL) were separated by electrophoresis in 1.5% and 2% agarose gel, respectively, stained with ethidium bromide (Figure 1). The primer sequences used are presented in Table 1.

### 2.5. Detection of phylogenetic groups

Phylogenetic grouping of ExPEC strains was done by multiplex PCR as described earlier targeting three genes, namely *chuA*, *yjaA*, and DNA fragment *TspE4.C2* (14). The assay was performed in a volume of 20 µL containing 10 µL of 2X PCR master mix (HiMedia) supplied with Taq DNA polymerase, buffer, $MgCl_2$, and dNTPs; 1 µL each of forward and reverse primers; 2 µL of DNA template;

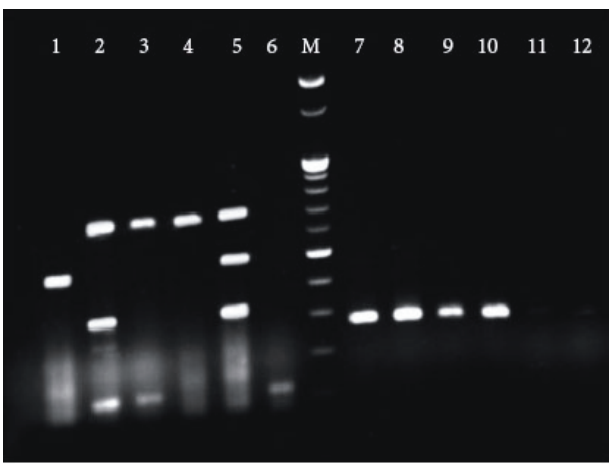


**Figure 1.** Detection of extraintestinal pathogenic *E. coli* by multiplex and uniplex PCR. M: 100-bp DNA ladder; Lane 1: *papC* (501 bp); Lane 2: *iss* (309 bp) and *iucD* (714 bp); Lane 3: *iucD* (714 bp); Lane 4: *iucD* (714 bp); Lane 5: *iss* (309 bp), *papC* (501 bp), and *iucD* (714 bp); Lane 7: *kpsMTII* (272 bp); Lane 8: *kpsMTII* (272 bp); Lane 9: *kpsMTII* (272 bp); Lane 10: *kpsMTII* (272 bp).

**Table 1.** Primer sequences used for detection of ExPEC virulence markers.

| Primer name | Oligonucleotide sequence | Target gene | Amplicon size (bp) | Reference |
|---|---|---|---|---|
| Set 1 | | | | Ewers et al. (12) |
| *astA f* | TGC CAT CAA CAC AGT ATA TCC | *astA* | 116 | |
| *astA r* | TCA GGT CGC GAG TGA CGG C | | | |
| *papC f* | TGA TAT CAC GCA GTC AGT AGC | *papC* | 501 | |
| *papC r* | CCG GCC ATA TTC ACA TAA | | | |
| *iss f* | ATC ACA TAG GAT TCT GCC G | *iss* | 309 | |
| *iss r* | CAG CGG AGT ATA GAT GCC A | | | |
| *irp 2 f* | AAG GAT TCG CTG TTA CCG GAC | *irp2* | 413 | |
| *irp 2 r* | AAC TCC TGA TAC AGG TGG C | | | |
| *iucD f* | ACA AAA AGT TCT ATC GCT TCC | *iucD* | 714 | |
| *iucD r* | CCT GAT CCA GAT GAT GCT C | | | |
| *tsh f* | ACT ATT CTC TGC AGG AAG TC | *tsh* | 824 | |
| *tsh r* | CTT CCG ATG TTC TGA ACG T | | | |
| *vat f* | TCC TGG GAC ATA ATG GTC AG | *vat* | 981 | |
| *vat r* | GTG TCA GAA CGG AAT TGT | | | |
| *cva A/B f* | TGG TAG AAT GTG CCA GAG CAA G | *cva A/B* *cvi cvaC* | 1181 | |
| *cvi cvaC r* | GAG CTG TTT GTA GCG AAG CC | | | |
| Set 2 | | | | Johnson and Stell (13) |
| *KpsMT II f* | GCG CAT TTG CTG ATA CTG TTG | *kpsMTII* | 272 | |
| *KpsMT II r* | CAT CCA GAC GAT AAG CAT GAG CA | | | |

and 2 µL of nuclease-free water to make the final volume of 20 µL. PCR conditions were set as initial denaturation (94 °C/5 min) followed by thirty cycles of denaturation (94 °C/30 s), annealing (55 °C/30 s), and extension (72 °C/30 s). Final extension was carried out at 72 °C/7 min. Five microliters of amplified product was separated in 2% agarose gel stained by ethidium bromide (Figure 2). The primer sequences used are presented in Table 2.

