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Distribution of serotypes and molecular characterization of avian pathogenic *Escherichia coli* isolated from chicken died of colibacillosis

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ABSTRACT

Avian pathogenic *Escherichia coli* (APEC), can inflicts not only severe losses to the poultry industry due to morbidity and condemnations but also can pose a serious public health and food biosafety concern by playing a key role as an acceptor and donor of transmissible antimicrobial resistance mechanisms. Our studies on 71 APEC strains isolated from chicken died of colibacillosis, in Mizoram, India, revealed 13 different serotypes with predominance of O83 (35.21%). Of the 71 serotyped APEC strains, 67 (94.37%) are characterized as multidrug resistant with antimicrobial resistance as high as against 16 antibiotics tested. These strains harboured combination of up to 8 antimicrobial resistance genes tetA (92.96%), intl (70.42%), sul1 (59.15%), sul2 (56.34%), Dfrla (53.52%), Aad A (50.70%) in more than 50% of the strains. In addition, 8 different virulence associated genes with combination up to 7 genes together and with maximum frequency of fimC (97.18%), hlyE (80.28%), tsh (61.97%), fyuA (60.56%), irp2 (59.15%) and iuCD (57.75%) were detected. This is the first report on prevalence and heterogeneity of serotypes, pattern of antibiotic resistance and virulence genes content among APEC strains from North East region of India.

Keywords: AMR, APEC, Colibacillosis, India, Virulence, Zoonosis

Colibacillosis is one of the most predominant causes of economic loss in the poultry industry worldwide. The disease is characterized by fibrinous inflammation in several visceral organs resulting pericarditis, perihepatitis, peritonitis, cellulitis, sinusitis, air-sacculitis, swollen head syndrome, arthritis/synovitis, omphalitis, salpingitis and yolk sac infection. Avian pathogenic *Escherichia coli* (*E. coli*), the etiological agent of colibacillosis is a pathotype that belongs to the extra-intestinal pathogenic *E. coli* (ExPEC) group (Ewers *et al*. 2003, Barnes *et al.* 2008). However, a clear definition of this pathotype in respect to serogroups, specific virulence genes or a specific virulence assay is still missing (Ewers *et al*. 2003).

The emergence of *E. coli* isolates with multiple antibiotic-resistant phenotypes, involving co-resistance to four or more unrelated families of antibiotics is considered as a serious health concern for both animal and human population (Maynard *et al*. 2003). Recent studies in different parts of India have reported antimicrobial residues in food animal products such as milk and chicken meat, indicating that antimicrobial usage is widespread in food animal production (Laxminarayan and Chaudhury 2016). Such practices lead to emergence of antibiotic resistant bacteria in high proportion and can extensively contaminate poultry meat at slaughtering, leading to serious public health concern (Lutful 2010, Chen and Jiang 2014). Further, the similarities between the virulence genes and phylogenetic backgrounds of avian and human ExPEC are of a great concern for zoonotic risk (Dziva *et al.* 2013). In humans, ExPEC causes several extra-intestinal diseases, including urinary tract infections, neonatal meningitis and sepsis. APEC are linked to these human diseases (Ewers *et al*. 2007, Mellata *et al*. 2009, Hussain *et al*. 2012, Nandanwar *et al*. 2014, Ranjan *et al*. 2017). Thus, poultry may play important source for dissemination of antimicrobial resistant *E. coli* in the community and environment (van den Bogaard *et al*. 2001).

In India, information regarding molecular epidemiology of *E. coli* is restricted mostly to faecal isolates from healthy poultry and very little is known about APEC in commercial flocks. To understand the diversity and molecular epidemiology of APEC and their potential to become a source for serious public health and food biosafety concern, we have investigated the prevalence and heterogeneity of serotypes, the pattern of antibiotic resistance and virulence genes content among APEC strains isolated from commercial chicken died of colibacillosis.

