Isolation, virulence gene profiling with molecular cloning of ibeA gene and antibiogram of *Escherichia coli* from respiratory tract infections of broiler chicken in Kashmir, India

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Received: November 5, 2016; Revised received: April 5, 2017; Accepted: August 13, 2017

**Abstract:** The present study has determined prevalence, serological diversity, virulence gene profile and *in vitro* antibiogram of *Escherichia coli* (*E. coli*) isolates from respiratory tract of broiler chicken in Kashmir valley along with molecular cloning of ibeA gene, an important zoonotic among invasion-associated genes responsible for neonatal meningitis in humans. A total of 224 broiler chickens with a history of respiratory tract infections, in a flock size of 2692 birds from organised and unorganised poultry farms of Kashmir valley, were screened for *E. coli* isolation. The prevalence of *E. coli* in birds with respiratory tract infection was found to be 6.38 per cent. Serogrouping revealed that the *E. coli* isolates were typeable into 10 serogroups with a prevalence rate of 8.8% for serogroup O2, along with few isolates rough or untypeable. The screening of *E. coli* isolates for virulence gene profile revealed the presence of *iss* in (89.53%), *lucDin* (85.46%), *tsh* in (51.74%), *cva/cvi* in (33.14%), *irp2* in (55.23%), *papCin* (33.72%), *vat* in (31.39%), *astAin* (38.95%), *timCin* (93.60%) and *ibeAin* (1.16%) isolates. The antibiogram screening of *E. coli* isolates revealed the majority of isolates to be resistant to tetracycline (94.71%), ampicillin (92.67%) and cephalaxin (79.67%). However, most of the isolates were sensitive to amikacin (74.41%) followed by amoxicillin-sulbactam (68.60%) and gentamicin (67.44%). The study thus, suggests that the *E. coli* isolates from respiratory tract infections of poultry harbour differential virulence gene profiles along with the genes of zoonotic interest and variable antimicrobial resistance, thus, opening the areas for devising dynamic, emergent and effective treatment line.

**Keywords:** Antiobigram, *Escherichia coli*, Prevalence, Virulence genes

**INTRODUCTION**

Respiratory diseases in avian species occur either as a primary disease or as secondary complications to viral diseases, causing considerable mortality, morbidity, decreased production and economic losses all over the world (Schouler et al., 2012). The bacterial pathogens associated with the respiratory tract infections in birds mainly include *Pasteurella*, *Staphylococcus*, *Haemophilus*, *Bordetella*, *Mycoplasma*, *Pseudomonas* and *Escherichia coli* (Ammar et al., 2017). *Escherichia coli* (*E. coli*), a rod-shaped, gram-negative, facultative anaerobic bacterium from *Enterobacteriaceae* family, is a ubiquitous organism in poultry production. It is a normal commensal bacterium of the gut and upper respiratory tract microflora of chicken which typically colonize within a few hours after hatching. The normal respiratory tract has the capability to remove *E. coli* and other bacteria from deep in the respiratory tract due to the action of ciliated epithelial cells, lining the trachea. However, any insult to the respiratory tract of chickens creates a climate for potential colonization of the respiratory tract by APEC (avian pathogenic *E. coli*) (Kabir, 2010). The respiratory infection caused by pathogenic *E. coli* strains is considered an initial step for colisepticemia development in birds and is widely prevalent in all age group of chickens (9.52 to 36.73%) with specially high prevalence rate in adult layer birds (36.73%) (Rahman et al., 2004).

Antimicrobial agents are the cornerstone for treatment of *E. coli* infections in poultry. Experience suggests that antimicrobial resistance genes readily emerge in the presence of the relevant selective antimicrobial pressure. APEC is a pathogen of zoonotic potential and the detection of antimicrobial resistance among avian *E. coli* is therefore a serious cause of concern for both avian species as well as humans (Kabir, 2010). The present study was undertaken with the objectives...
to determine the prevalence, distribution of virulence genes (iss, iucD, irp2, tsh, astA, papC, vat, cva/cvi, fimC and ibeA) and in-vitro antibiogram of E. coli isolates.