### 2.6. Detection of ESBL strains

ESBL production in the *E. coli* strains was detected in two steps: initial screening of ESBL production, followed by phenotypic confirmation in accordance with the recommendations of the CLSI (15). For initial screening, fresh *E. coli* cultures grown overnight in brain heart infusion (BHI) broth were smeared on Mueller Hinton agar (MHA) plates and antimicrobial disks of cefpodoxime (10 µg), ceftazidime (30 µg), aztreonam (30 µg), ceftriaxone

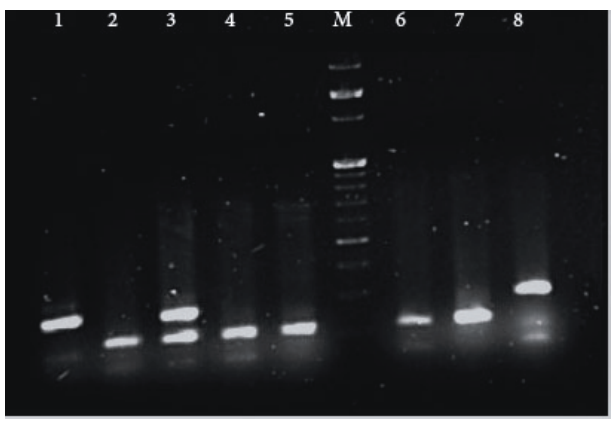


**Figure 2.** Detection of *E. coli* phylogeny groups by multiplex PCR. M: 100-bp DNA ladder; Lanes 1 and 8: amplification of *chuA* (279 bp) (phylogroup D); Lanes 2, 4, 5, 6, and 7: amplification of *yjaA* (211 bp) (phylogroup A); Lane 3: amplification of *chuA* (279 bp) and *yjaA* (211 bp) (phylogroup B2).

**Table 2**. Primer sequences used for detection of *E. coli* phylogenetic groups.

| Primer name | Primer sequence (5′-3′) | Target gene | Amplicon size (bp) | Reference |
|---|---|---|---|---|
| ChuA.1 | GACGAACCA ACGGTCAGGAT | chuA | 279 | Clermont et al. (14) |
| ChuA.2 | TGCCGCCAGTACC AAAGACA | | | |
| YjaA.1 | TGAAGTGTCAGGAGACGCT G | yjaA | 211 | |
| YjaA.2 | ATGGAGAATGCGTTCCTCAAC | | | |
| TspE4C2.1 | GAGTAATGTCGGGGCATTCA | TspE4.C2 | 152 | |
| TspE4C2.2 | CGCGCCAACAAAGTATTACG | | | |

(30 µg), and cefotaxime (30 µg) were placed aseptically. Plates were allowed to dry and incubated at 37 °C for 24 h. Inhibition zones were measured and interpreted as per the CLSI guidelines. Antimicrobial disks of cefotaxime (30 µg) alone and cefotaxime (30 µg) with clavulanic acid (10 µg) were used for phenotypic confirmation of ESBL production. A difference of ≥5 mm in the zones of inhibition of cephalosporin disks and cephalosporin with clavulanic acid indicated ESBL positivity in *E. coli*.

**2.7. Antimicrobial susceptibility testing**

Antimicrobial susceptibility and resistance patterns of *E. coli* were studied by disk diffusion method. Antimicrobial disks were procured from HiMedia Laboratories Pvt. Ltd. (Mumbai, India). Antimicrobial disks used were amoxycillin/clavulanic acid (30 µg), ampicillin (10 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), colistin (10 µg), gentamicin (10 µg), nalidixic acid (30 µg), neomycin (30 µg), tetracycline (30 µg), and trimethoprim (5 µg). *E. coli* cultures grown overnight in BHI broth were streaked on MHA and antimicrobial disks were placed. After incubation at 37 °C for 24 h, *E. coli* isolates were grouped as resistant, intermediate, and sensitive according to the manufacturer's instructions.

**2.8. Detection of β-lactam genes**

ESBL-positive *E. coli* isolates were further investigated by multiplex PCR for the presence of genes encoding β-lactamases. Previously used oligonucleotide sequences and PCR protocol were followed (16). PCR was performed in a volume of 25 µL containing 12.5 µL of PCR master mix (HiMedia), 1 µL each of forward and reverse primers, 2 µL of DNA template, and 2.5 µL nuclease-free water to make a final volume of 25 µL. Multiplex PCR was set with the following conditions: initial denaturation (95 °C/15 min), followed by thirty cycles of denaturation (94 °C/30 s), annealing (62 °C/90 s), and extension (72 °C/60 s). Final extension was set at 72 °C for 10 min. Electrophoresis of PCR products was carried out in 1.5% agarose gel stained with ethidium bromide (Figure 3). Primer sequences used are mentioned in Table 3.