MATERIALS AND METHODS

Collection of samples: Outbreaks of Colibacillosis in 18 different poultry farms located in Aizawl district of Mizoram state was confirmed on the basis of clinico-pathological

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studies followed by isolation and identification of *E. coli* in affected birds. Detailed post mortem examination was performed on total 206 dead birds from affected farms and gross lesions were recorded. Heart blood and swab samples from liver were collected aseptically for bacteriological examination.

Isolation, identification and serotyping of E. coli: Collected samples from dead birds bearing characteristics lesions of colibacillosis were immediately inoculated on MacConkey (HiMedia, Mumbai, India) agar plates and incubated at 37°C for 18–24 h. Bright pink single colonies were picked up and sub cultured on eosin methylene blue (EMB) agar (HiMedia, Mumbai, India) plates to observe the characteristic metallic sheen of *E. coli*. The well separated pure colonies were picked up on nutrient agar slants as pure culture and subjected for standard morphological and biochemical tests. A total of 123 isolates after confirmation by morphological and biochemical tests and detection of 16S ribosomal DNA gene of *E. coli* were serotyped at National Salmonella and Escherichia Centre, Central Research Institute, Kasauli, India, based on their somatic (O) antigens.

Antimicrobial susceptibility testing: Antimicrobial susceptibility testing was performed by the Kirby-Bauer disc diffusion method using Mueller-Hinton agar (HiMedia Laboratories, Mumbai, India, MV1084), as per the recommendation of Clinical Laboratory Standard Institute (CLSI, 2013) using 18 commercially available antibiotic discs (Supplementary Table 1). Zones of inhibition were measured using zone size interpretative chart furnished by the manufacturer.

Preparation of E. coli *DNA for PCR assay:* For rapid detection of antimicrobial resistance and virulence associated genes, isolated bacterial cultures were inoculated into 2 ml Luria Bartani (L-B) broth and incubated at 37°C under constant shaking for 18 h. After incubation, 1 ml broth culture was taken in a 1.5 ml micro centrifuge tube and centrifuged at 5,867 g for 10 min. The pellet was washed twice in sterile normal saline solution (NSS) (0.85% NaCl) and re-suspended in 400 µl of nuclease-free sterile distilled water and boiled for 10 min followed by immediate chilling. Cell debris was removed by centrifugation at 2,292 g for 5 min. The supernatant was used as template DNA for PCR.

Detection of 16S ribosomal DNA gene of E. coli*:* All the *E. coli* isolates identified by morphological and biochemical test were further confirmed by detection of 16S ribosomal DNA gene of *E. coli*, using the primer EC16-F: GACCTCGGTTTAGTTCACAGA and EC16-R: CACACGCTGACGCTGACCA8 (Candrian *et al.* 1991). The amplified PCR product of 585 bp was visualized under UV transilluminator (Alpha Imager) in 1.5% agarose gel electrophoresis.

Detection of antimicrobial resistance genes PCR: The presence of antibiotic resistance genes in 71 serotyped *E. coli* strains was analyzed by PCR. PCR reactions were performed in a total volume of 25 µl, containing 2.5 µl of 10X Dream Taq Buffer, 0.2 mM of each dNTP, 0.1–1 mM of forward and reverse primer, 1.25U of Dream Taq DNA polymerase (Fermentas), and 5 µl (40–260 ng/µl) of DNA. Amplification reactions were carried out using a thermocycler (Eppendrof Mastercycler, Hamburg, Germany) as follows: 3 mins at 95°C, 35 cycles each consisting of 1 min at 94°C, 90s at specific annealing temperature as described earlier (Supplementary Table 2) and 1 min at 72°C, followed by a final extension step of 10 min at 72°C. Amplified samples were analyzed by electrophoresis in 1.5% agarose gel and stained by ethidium bromide. A molecular weight marker with 100 bp DNA ladder (Fermentas) was used as a size standard.