MATERIALS AND METHODS

Sampling and isolation of E. coli isolates: 224 each of nasal swabs, tracheal swab and air sacs were collected adopting standard aseptic measures, for isolation of E. coli from clinically affected chickens with a history of respiratory infection from Govt. Poultry Farm, Hariparbat, Central Veterinary Hospital, Gowkadal and other unorganised farms of Kashmir Valley. Isolation of E. coli was performed by plating the sample on MacConkey agar (Hi Media, Mumbai, India) and incubating at 37°C overnight. Rose pink colonies from each sample were picked and subcultured on nutrient agar slants as pure culture and observed characteristic metallic sheen. The well separated colonies were subcultured on eosine methylene blue agar (EMB) (Hi Media, Mumbai, India) and incubating at 37°C overnight. Rose pink colonies from each sample were picked and subcultured on nutrient agar slants as pure culture and observed characteristic metallic sheen. The well separated colonies were subcultured on eosine methylene blue agar (EMB) (Hi Media, Mumbai, India) and incubating at 37°C overnight.

Serogrouping: A total of 172 variable representatives (having same virulence gene profile only one representative E. coli isolate from the group was chosen) among the multiple E. coli isolates were sent to National Salmonella and Escherichia Centre, Central Research Institute, Kasauli-173204, Himachal Pradesh, for serogrouping.

Extraction of bacterial DNA: E. coli isolates were grown in nutrient broth (Hi-Media) at 37°C overnight. Organisms from 1.5 ml growth were pelleted by centrifugation at 10,000 rpm for 10 min. The bacterial pellet was re-suspended in 150 μl of sterile distilled water. The bacteria were lysed by boiling for 10 min in a water bath. The lysate was centrifuged and the supernatant was used directly as template for PCR.

Molecular profiling of virulence genes: All the E. coli isolates (n= 172) were screened by multiplex polymerase chain reaction (m-PCR) using thermo cycler (eppendorf, Germany) with initial denaturation for 3 minutes at 94°C, followed by 25 cycles of denaturation at 94°C for 30 seconds but annealing at 58°C for 30 seconds for iss, iucD, tsh, and cva/cvi (Dozois et al., 1992; Franck et al., 1998) and annealing at 60°C for 30 seconds for irp2, papC and vat virulence genes with extension at 68°C for 3 minutes and a final extension at 72°C for 10 minutes for all (Dozois et al., 1992; Franck et al., 1998). While astA (2mM MgCl2 concentration with initial denaturation for 2 minutes at 94°C, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 min and extension at 72°C for 2 min and final extension was carried out at 72°C for 10 min) (Yamamoto and Echeverria, 1996), fimC (2mM MgCl2 concentration with initial denaturation for 3 minutes at 94°C, followed by 27 cycles of denaturation at 94°C for 1 minute, annealing at 59°C for 1 minute and extension at 72°C for 90 sec and final extension carried out at 72°C for 10 min.) (Janben et al., 2001) and ibeA genes