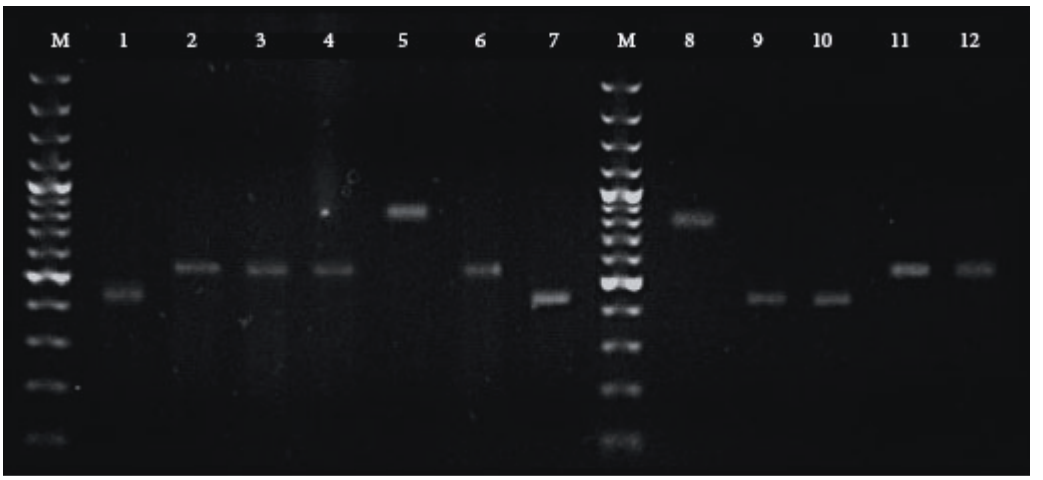


**Figure 3.** Detection of beta-lactam genes in the *E. coli* isolates. M: 100-bp DNA ladder; Lanes 1, 7, 9, and 10: *blaTEM* (445 bp); Lanes 2, 3, 4, 6, 11, and 12: *bla*CTXM (593 bp); Lanes 5 and 8: *bla*OXA (813 bp).

## 3. Results

### 3.1. Rate of *E. coli* contamination

The overall prevalence of *E. coli* observed in this study was 41.59%. A total of 146 *E. coli* strains were recovered from 351 samples. Prevalence of *E. coli* in different samples was recorded as follows: layers (64%), raw chicken (59.26%), turkeys (41.18%), broilers (35.80%), ducks (17.24%), and water (28%). Out of seven water samples, two were from a drinking water source at the layer farm and five were from carcass washings. Out of 146 isolates, 19 (13.01%) were confirmed as ExPEC strains by PCR. Maximum numbers of ExPEC strains were isolated from broilers (25%), followed by layers (15.87%) and raw chicken (3.12%). ExPEC strains could not be isolated from turkey, duck, and water samples (Table 4).

### 3.2. Phylogenetic distribution of ExPEC

Out of 19 ExPEC strains, nine (47.36%), seven (36.84%), and three (15.78%) represented phylogenetic groups B2, A, and D, respectively. Irrespective of the phylogenetic groups of ExPEC, other detected genes specific for extraintestinal strains were *iucD* and *kpsMTII* (17, 89.47% each), *papC* and *astA* (7, 36.84% each), *iss* (2, 10.53%), and *irp2* and *tsh* (1, 5.26% each). Prevalence of virulent genes in *E. coli* other than ExPEC strains was detected in the order of *iucD* (19.18%), *astA* (13.70%), *iss* (8.90%), *papC* (6.16%), *tsh* (4.11%), and *irp2* (2.74%). The *vat* and *cva* genes were not detected in any of the *E. coli* isolates.

### 3.3. ESBL positivity

Screening of all 146 *E. coli* isolated from different samples revealed 23 (15.15%) isolates as ESBL-positive strains. Sample-wise distribution of ESBL-positive isolates was recorded as follows: broilers (15.87%), layers (15.62%), ducks (20%), water (57.14%), and raw chicken (9.38%). Except turkey, ESBL-positive *E. coli* were isolated from all samples. It was noted that water used for carcass washing at retail chicken shops harbored maximum ESBL-positive *E. coli* as compared to other samples. Out of 19 ExPEC strains, four (21.05%) were ESBL producers, two each from cloacal swabs of broiler and layer birds.

### 3.4. Antimicrobial resistance pattern

A high degree of resistance to nalidixic acid, tetracycline (95.89% each), trimethoprim (89.04%), colistin (82.88%), and ciprofloxacin (54.11%), including β-lactam antibiotics ampicillin (84.93%) and amoxicillin/clavulanic acid

**Table 3.** Primer sequences used for detection of beta-lactam genes in *E. coli*.

| Primer name | Primer sequence (5′-3′) | Target gene | Amplicon size (bp) | Reference |
|---|---|---|---|---|
| SHV f | CTT TAT CGG CCC TCA CTC AA | $bla_{SHV}$ | 237 | Fang et al. (16) |
| SHV r | AGG TGC TCA TCA TGG GAA AG | | | |
| TEM f | CGC CGC ATA CAC TAT TCT CAG AAT GA | $bla_{TEM}$ | 445 | |
| TEM r | ACG CTC ACC GGC TCC AGA TTT AT | | | |
| CTXM f | ATG TGC AGY ACC AGT AAR GTK ATG GC | $bla_{CTX-M}$ | 593 | |
| CTXM r | TGG GTR AAR TAR GTS ACC AGA AYC AGC GG | | | |
| OXA f | ACA CAA TAC ATA TCA ACT TCG C | $bla_{OXA}$ | 813 | |
| OXA r | AGT GTG TTT AGA ATG GTG ATC | | | |