Detection of virulence genes by multiplex PCR: PCR was carried out to detect Haemolysin A (hlyA), Enteroaggregative toxin (astA), P fimbriae (papC), Aerobactin (iuCD), Temperature sensitive haemagglutinin (tsh), Haemolysisn E (hlyE), Iron acquisition related factors ferric yersiniabactin (fyuA), Iron repressible protein (irp2), Colonization factors F1 - fimbriae (fimC), Cytotoxic necrotizing factor (cnf- sense), all intimin genes (SK), EHEC-hemolycin (eha) and Shiga toxin 1&2 (Lin) (stx) genes in all the isolates using published oligonucleotide primers and thermal profile (Supplementary Table 3). The PCR reaction was performed in a thermal cycler (Eppendorf, Germany) and amplified products were visualized in 1.5% agarose gel electrophoresis and the size of the PCR product was compared to the GeneRulerTM 100 bp DNA Ladder (Fermentas Life Sciences, Ottawa, ON, Canada).

Cloning and sequencing of virulence and antimicrobial resistance genes: Five from each of the virulence and antimicrobial resistance genes were cloned in pTZ57R/T vector using InsT/AcloneTM PCR product cloning kit (Fermentas Life Sciences, Ottawa, ON, Canada). The recombinant plasmids containing gene fragments were sequenced at DNA sequencing facility, South campus, Delhi University, New Delhi, India. The generated sequences were analyzed (data not included) and accession no. allotted to the sequences (Supplementary Table 4).

RESULTS AND DISCUSSION

We have initially investigated a total of 123 APEC strains isolated from 18 different commercial farms affected with collibacillosis. Only 71 (57.72%) isolates out of 123 were serotyped into 13 different serotypes based on their somatic (O) antigens and rest 52 (42.28%) isolates were untypable (Table 1). The most common types of O antigen was O83 (35.21%), followed by O88 (16.90%), O119 (14.08%), O17 (8.45%), O8 (7.04%), O34 and O126 (each 4.23%), O11 (2.82%), O2, O7, 049, O84 and O149 (each 1.41%).

There are more than 180 forms of O antigen recognized for *E. coli* till date and each serotype of *E. coli* has been considered to have an important role in clinical presentation of diseases (Wang *et al*. 2012, Mellata 2013). Earlier studies on serotyping of APEC isolates have revealed that APEC are usually belongs to certain O serogroups, especially of O1, O2, O8, O15, O18, O35, O78, O88, O109, and O115 or is often non-typable (McPeake *et al.* 2005). Among these

Table 1. Antibiotic susceptibility pattern of *E. coli* isolate from chicken died of colibacillosis