<table>
<thead>
<tr>
<th>Primer</th>
<th>Target gene</th>
<th>Primer Sequence 5'-3'</th>
<th>Primer Conc. (μM)</th>
<th>Product size</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Iss</td>
<td>F-ATCACATAGGATTCTGGCC</td>
<td>0.5</td>
<td>309bp</td>
<td>Dozois et al. (1992)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R-CAGCCGAGATAGTATGGCCA</td>
<td></td>
<td></td>
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<tr>
<td>2</td>
<td>iucD</td>
<td>F-CGCGCGTGCTGGGTAAG</td>
<td>0.5</td>
<td>714bp</td>
<td>Franck et al. (1998)</td>
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<tr>
<td></td>
<td></td>
<td>R-CAGGCCGTTCACAAAGTATCCTG</td>
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<td></td>
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<tr>
<td>3</td>
<td>Tsh</td>
<td>F-CTATCTCTGCAAGGAAGTC</td>
<td>0.5</td>
<td>824bp</td>
<td>Dozois et al. (1992)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R-CCTCCGATGCTTCGAACGT</td>
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<tr>
<td>4</td>
<td>cva/cvi</td>
<td>F-TGTTAGAATGTGGCAGAGCAAG</td>
<td>0.5</td>
<td>1181bp</td>
<td>Dozois et al. (1992)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R-GAGCTGTGGTGAAGGCAAGCC</td>
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<tr>
<td>5</td>
<td>irp2</td>
<td>F-AAGGATTCTCGTGTACCCCGAC</td>
<td>0.5</td>
<td>413bp</td>
<td>Dozois et al. (1992)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R-AACTCTCGTATACAGGTG GC</td>
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<tr>
<td>6</td>
<td>papC</td>
<td>F-TGATATACAGCACTAGTAGC</td>
<td>0.5</td>
<td>501bp</td>
<td>Frank et al. (1998)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R-CGCGCCATATCTCACATAA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Vat</td>
<td>F-TCTGGGAGCAATATGGTACG</td>
<td>0.5</td>
<td>981bp</td>
<td>Dozois et al. (1992)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R-GTGTCAGAACCAGTATTGT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>astA</td>
<td>F-CCATCAACACAGTATATC</td>
<td>0.5</td>
<td>111bp</td>
<td>Yamamoto and Echeverria (1996)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R-GTCGCCAGTGCACGGGTTCGT</td>
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<td></td>
<td></td>
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<tr>
<td>9</td>
<td>fimC</td>
<td>F-GGGTAAAGGAAATGGCGAT</td>
<td>0.25</td>
<td>497bp</td>
<td>Janben et al. (2001)</td>
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<tr>
<td></td>
<td></td>
<td>R-CGTCATTCTTGGGGTTAGTC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>ibeA</td>
<td>F-TGACACGTGGTTTCGTTTTT</td>
<td>0.5</td>
<td>813bp</td>
<td>Germon et al. (2005)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R-TGGTCAATCTCCTGGCGA</td>
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</tbody>
</table>
were screened separately by PCR(1.5mM MgCl₂ concentration with initial denaturation for 5 minutes at 95°C, followed by 29 cycles of denaturation at 95°C for 30 seconds, annealing at 52°C for 30 seconds and extension at 72°C for 1 minute and final extension at 72°C for 10 minutes)(Germon et al., 2005). The primer sequences used are detailed in Table1. Amplified PCR products were analyzed by electrophoresis in 2% (w/v) agarose gel containing ethidium bromide (0.5 μg/ml) (Sambrook and Russel, 2001). The products were visualized under UVillumination and documented with Gel Doc System (Ultracam Digital Imaging, Ultra Lum, Inc, Claremont, CA).

**Cloning and DNA sequencing of ibeA gene:** Chromosomal DNA was prepared using a MinElute PCR Purification Kit (Qiagen, Hilden, Germany) as per manufacturer’s instructions. After purification of the representative ampiclon (813bp), it was cloned into pDrive Vector (Qiagen, Hilden, Germany) using PCR cloning kit (Qiagen, Hilden, Germany). The plasmid from the clone with desired insert was extracted using QIAprep Miniprep Kit (Qiagen, Hilden, Germany). Recombinant plasmids were confirmed for the presence of inserted ibeA gene by PCR using gene specific primers 5'-TGTTCAAATCCTGGCTGGAA and 3'-CTGTTCTCGGTGAA-A-5’ with same PCR conditions as used during amplification of ibeA (Germon et al.2005). The recombinant plasmids were further analyzed by restriction enzyme digestion, by digesting 1 μg of plasmid DNA with 5U of an EcoRI (Fermentas Life Sciences) for 2 hrs. in a total volume of 20 μl at 37°C. The digested products and the the molecular weight marker were subjected toagarose gel electrophoresis under standard conditions, to concur the presence of insert and that a frameshift during recombination hasn’t taken place (Fig 6). The representative plasmid clone was sequenced from both sides using M13 universal primers on ABI 377 Perkin Elmer Automated DNA Sequencer (Merck Specialities Pvt Ltd, Bangalore, India). The data obtained was analysed using DNASTAR package analysis software.