**Table 4.** Sample-wise detection of *E. coli* ESBL-positive and ExPEC strains.

| Sr. no. | Type of sample | Number of samples | No. of *E. coli* | ESBL-type *E. coli* | ExPEC |
|---|---|---|---|---|---|
| 1 | Broiler | 176 | 63 (35.79%) | 10 (15.87%) | 10 (15.87%) |
| 2 | Layer | 50 | 32 (64.00%) | 05 (15.62%) | 08 (25.00%) |
| 3 | Turkey | 17 | 07 (41.18%) | -- | -- |
| 4 | Duck | 29 | 05 (17.24%) | 01 (20.00%) | -- |
| 5 | Water | 25 | 07 (28.00%) | 04 (57.14%) | -- |
| 6 | Raw chicken meat | 54 | 32 (59.26%) | 03 (09.37%) | 01(3.12%) |
| Total | | 351 | 146 (41.59%) | 23 (15.75%) | 19 (13.01%) |

(81.51%), was expressed by the *E. coli* isolates. All *E. coli* isolated from raw chicken samples were 100% resistant to nalidixic acid and tetracycline. Similarly, all isolates recovered from turkey and layer birds were resistant to nalidixic acid; isolates from water samples were found resistant to tetracycline and ampicillin. EXPEC strains were also resistant to nalidixic acid (100%) and tetracycline (100%), followed by neomycin and colistin (94.74% each), trimethoprim (89.46%), and ciprofloxacin, ampicillin, and amoxicillin/clavulanic acid (78.95% each). The results of antimicrobial resistance of *E. coli* are shown in Table 5.

### 3.5. Detection of β-lactam genes

Out of 15 (11 commensal and four ExPEC) ESBL-positive *E. coli* screened, β-lactam genes were detected in 12 isolates. *bla*CTX-M, *bla*TEM, and *bla*OXA genes were present in six (26.08%), four (17.39%), and two (8.69%) isolates, respectively. *bla*SHV was not detected in any of the isolates. Out of four ESBL-positive ExPEC strains, β-lactam genes were present in three isolates. Sample-wise detection of ExPEC, their corresponding phylogenetic groups, resistance patterns, and presence of β-lactam genes are depicted in Table 6.

## 4. Discussion

The aim of this study was to learn the prevalence of extraintestinal pathogenic *E. coli* and ESBL-type strains being excreted in the feces of poultry and also to learn the rate of contamination of raw chicken by multidrug-resistant *E. coli* strains. We have isolated 146 *E. coli* from 351 samples comprising cloacal swabs of different poultry species, raw chicken sold in a retail market, and water samples used at a poultry farm as well as for carcass washing at chicken markets. Our findings affirm the role of poultry in fecal excretion of multidrug-resistant as well as ESBL-positive *E. coli* strains. Healthy broiler and layer birds may be possible reservoirs of ExPEC strains as these strains were isolated from 13.01% samples. The potential role of ExPEC in poultry colibacillosis and human urinary tract infections was documented earlier (17). The high prevalence of *E. coli* recorded in this study could be attributed to the fact that commensal strains of *E. coli* are normal inhabitants of the intestinal tract of poultry and fecal shedding is common. However, its detection in raw chicken and water used for carcass washing is indicative of carcass contamination during slaughtering and dressing. Similarly, detection of ExPEC strains in broilers, layers, and raw chicken is alarming from a public health perspective.

The zoonotic potential of *E. coli* of poultry origin to cause extraintestinal human infection has been investigated recently (18). Based on the criteria suggested earlier, 19 isolates containing *papC*, *iucD*, and *kpsMTII* marker genes were grouped as ExPEC strains. The combination of ExPEC-defining markers observed in our study was *iucD* + *kpsMTII* (12/19), *papC* + *iucD* (2/19), *papC* + *kpsMTII* (2/19), and *papC* + *iucD* + *kpsMTII* (3/19). Although many strains could not be grouped as ExPEC as per the criteria stated earlier, virulence genes like *iucD*, *astA*, *iss*, *papC*, *tsh*, and *irp2* were randomly present. The *vat* and *cva* genes were absent in all the isolates. A study from a Finnish retail market revealed that 22% of marinated and nonmarinated poultry meat products were contaminated with ExPEC and virulence genes were most frequent among isolates of phylogroup B2 (19). Most of the ExPEC strains of our study were also from phylogroup B2 and commonly detected virulence genes in them were *iucD* and *kpsMTII*.

The frequency of virulent genes associated with avian pathogenic *E. coli* (APEC) studied in Egypt revealed the presence of *tsh* and *papC* genes in *E. coli* from infected and healthy broiler birds. Variation in the distribution of virulent genes representing ExPEC has been reported earlier and was also observed in this study. The *papC* gene

**Table 5.** Resistance patterns of the *E. coli* isolates including ExPEC strains.

| Source and no. of isolates | Resistance to antimicrobials used (%) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | C (30 µg) | CIP (5 µg) | NA (30 µg) | TE (30 µg) | G (10 µg) | AMP (10 µg) | AMC (30 µg) | CL (10 µg) | N (30 µg) | TR (5 µg) |
| Broiler (63) | 6.35 | 52.38 | 93.65 | 95.24 | 33.33 | 87.30 | 87.30 | 88.89 | 85.72 | 87.30 |
| Layer (32) | 00.00 | 62.50 | 100.0 | 96.88 | 12.50 | 65.62 | 56.25 | 81.25 | 75.00 | 87.50 |
| Turkey (07) | 00.00 | 57.14 | 100.0 | 71.44 | 00.00 | 85.71 | 85.71 | 14.29 | 71.43 | 85.71 |
| Duck (05) | 00.00 | 60.00 | 80.00 | 100.0 | 00.00 | 80.00 | 80.00 | 20.00 | 80.00 | 100.0 |
| Water (07) | 28.00 | 57.14 | 85.71 | 100.0 | 28.57 | 100.0 | 71.43 | 71.43 | 85.71 | 85.71 |
| Raw chicken meat (32) | 00.00 | 46.87 | 100.0 | 100.0 | 37.50 | 96.88 | 96.88 | 100.0 | 87.50 | 93.76 |