Isolate	Serotype	Resistant against	Total
Th3	O2		0
St10(Lu)	O7	AMX, AMP, CN, COT, NA, PI, S, SF, TE, TR	10
Dn13	O8	AMX, AMP, CN, CTX, CIP, COT, NA, PI, S, SF, TE, TR	12
CF8	O8	AT, CN, CTX, CAZ, CIP, COT, NA, PI, SF, TE, TR	11
St16	O8	AT, CAZ, S	3
St12	O8	CTX, CIP, NA	3
St13(Li)	O8	CTX, CIP, NA, S, TE	5
Dn12	O11	AMX, AMP, CN, CTX, CAZ, CIP, COT, NA, PI, S, SF, TE, TR	13
St14	O11	AMX, AMP, AT, CN, CFM, CTX, CAZ, CTR, COT, NA, PI, SF, TE, TR	14
Dn13	O17	AMX, AMP, CN, CTX, CIP, COT, NA, PI, S, SF, TE, TR	12
Dn13a	O17	AMX, AMP, CTX, CAZ, CIP, COT, NA, PI, S, SF, TE, TR	12
Dn8	O17	AMX, AMP, CIP, COT, NA, PI, S, SF, TE, TR	10
DN11	O17	AMX, AMP, CN, CFM, CIP, COT, NA, PI, S, SF, TE, TR	12
St7	O17	AMX, AMP, CN, CIP, COT, NA, PI, SF, TE, TR	10
St23(Ylk)	O17	AMP, CAZ, COT, GEN, NA, SF, TE, TR	8
St17	O34	AT, CFM, CTX, CAZ, CTR, CIP, NA	7
Dn14	O34	AMX, AMP, CN, CTX, CIP, COT, NA, PI, S, SF, TE, TR	12
CF2	O34	AMP, AT, CN, CTX, CAZ, CIP, COT, NA, PI, SF, TE, TR	12
Se5	O49	AT, CAZ, CIP, S	4
Dn10	O83	AMX, AMP, NA, TE, TR	5
Lma	O83	AMX, AMP, CIP, COT, NA, PI, TR	7
LM (Li)	O83	AMX, AMP, CTX, CIP, NA, PI, S, SF, TE, TR	10
SP3	O83	AMX, AMP, PI, SF, TE	5
St10(Li)	O83	AMX, AMP, CN, COT, NA, PI, SF, TR	8
Th2	O83	NA, TE	2
Zm1(Li)	O83	CN, NA, TE	3
Sta	O83	AMX, AMP, CFM, CTX, CIP, COT, NA, PI, SF, TR	10
St1 (Li)	O83	AMX, AMP, CTX, COT, NA, PI, S, SF, TE, TR	10
St11	O83	AMX, CIP, COT, NA, PI, S, SF, TE, TR	9
SP5	O83	AMX, AMP, COT, GEN	4
St13	O83	CTX, CIP, NA, PI, TE	5
St13(Lu)	O83	NA	1
Zm1	O83	PI	1
Zm1(Lu)	O83	NA, TE	2
Zm2	O83	AMX, PI	2
BN	O83	AMX, AMP, CIP, COT, NA, PI, S, SF, TE, TR	10
ST1	O83	AMX, AMP, CTX, COT, NA, PI, SF, TE, TR	9
St2 (Li)	O83	AMX, AMP, CN, COT, NA, PI, S, SF, TE, TR	10
St7 HS	O83	AMX, AMP, CN, CIP, COT, NA, PI, TE, TR	9
SP8	O83	AMX, AMP, AT, CN, CFM, CTX, CAZ, CTR, CIP, COT, NA, PI, S, SF, TE, TR	16
Se6	O83	AMP, CAZ, IPM, NA, PI	5
Sp7(Li)	O83	AMX, AMP, CN, CTX, COT, NA, PI, S, SF, TE, TR	11
St5	O83	AMX, AMP, CIP, COT, NA, PI, SF, TR	8
Sp2	O83	AMX, AMP, CIP, COT, NA, PI, S, SF, TE, TR	10
Dn12a	O84	AMX, AMP, CN, CTX, CAZ, CIP, COT, NA, PI, S, SF, TE, TR	13
Dn4A	O88	AMX, AMP, CIP, NA, PI, TE	6
Th1(Li)	O88	AMX, AMP, COT, NA, PI, SF, TE, TR	8
SP3(Li)	O88	AMX, AMP, PI, TE	4
St7 (Slp)	O88	AMX, AMP, CN, CTX, CIP, COT, NA, PI, SF, TE, TR	11
St4	O88	AMX, AMP, CTX, CIP, COT, GEN, NA, PI, S, SF, TE	11
St19a (HS)	O88	CTX, NA, TE	3
Swab1P	O88		0
CF	O88	AMX, AMP, CN, CTX, CAZ, GEN, NA, PI, TE	9
CF3	O88	AMP, AT, CN, CTX, CAZ, CIP, COT, NA, PI, S, SF, TE, TR	13
DN6	O88	AMX, AMP, CIP, COT, NA, PI, S, SF, TE, TR	10
SP4	O88	AMX, AMP, CN, COT, NA, PI, S, SF, TE, TR	10
SP8 (Int)	O88	AMX, AMP, CN, CFM, CTX, CAZ, CTR, CIP, COT, NA, PI, S, SF, TE, TR, AT	16
Dn13(Li)	O119	AMX, AMP, CTX, CIP, COT, NA, PI, S, SF, TE, TR	11

(*Contd...*)

(*Table 1 Contd...*)