**Antimicrobial susceptibility determination : In vitro** antibiotic sensitivity pattern of the E. coli isolates to antimicrobial agents was determined by disc diffusion method as described by Bauer et al., (1966) utilizing Muller Hinton Agar plates (HiMedia) by placing 20 mm antibiotic discs of 14 commonly used antimicrobial agents and measuring the diameter of zone of inhibition.

**RESULTS**

In the present study out of 672 samples (224 tracheal swabs,224 air sacs and 224 nasal swabs) from 224 birds suspected to have respiratory tract infections (RTI’s), 493 samples (171 nasal swabs, 163 tracheal swabs and 159 air sacs) carried E. coli. Multiple E. coli isolates from a single dead bird sample having same virulence gene profile(n=224) were considered to be same strain, hence only one representative E. coli isolate from them(i.e.; n=172) were selected for further study resulting in average 6.38% prevalence of mortality by RTI’s associated with E.coli, with the higher prevalence of 8.63% in unorganized sector and a lower prevalence of 3.6% in organized sector (Table 2).

Wide serological diversity typed into 10 different serogroups viz; O1, O2, O5, O11, O20, O27, O29, O66, O75 and O104 along with 12 rough (R) isolates was revealed, with a single top frequency of 8.88% for serogroup O2 and 33.33% foruntypable. All the 172 E. coli isolates were screened for ten virulence genes.The representative gene profile is depicted in Figures 1t05:The prevalence rate of invasion-associated genes was found highest with fimC (93.60%) followed by iss(89.53%), tucD(85.46%), irp2 (55.23%), tsh (51.74%), astA (38.95%), papC (33.72%), vat (31.39%), cva/cvi(33.14%),and ibeA (1.16%). None of the isolates possessed all the ten virulence genes targeted in this study. E. coli isolates showed high resistance against tetracycline (94.71%) followed by ampicillin (92.67%), cephalaxin (79.67%), co-trimoxazole (67.74%), enrofloxacin (62.11%), amoxyclin (59.88%), and streptomycin (59.63%). A relatively higher sensitivity was recorded against amikacin (74.41%), amoxacin-sulbactam (68.60%) and gentamicin (67.44%), nitrofurantoin (63.95%), chloramphenicol (61.62%) and sulphadiazine (51.16%). All the isolates showed multidrug resistance pattern (Fig 7).

**Table 2.** Prevalence of E. coli in chicken with respiratory tract infection

<table>
<thead>
<tr>
<th>S. No. Source</th>
<th>Flock size</th>
<th>No. of birds infected with RTI and other associated disease</th>
<th>No. of birds exclusively with RTI</th>
<th>Prevalence of RTI (%)</th>
<th>Positive samples for E. coli</th>
<th>Prevalence of E. coli (%)</th>
<th>Occurrence of E. coli (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Private sector</td>
<td>1471</td>
<td>747</td>
<td>161</td>
<td>10.94</td>
<td>127</td>
<td>8.63</td>
<td>78.8</td>
</tr>
<tr>
<td>2. Govt. sector</td>
<td>1221</td>
<td>115</td>
<td>63</td>
<td>5.15</td>
<td>45</td>
<td>3.6</td>
<td>71.4</td>
</tr>
<tr>
<td>Total</td>
<td>2692</td>
<td>862</td>
<td>224</td>
<td>8.32</td>
<td>172</td>
<td>6.38</td>
<td>76.78</td>
</tr>
</tbody>
</table>

RTI = Respiratory tract infection

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The recombinant plasmids subjected to DNA sequencing, to obtain the nucleotide sequence of *ibe*A were subjected to homology assessment by Basic Local Alignment Search Tool (BLAST), NCBI GenBank database resulted in 99% homology to invasion protein complete cds *ibe*A genes of JKE60 (Accession No. FJ158545.1), and BEN 2908 (Accession No. AY248744.1) *E. coli* strains. The sequence was submitted to GenBank (Accession No. KF640638).