C - Chloramphenicol, CIP - ciprofloxacin, NA - nalidixic acid, TE - tetracycline, G - gentamicin, AMP - ampicillin, AMC - amoxicillin/clavulanic acid, CL - colistin, N - neomycin, TR - trimethoprim.

**Table 6.** Detection of ExPEC with phylogrouping, antibiogram, and ESBL production.

| Source of sample | Serial no. of sample | Sample code | ExPEC genes detected | Phylogroup | Antibiogram | | | ESBL including *Bla* genes |
|---|---|---|---|---|---|---|---|---|
| | | | | | S | I | R | |
| Cloacal swabs of broilers | 1 | CS12 | *iucD*, *kpsMTII* | B2 | C | - | CIP, NA, TE, G, AMP, AMC, CL, N, TR | - |
| | 2 | CS14 | *iss*, *papC*, *iucD*, *kpsMTII* | A | G, TR | C | CIP, NA, TE, AMP, AMC, CL, N | - |
| | 3 | CS23 | *astA*, *iss*, *iucD*, *kpsMTII* | A | C | - | CIP, NA, TE, G, AMP, AMC, CL, N, TR | - |
| | 4 | CS24 | *astA*, *iucD*, *kpsMTII* | A | G | - | CIP, C, NA, TE, G, AMP, AMC, CL, N, TR | - |
| | 5 | CS26 | *astA*, *iucD*, *kpsMTII* | A | G | C | CIP, NA, TE, AMP, AMC, CL, N, TR | - |
| | 6 | CS64 | *astA*, *iucD*, *kpsMTII* | B2 | C | G | CIP, NA, TE, AMP, AMC, CL, N, TR | - |
| | 7 | CS72 | *iucD*, *kpsMTII* | B2 | C | - | CIP, NA, TE, G, AMP, AMC, CL, N, TR | + *blaTEM* |
| | 8 | CS93 | *astA*, *papC*, *iucD*, *kpsMTII* | D | C, G | - | CIP, NA, TE, AMP, AMC, CL, N, TR | - |
| | 9 | CS95 | *papC*, *iucD*, *kpsMTII* | B2 | C, G | AMP | CIP, NA, TE, AMC, CL, N, TR | + *blaOXA* |
| | 10 | AF4 | *papC*, *kpsMTII* | D | C | - | CIP, NA, TE, G, AMP, AMC, CL, N, TR | - |
| Cloacal swabs of layers | 11 | LS11 | *iucD*, *kpsMTII* | D | C, G | AMP, AMC | CIP, NA, TE, CL, N, TR | - |
| | 12 | LS27 | *papC*, *kpsMTII* | B2 | C | CIP, G | NA, TE, AMP, AMC, CL, N, TR | - |
| | 13 | LS29 | *irp2*, *papC*, *iucD* | A | CL, C | CIP, G | NA, TE, AMP, AMC, N, TR | + *blaCTXM* |
| | 14 | LS30 | *papC*, *iucD* | A | C, G | CIP, AMP, AMC, N | NA, TE, CL, TR | - |
| | 15 | LS35 | *astA*, *iucD*, *kpsMTII* | B2 | C, G, AMP | AMC | CIP, NA, TE, CL, N, TR | - |
| | 16 | LS38 | *iucD*, *kpsMTII* | B2 | C | AMC | CIP, NA, TE, G, AMP, CL, N, TR | - |
| | 17 | LS42 | *astA*, *iucD*, *kpsMTII* | B2 | C, G. TR | CIP | NA, TE, AMP, AMC, CL, N | + |
| | 18 | LS46 | *iucD*, *tsh*, *kpsMTII* | B2 | C | G | CIP, NA, TE, AMP, AMC, CL, N, TR | - |
| Chicken samples | 19 | C22 | *iucD*, *kpsMTII* | A | - | C | CIP, NA, TE, G, AMP, AMC, CL, N, TR | - |

S-sensivitive; I - intermediate; R - resistant.

was predominantly present in the APEC strains (20). Frequency of detection of the *iss* gene was less in the *E. coli* isolates of healthy birds than diseased one (21). Isolates of our study were recovered from healthy birds, which showed a predominance of *iucD*, *papC*, and *kpsMTII* genes.

Chicken intestines can serve as a reservoir for *E. coli* strains capable of causing extraintestinal infections in human and thus the detection of ExPEC in poultry has public health significance. The cloacal swabs collected and investigated during this study were from healthy birds and none of the samples were from ill birds. This indicates the role of healthy birds in fecal shedding of ExPEC strains in the poultry environment. Poultry-associated *E. coli* often possesses virulence genes similar to those of human ExPEC, suggesting the potential to cause human disease. We could confirm only one strain as ExPEC out of 32 *E. coli* isolates of raw chicken origin. A high degree of ExPEC contamination of retail chicken meat, poultry carcasses, and turkey has also been recorded by other investigators (22). We could not detect any ExPEC strain in the fecal samples of turkey. In a previous study, ExPEC strains were isolated from intensive poultry farms in China (23).