Isolate	Serotype	Resistant against	Total
Dn14(Li)	O119	AMX, AMP, CN, CIP, COT, NA, PI, S, SF, TE, TR	11
St18	O119	AMX, AMP, CN, CIP, COT, NA, PI, S, SF, TE, TR	11
Dn7	O119	AMX, AMP, CIP, COT, NA, PI, S, SF, TE, TR	10
Dn13a (Li)	O119	AMX, AMP, CTX, CIP, COT, NA, PI, S, SF, TE, TR	11
St7 (Ovd)	O119	AMX, AMP, CN, CTX, CIP, COT, NA, PI, SF, TE, TR	11
St19(Ova)	O119	CTX, CAZ, GEN, NA, PI, S, TE	7
St19(HS)	O119	CAZ, CTX, GEN, NA, TE	5
Zm5	O119	CN, CTX, CAZ, CIP, NA, PI, TE	7
CF4	O119	AMX, AMP, AT, CN, CFM, CTX, CAZ, CTR, COT, NA, PI, SF, TE, TR	14
Zm4 (Li)	O126	AMX, AMP, CN, CTX, COT, NA, PI, SF, TE, TR	10
St10	O126	AMX, AMP, CN, COT, NA, PI, S, SF, TE, TR,	10
St8	O126	AMX, AMP, CTX, CIP, COT, GEN, NA, PI, S, SF, TE, TR	12
Sp4	O149	AMX, AMP, CN, COT, NA, PI, S, SF, TE, TR	10

O1, O2, and O78 are most frequently isolated from colibacillosis in many countries worldwide indicating their involvement in extraintestinal infections. Similar finding was recorded from Jammu and Kashmir with predominance of O78 (17.8%), O76 (15.59%), O8 (14.45%), and O1 (12.17%) serotypes (Amin *et al*. 2017; Younis *et al*. 2017). Our studies from North East Region (NER) of India, has recorded predominance of serotype O83 (35.21%) followed by O88 (16.90%) along with maximum combination of antimicrobial resistance and virulence associated genes (Table 2a & 2b, Table 3a & 3b). The most prevalent serotypes of Northernmost region of India was not even detected in North Eastern region. Apart from revealing the variability in distribution of different serogroups of APEC in different geographical regions, these findings has differed the earlier findings of linking certain specific serotypes with APEC. Several studies on O-serotyping of the uropathogenic *E. coli* (UPEC) of human population revealed O6 and O2 as the most common serotype associated with UTI (Blanco *et al.* 1996). Although one strain has been identified as O2, none of the APEC strains in the present study were of O6 serotype. However, to best of our knowledge no information regarding distribution and prevalence of O-serotype of ExPEC, causing extra-intestinal diseases in human population of NER, India, is available to compare and rule out their possible link with APEC prevailing in poultry population of the region.

All the 71 isolates were further investigated for antimicrobial susceptibility and detection of antimicrobial resistance and virulence associated genes. The antimicrobial sensitivity test reveals 67 (94.37%) APEC strains as multidrug resistant (Table 1). Two of the isolates (SP8, SP8int) belonging to the serotype O83 and O88 respectively revealed resistance against 16 antibiotics tested. The most common resistance phenotypes were against: nalidixic acid (87.32%), tetracycline (78.87%), Piperacillin (77.46%), ampicillin (70.42%), Amoxicillin (69.01%), Trimethoprim (64.79%), Co-Trimoxazole (64.79%), Sulphafurazole (63.38%), Ciprofloxacin (54.93%), Cefotaxime (47.89%), streptomycin (47.89%), Cefalexin (42.25%), Ceftazidime (26.76%), Aztreonam (14.08%), Gentamicin (9.86%), Ceftriaxone (7.04%) and Imipenem (1.40%).

There are very few reports related to the molecular basis of resistance in APEC strains are available in India. Therefore, the presence of genes associated with resistance to streptomycin (aadA1), tetracycline [tet(A), tet(B)],

Table 2a. Prevalence of antimicrobial resistance genes in APEC field strains (n=71), as detected by PCR in association to serogroups

Serogroup	tetA	Intl-1	Sul1	Sul2	Dfrla	AadA	blaTEM	blacMY	tetB	Intl-2	blaSHV	blaCTX	1CS	Ti
All strains (n= 71)	66	50	42	40	38	36	31	12	6	3	0	0	0	0
O2 (n=1)	1	–	–	–	–	–	–	–	–	–	–	–	–	–
O7 (n=1)	1	1	1	1	1	–	1	–	–	–	–	–	–	–
O8 (n=5)	5	3	3	3	3	3	2	1	–	–	–	–	–	–
O11 (n=2)	2	1	1	1	1	2	1	1	–	–	–	–	–	–
O17 (n=6)	6	5	4	4	5	3	3	1	–	–	–	–	–	–
O34 (n=3)	3	3	2	3	2	3	1	–	–	–	–	–	–	–
O49 (n=1)	1	1	1	–	1	–	–	–	–	–	–	–	–	–
O83 (n=25)	21	17	14	13	12	12	11	2	4	2	–	–	–	–
O84 (n=1)	1	1	1	1	1	1	–	–	–	–	–	–	–	–
O88 (n=12)	11	8	5	8	6	5	6	4	2	1	–	–	–	–
O119 (n=10)	10	7	8	5	5	5	3	2	–	–	–	–	–	–
O126 (n=3)	3	3	1	1	1	2	3	–	–	–	–	–	–	–

Table 2b. Percentage of strains with the given pair of antimicrobial resistance genes among 71 serotyped APEC isolated from chicken died of colibacillosis

AMR genes	tetA	Intl-1	Sul1	Sul2	Dfrla	AadA	bla TEM	blac MY	tetB	Intl-2	bla SHV	bla CTX	1CS	Ti
tetA														
Intl -1	47 (68.20%)													
Sul1	40 (58.34%)	36 (50.70%)												
Sul2	37 (52.11%)	33 (48.48%)	25 (35.21%)											
Dfrla	37 (52.11%)	36 (50.70%)	29 (40.85%)	23 (32.39%)										
AadA	34 (47.89%)	31 (43.66%)	29 (40.85%)	21 (29.58%)	19 (26.76%)									
blaTEM	28 (39.44%)	26 (38.62%)	15 (21.13%)	11 (19.49%)	9 (12.68%)	6 (8.45%)								
blacMY	12 (18.90%)	6 (8.45%)	3 (4.23%)	2 (2.82%)	2 (2.82%)	2 (2.82%)	2 (2.82%)							
tetB	3 (4.23%)	1 (1.41%)	1 (1.41%)	0	0	0	0	0						
Intl-2	3 (4.23%)	2 (2.82%)	2 (2.82%)	1 (1.41%)	1 (1.41%)	1 (1.41%)	0	0	0					
blaSHV	0	0	0	0	0	0	0	0	0	0				
blaCTX	0	0	0	0	0	0	0	0	0	0	0			
1CS	0	0	0	0	0	0	0	0	0	0	0	0		
Ti	0	0	0	0	0	0	0	0	0	0	0	0	0	

Table 3a. Prevalence of virulence associated genes in APEC field strains (n=71), as detected by PCR in association to serogroups