ExPEC isolates of our study belong to three phylogenetic groups: B2 (47.36%), A (36.84%), and D (15.78%). A recent report from India demonstrated the significance of commercial broiler chicken in the transmission of multidrug-resistant commensal and pathogenic *E. coli* including ExPEC strains into the environment. The *E. coli* isolates of poultry origin belonged to phylogroups A and B1 (36% each), D (9%), C (8%), F (7%), and E and B2 (2%, each). Low prevalence of ExPEC was attributed to the collection of samples from healthy birds, which resulted in the low prevalence of phylogenetic group B2 (24). In our study, all the samples were collected from healthy birds; however, the occurrence of ExPEC and phylogroups B2 was recorded high. We could not detect phylogenetic group B1 in any of the *E. coli* isolates. ExPEC strains with the potential to cause infections mostly belong to phylogroups B2 and D (25). ExPEC isolates of our study were representatives of phylogroups B2 and D, which indicates their pathogenic potential and probable risk to humans. ExPEC strains liable for extraintestinal infections are distinct from commensal and diarrheagenic *E. coli* (DEC). Commensal *E. coli* are representative of A or B1, whereas DEC strains may be from A, B1, D, or ungrouped lineages. ExPEC strains are predominantly derived from groups B2 and D (26). *E. coli* strains of each pathotype show variation in the distribution of phylogenetic groups due to virulence characteristics. Variation has also been recorded in the phylogenetic grouping of ExPEC strains of poultry origin (27).

ESBL production was studied in 146 *E. coli* isolates comprising ExPEC and commensal strains. Overall, 23 (15.75%) *E. coli* isolates were phenotypically confirmed as ESBL producers. The difference in the zone diameter during phenotypic confirmation ranged from a minimum of 5 mm to a maximum of 20 mm. Water samples harbored maximum ESBL positive isolates as compared to other samples. Out of four ESBL-positive isolates from water, three were from carcass washing and one was from a water storage tank at the poultry farm. Out of 19 ExPEC strains, four (21.05%) were ESBL producers. Other than turkey, ESBL-positive *E. coli* was isolated from broilers, layers, ducks, raw chicken, and water samples in our study. ESBL-producing *E. coli* is considered as a global public health threat of emerging nature. An extensive study on *E. coli* of animal and human origin recovered from different states of India at the National *Salmonella* and *Escherichia* Centre, Kasauli, India revealed 15.3% *E. coli* as ESBL-positive strains and about 13.5% ESBL-positive *E. coli* were from poultry sources (28). Our findings are in close association with these observations.

Poultry and chicken meat has been considered as an important source of ESBL-producing *E. coli* for humans. Significant genetic similarities were observed in ESBL-positive *E. coli* isolated from chicken meat and humans (29). Although *E. coli* is an opportunistic pathogen for poultry, its zoonotic potential and risks associated with ESBL production need to be evaluated systematically. A study from Dutch broiler and layer farms revealed very high (65% and 81%, respectively) prevalence of ESBL-type *E. coli* (30).

Multiple antimicrobial resistance was commonly observed in the *E. coli* isolates irrespective of their sources in our study. Similarly, little variation was observed in the sensitivity and intermediate resistance patterns of ExPEC strains belonging to different phylogroups. All the strains including ExPEC were highly resistant to nalidixic acid, tetracycline, colistin, neomycin, and trimethoprim. Sensitivity was observed against chloramphenicol and gentamicin only. Turkey and duck origin *E. coli* were highly sensitive to chloramphenicol and gentamicin. Multiple antibiotic resistance (MAR) indices for individual ExPEC strains were calculated. The MAR index for ExPEC isolated from broiler birds was in the range of 0.7–0.9, whereas for ExPEC of layer origin it was 0.4–0.9 A single ExPEC isolated from chicken meat was also a multidrug-resistant strain with MAR index of 0.9. MAR index values above 0.3 are indicative of a high-risk source of contamination. These observations are alarming in the context of increasing antimicrobial resistance in veterinary pathogens of public health importance. Tetracycline, ciprofloxacin, and ESBL-type resistant *E. coli* have been recently isolated from chicken meat, broiler ceca, and dairy cattle from India (24,31).

Chromosomal or plasmid-encoded β-lactamases are mainly responsible for resistance to β-lactams in *E. coli* and other bacteria of the family Enterobacteriaceae. ESBLs are associated with resistance against most of the β-lactam antibiotics. The increased incidence of broad-spectrum β-lactamase-producing *E. coli* of human and animal origin has been documented globally (32). Of the 23 ESBL-positive isolates of our study, β-lactam genes encoding broad spectrum β-lactamases, namely *bla*CTX-M, *bla*TEM, and *bla*OXA, were detected in 52.17% isolates. *bla*CTXM was the predominant one, followed by *bla*TEM. Beta-lactam genes *bla*CTXM-15 (79%), *bla*TEM (63%), and *bla*SHV (32%) have been recently detected in *E. coli* isolated from raw chicken, fecal samples of broilers, and free range chickens from India (24). A study from Turkey also revealed the presence of *bla*TEM (24.24%) and *bla*CTXM (92%) in ESBL-positive *E. coli* isolated from a poultry slaughterhouse (33).