Serogroup	fimC	hlyE	tsh	fyuA	irp2	iuCD	astA	papC	cnf-sense	SK	eha	stx	hlyA
All strains (n= 71)	69.00	57.00	44.00	43.00	42.00	41.00	7.0	3.00	–	–	–	–	–
O2 (n=1)	–	1.0	–	–	–	–	1.0	–	–	–	–	–	–
O7 (n=1)	1.0	1.0	–	1.0	1.0	1.0	0.0	–	–	–	–	–	–
O8 (n=5)	5.0	3.0	5.0	5.0	5.0	3.0	0.0	–	–	–	–	–	–
O11 (n=2)	2.0	2.0	1.0	1.0	1.0	1.0	0.0	–	–	–	–	–	–
O17 (n=6)	5.0	5.0	4.0	4.0	4.0	5.0	0.0	1.0	–	–	–	–	–
O34 (n=3)	3.0	3.0	2.0	2.0	2.0	2.0	0.0	–	–	–	–	–	–
O49 (n=1)	1.0	1.0	–	–	–	1.0	0.0	–	–	–	–	–	–
O83 (n=25)	25.0	20.0	13.0	16.0	16.0	12.0	5.0	1.0	–	–	–	–	–
O84 (n=1)	1.0	1.0	1.0	1.0	1.0	1.0	0.0	–	–	–	–	–	–
O88 (n=12)	12.0	9.0	8.0	5.0	4.0	6.0	1.0	1.0	–	–	–	–	–
O119 (n=10)	10.0	8.0	8.0	5.0	5.0	6.0	–	–	–	–	–	–	–
O126 (n=3)	3.0	3.0	1.0	2.0	2.0	2.0	–	–	–	–	–	–	–
O149 (n=1)	1.0	–	1.0	1.0	1.0	1.0	–	–	–	–	–	–	–

Table 3b. Percentage of strains with the given pair of virulence associated genes among 71 serotyped APEC isolated from chicken died of colibacillosis

Virulence gene	fimC	hlyE	tsh	fyuA	irp2	iuCD	astA	papC
fimC								–
hlyE	55 (77.46%)							–
tsh	43 (60.56%)	32 (45.07%)						–
fyuA	43 (60.56%)	33 (46.48%)	27 (38.03%)					–
irp2	41 (57.75%)	32 (45.07%)	27 (38.03%)	27 (38.03%)				–
iuCD	40 (56.34%)	35 (49.30%)	25 (35.21%)	21 (29.58%)	21 (29.58%)			–
astA	6 (8.45%)	4 (5.63%)	1 (1.41%)	1 (1.41%)	1 (1.41%)	0		–
papC	3 (4.23%)	1 (1.41%)	0	0	0	0	0	–

trimethoprim (dfrA1), sulfonamides (sul1, sul 2) and ampicillin/beta-lactams (blaSHV, blaCMY, blaCTX, blaTEM) were determined by PCR in the present study (Table 2a & 2b). Of the 71 APEC strains, 66 (92.96%) were found to harbour the tetA gene, which confer resistance to tetracycline. Another tet gene of class B was detected in 6 strains (8.45%) and 3 of them carried both the genes. The prevalence of trimethoprim resistance conferring dihydrofolate reductase gene (dfrA) was detected in 38 (53.52%) strains. Currently, three different types of dihydropteroate synthase (DHPS) gene (sul1, sul2 and sul3), which confers resistance to sulphonamides have been identified (Sko¨ld 2000). The present study has detected, the sul1 and sul2 genes in total 42 (59.15%) and 40 (56.34%) strains respectively and 27 (38.03%) of those strains showed both the genes. The aadA gene, which encodes resistance to streptomycin and spectinomycin via an adenylyl transferase was detected in 36 (50.70%) strains. Among the ESBL, blaTEM was detected in 32 strains, blaCMY in 12 strains, while none of the strain harboured blaSHV, and blaCTX (Table 2a and 2b).

A substantial proportion of resistance determinants in Gram negative bacteria reside on class 1 integrons that are capable of capturing and expressing genes contained in cassette-like structures (Fluit and Schmitz 1999). Therefore, all the 71 strains were also screened for the presence of class 1 and 2 integrons, viz, intl1 and intl2, as well as its gene cassettes 5CS/ 3CS and TiB/TiF. The intl1 was detected in 50 strains (70.42%), while intl2 was detected only in 3 (4.23%) of the strains. Two of the strain (2.81%) had both class 1 and class 2 integrase genes and one strain (1.41%) had only the class 2 integrase (intI2). Integrons have been identified as a primary source of resistance genes and were suspected to serve as reservoirs of antimicrobial resistance genes within microbial populations. We have observed the class 1 integrase (intI1) in all the APEC strains, which are bearing 3 or more antimicrobial resistance genes. Two of the strains, which carried 5–6 antimicrobial resistance genes, had both intl1 and intl2. Earlier studies have revealed the prevalence of integrons as 49% in Australia (White *et al*. 2000), 54% in Taiwan (Chang *et al*. 2000), 54.6% in Korea (Yu *et al*. 2003) and 60% in European countries (Martinez-Freijo *et al*. 1998) in clinical *E. coli* isolates, while present study has recorded 70.42% prevalence of integrons in the APEC isolates.