In conclusion, ExPEC strains of this study predominantly belonged to phylogenetic groups B2 and A. High percentages of *E. coli* were ESBL-positive strains harboring broad-spectrum β-lactamase genes *bla*CTXM and *bla*TEM. *E. coli* excreted in the feces of poultry were multidrug-resistant strains with MAR indexes of >0.4. ExPEC and ESBL-type *E. coli* may possess a zoonotic risk of indirect transmission from food and environmental sources. Thus, broilers and layer birds could be considered as reservoirs of ESBL-positive *E. coli*, including ExPEC strains belonging to phylogenetic groups B2 and A.

**Acknowledgment**
The authors are thankful to the Associate Dean, KNP College of Veterinary Science Shirwal, for financial support and the In Charge CIF for providing laboratory facilities for this research.

## References

1. Moyaert H, Jong AD, Simjee S, Thomas V. Antimicrobial resistance monitoring projects for zoonotic and indicator bacteria of animal origin: common aspects and differences between EASSA and EFSA. Vet Microbiol 2014; 171: 279-283.
2. Economou V, Gousia P. Agriculture and food animals as a source of antimicrobial-resistant bacteria. Infect Drug Resist 2015; 8: 49-61.
3. Bauchart P, Germon P, Bree A, Oswald E, Hacker J, Dobrindt U. Pathogenomic comparison of human extraintestinal and avian pathogenic *Escherichia coli*--search for factors involved in host specificity or zoonotic potential. Microb Pathog 2010; 49: 105-115.
4. Clermont O, Olier M, Hoede C, Diancourt L, Brisse S, Keroudean M, Glodt J, Picard B, Oswald E, Denamur E. Animal and human pathogenic *Escherichia coli* strains share common genetic backgrounds. Infect Genet Evol 2011; 11: 654-662.
5. Johnson JR, Murray AC, Gajewski A, Sullivan M, Snippes P, Kuskowski MA, Smith K. Isolation and molecular characterization of nalidixic acid-resistant extraintestinal pathogenic *Escherichia coli* from retail chicken products. Antimicrob Agents Chemother 2003; 47: 2161-2168.
6. Belanger L, Garenaux A, Harel J, Boulianne M, Nadeau E, Dozois CM. *Escherichia coli* from animal reservoirs as a potential source of human extraintestinal pathogenic *E. coli*. FEMS Immunol Med Microbiol 2011; 62: 1-10.
7. Ewers C, Antao E M, Diehl I, Philipp HC, Wieler LH. Intestine and environment of the chicken as reservoirs for extraintestinal pathogenic *Escherichia coli* strains with zoonotic potential. Appl Environ Microbiol 2009; 75: 184-192.
8. Smet A, Martel A, Persoons D, Dewulf J, Heyndrickx M, Cloeckaert A, Praud K, Claeys G, Catry B, Herman L et al. Comparative analysis of Extended-spectrum-b-lactamase (ESBL)-carrying plasmids from different members of Enterobacteriaceae isolated from poultry, pigs and humans: evidence for a shared b-lactam resistance gene pool? J Antimicrob Chemother 2009; 63: 1286-1288.
9. Dutta S, Guin S, Ghosh S, Pazhani GP, Rajendran K, Bhattacharya MK, Takeda Y, Nair GB, Ramamurthy T. Trends in the prevalence of diarrheagenic *Escherichia coli* among hospitalized diarrheal patients in Kolkata, India. PLoS One 2013; 8: e56068.
10. Kar D, Bandyopadhyay S, Dimri U, Mondal DB, Nanda PK, Das AK, Batabyal S, Dandapat P, Bandyopadhyay S. Antibacterial effect of silver nanoparticles and capsaicin against MDR-ESBL producing *Escherichia coli*: an in vitro study. Asian Pac J Trop Dis 2016; 6: 807-810.
11. Quinn PJ, Carter ME, Markey B, Carter GR. *Escherichia coli* species. In: Clinical Veterinary Microbiology. London, UK: Wolfe Publishing; 1994. pp. 254-258.
12. Ewers C, Janben T, Kiegling S, Philipp HC, Wieler LH. Rapid detection of virulence-associated genes in avian pathogenic *Escherichia coli* by multiplex polymerase chain reaction. Avian Dis 2005; 49: 269-273.
13. Johnson JR, Stell AL. Extended virulence genotypes *of Escherichia coli* strains from patients with urosepsis in relation to phylogeny and host compromise. J Infect Dis 2000; 181: 261-272.

14. Clermont O, Bonacorsi S, Bingen E. Rapid and simple determination of the *Escherichia coli* phylogenetic group. Appl Environ Microbiol 2000; 66: 4555-4558.

15. Clinical Laboratory Standard Institute. Performance Standards for Antimicrobial Susceptibility Testing: Twenty-Forth Informational Supplement (M 100-S 23). Wayne, PA, USA: CLSI; 2014.