PCR assay to detect 13 different virulence associated genes has yielded detection of 8 different genes with highly varying frequency (Table 3a & 3b). While none of the 71 isolates harboured cnf Sense, SK, eha, stx and hlyA genes; fimC gene was detected in 69 of the strain (97.18%) followed by hlyE gene in 57 strain (80.28%), tsh gene in 44 strain (61.97%), fyuA gene in 43 strain (60.56%), irp2 gene in 42 strain (59.15%), iuCD gene in 41 strain (57.75%), astA gene in 7 strain (9.86%) and papC gene only in 3 strains (4.23%). Maximum number of total 7 genes together were detected in the isolate Zm1(Lun) followed by 6 genes together in 23 strains, 5 genes in 14 strain, 4 genes in 10 strain, 3 genes in 7 strain, 2 genes in 14 strains.

APEC strains are very diverse, and their diversity is attributed to the diversity of their virulence factors and serotypes (Mellata *et al*. 2009). Although the underlying mechanisms by which APEC cause disease is still not fully understood (Ewers *et al*. 2004), a close relationship between virulence factors and pathogenicity of APEC has been reported (Janben *et al*. 2001, Dziva and Stevens 2008, Tyler *et al*. 2008). Genes coding for adhesins, toxins or iron acquisition systems have been described to be of particular importance during the pathogenesis of septicaemia. The fyuA (ferric yersiniabactin uptake) and irp2 (iron-repressible protein) gene, coding for proteins involved in iron acquisition are found in human septicaemic and entero-aggregative *E. coli* isolates (Karch *et al*. 1999) and has recently been described in avian *E. coli* isolates (Gophna *et al*. 2001). We have detected the prevalence of fimC (97.18%), hlyE (80.28%), tsh (61.97%), fyuA (60.56%), irp2 (59.15%) and iuCD gene (57.75%) significantly in higher frequency. According to the genetic criteria, the pathogenicity of APEC strain is determined by presence of at least five virulence genes (De Carli *et al*. 2015). Of the 71 APEC strains analysed, 39 (54.93%) carried 5 or more than 5 virulence genes. Isolate Zm1(Lu), belonging to serogroup O83 carried as high as 7 virulence genes and 23(32.39%) other strains carried 6 virulence genes of different combination (Table 3a & 3b). Combination of tsh, hlyE, fyuA, irp2, fimC genes together was observed in 22 (30.99%), while astA, papC and iuCD was detected in lower frequency. Earlier many studies reported that a high proportion of APEC isolated from chickens, turkeys and pigeons possessed stx genes and thus these birds were considered to be the reservoirs of STEC (Grossmann *et al*. 2005, Dutta *et al*. 2011). However, we have not found the cnf-sense, SK, eha, stx and hlyA genes in any of the 71 strains.

Indiscriminate use of antibiotics, not only as therapeutics but also as growth promoter and disease preventive measures in poultry (Osti *et al*. 2017) is leading to evolution of multidrug resistant *E. coli* strains. Further, transference of these resistance determinants by mobile genetic elements including plasmids, transposons, and gene cassettes in integrons (Carattoli 2001) and the alteration in mar locus regulation (McPeake *et al*. 2005, Lutful 2010, Amin *et al*. 2017) are contributing to expansion of these multi drug resistant bacteria. This study has demonstrated significantly high distribution of antimicrobial resistance genes in combination with the virulence genes in the multidrug resistant APEC strains isolated from chicken died of colibacillosis. The fact that this flora may play a key role as an acceptor and donor of transmissible antimicrobial resistance mechanisms may also result in a serious public health and food biosafety concern.

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