16. Fang H, Ataker F, Hedin G, Dornbusch K. Molecular epidemiology of extended-spectrum β-lactamases among *Escherichia coli* isolates collected in a Swedish hospital and its associated health care facilities from 2001 to 2006. J Clin Microbiol 2008; 46: 707-712.

17. Moulin-Schouleur M, Répérant M, Laurent S, Brée A, Mignon-Grasteau S, Germon P, Rasschaert D, Schouler C. Extraintestinal pathogenic *Escherichia coli* strains of avian and human origin: link between phylogenetic relationships and common virulence patterns. J Clin Microbiol 2007; 45: 3366-3376.

18. Mitchell NM, Johnson JR, Johnston B, Curtiss R, Mellata M. Zoonotic potential of *Escherichia coli* isolates from retail chicken meat products and eggs. Appl Environ Microbiol 2015; *81*: 1177-1187.

19. Lyhs U, Ikonen I, Pohjanvirta T, Raninen K, Perko-Mäkelä P, Pelkonen S. Extraintestinal pathogenic *Escherichia coli* in poultry meat products on the Finnish retail market. Acta Vet Scand 2012; 54: 64-69.

20. Rodriguez-Siek KE, Giddings CW, Doetkott C, Johnson TJ, Fakh MK, Nolan LK. Comparison of *Escherichia coli* isolates implicated in human urinary tract infection and avian colibacillosis. Microbiology 2005; 151: 2097-2110.

21. Delicato ER, de Brito BG, Gaziri LC, Vidotto MC. Virulence-associated genes in *Escherichia coli* isolates from poultry with colibacillosis. Vet Microbiol 2003; 94: 97-103.

22. Aslam M, Toufeer M, Bravo CN, Lai V, Rempel H, Manges A, Diarra MS. Characterization of Extraintestinal Pathogenic *Escherichia coli* isolated from retail poultry meats from Alberta, Canada. Int J Food Microbiol 2014; 177: 49-56.

23. Gai W, Wang J, Cui Z, Qu Z, Cui J, Du X, Huang X, Zhao J. Molecular classification and drug resistance analysis of *Escherichia coli* isolated from poultry in China. Int J Clin Exp Med 2015; 8: 836-844.

24. Hussain A, Shaik S, Ranjan A, Nandanwar N, Tiwari SK, Majid M, Baddam R, Qureshi IA, Semmler T, Wieler LH et al. Risk of transmission of antimicrobial resistant *Escherichia coli* from commercial broiler and free-range retail chicken in India. Front Microbiol 2017; 8: 2120.

25. Smith JL, Fratamico PM, Gunther NW. Extraintestinal pathogenic *Escherichia coli.* Foodborne Pathog Dis 2007; 4: 134-163.

26. Johnson JR, Russo TA. Extraintestinal pathogenic *Escherichia coli*: "The other bad *E. coli*". J Lab Clin Med 2002; 139: 155-162.

27. Unno T, Han D, Jang J, Widmer K, Ko G, Sadowsky MJ, Hur HG. Genotypic and phenotypic trends in antibiotic resistant pathogenic *Escherichia coli* isolated from humans and farm animals in South Korea. Microbes Environ 2011; 26: 198-204.

28. Thakur R, Kumar Y, Singh V, Gupta N, Vaish VB, Gupta S. Serogroup distribution, antibiogram patterns and prevalence of ESBL production in *Escherichia coli.* Indian J Med Res 2016; 143: 521-524.

29. Kluytmans JA, Overdevest IT, Willemsen I, Kluytmans-van den Bergh MF, van der Zwaluw K, Heck M, Rijnsburger M, Vandenbroucke-Grauls CM, Savelkoul PH, Johnston BD et al. Extended-spectrum β-lactamase-producing *Escherichia coli* from retail chicken meat and humans: comparison of strains, plasmids, resistance genes, and virulence factors. Clin Infect Dis 2013; 56: 478-487.

30. Blaak H, van Hoek AHAM, Hamidjaja RA, van der Plaats RQJ, Kerkhof-de Heer L, de RodaHusman AM, Chets FM. Distribution, numbers, and diversity of ESBL-producing *E. coli* in the poultry farm environment. PLoS One 2015; 10: e0135402.

31. Kar D, Bandyopadhyay S, Bhattacharyya D, Samanta I, Mahanti A, Nanda PK, Mondal B, Dandapat P, Das AK, Dutta TK et al. Molecular and phylogenetic characterization of multidrug resistant extended spectrum beta-lactamase producing *Escherichia coli* isolated from poultry and cattle in Odisha, India. Infect Genet Evol 2015; 29: 82-90.

32. Smet A, Martel A, Persoons D, Dewulf J, Heyndrickx M, Catry B, Herman L, Haesebrouck F, Butaye P. Diversity of extended-spectrum beta-lactamases and class C beta-lactamases among cloacal *Escherichia coli* isolates in Belgian broiler farms. Antimicrob Agents Chemother 2008; 52: 1238-1243.

33. Ünal N, Karagöz A, Aşkar S, Dilik Z, Yurteri B. Extended-spectrum β-lactamases among cloacal *Escherichia coli* isolates in healthy broilers in Turkey. Turk J Vet Anim Sci 2017; 41: 72-76.