**DISCUSSION**

In the present investigation overall prevalence of *E. coli* in chicken with respiratory tract infection was recorded as 6.38 per cent. These findings are in agreement with the findings of Dave *et al.* (2004) from Gujarat and Sarpe *et al.* (2009) from Maharashtra who reported a prevalence rate of 5.12 and 11.34 per cent of *E. coli,* respectively in respiratory infections in poultry. The present investigation revealed a wide serological diversity in *E. coli* isolates with 10 different serogroups besides 15 untypeable and 12 rough isolates. In the present study, Serogroup O2 (8.88%) was the most predominant serogroup isolated while the serogroup O1 was least encountered with one (2.22%) isolate. These findings are in concurrence with the reports of Ewers *et al.* (2004) from Germany and Ya-
Fig. 3. Agarose gel electrophoresis of amplicon of astA virulence gene. Lane 1, 2, 3: Sample +ve for astA gene. Lane M: Molecular weight marker, Lane N: Negative control, Lane P: Positive control.

Fig. 4. Agarose gel electrophoresis of amplicon of fimC virulence gene. Lane 1: Sample +ve for fimC gene. Lane M: Molecular weight marker, Lane N: Negative control, Lane P: Positive control.

Fig. 5. Agarose gel electrophoresis of PCR amplicons of ibeA virulence gene. Lane 1 and 2: Sample +ve for ibeA gene, Lane M: Molecular weight marker, Lane N: Negative control, Lane P: Positive control.
guchi et al. (2007) from Japan and Circella et al. (2009) from Italy, Sharada et al. (2010) India and Wang et al. (2010) China. A good number (33.33%) of E. coli isolates were untypeable corroborating with the findings of Altekruse et al. (2002) and Rodriguez et al. (2005) from USA who reported 26.6 and 29.5 per cent E. coli isolates as untypeable. In the present study, the antimicrobial sensitivity test of the E. coli isolates from respiratory tract of infected birds revealed highest (94.71%) resistance to tetracycline. This is in agreement with findings of Yang et al. (2004) from China, Zhao et al. (2005) from USA and Salehi and Bonab (2006) from Iran who reported 100, 87 and 94 per cent of avian pathogenic E. coli isolates resistant to tetracycline. This percentage is higher than the findings of Kim et al. (2007) from Korea and Raji et al. (2007) from Nigeria who reported 84.2 and 60 per cent of avian pathogenic E. coli isolates resistant to tetracycline. Indiscriminate use of tetracycline for prophylactic and therapeutic purposes in poultry could be the probable cause. These findings are more or less similar to findings of Salehi and Bonab (2006) from Iran who reported similar antimicrobial resistance pattern of avian pathogenic E. coli isolates except that of ampicillin. Regarding the resistance of ampicillin in this study, findings corroborate with the findings of Saidi et al. (2013) from Zimbabwe. From the present study, lowest resistance of E. coli isolates was observed to amikacin (25.6%) followed by amoxicillin-sulbactam (31.4%) and gentamicin (32.5%). These findings corroborate with the findings of Salehi and Bonab (2006) from Iran but disagree with
Omer et al. (2010) from Sudan who found high number of *E. coli* isolates were resistant to amoxicillin-sulbactam.

**Conclusion**

The prevalence and occurrence of *E. coli* in respiratory tract infections in Kashmir Valley was found to be 6.38 and 76.78 percent respectively. Wide serological diversity was revealed among *E. coli* isolates and were typed into 10 different serogroups viz; O1, O2, O5, O11, O20, O27, O29, O66, O75 and O104 and 12 isolates were rough (R). Most frequent serogroup detected among *E. coli* isolates was O2 (8.88%). A good number of *E. coli* isolates were found to be untypable (33.33%). Overall prevalence of *iss, iccD, tsh, cva/cvi, irp2, papC, vat, astA, fimC* and *ibeA* genes was found to be 89.53, 85.46, 51.74, 33.14, 55.23, 33.72, 31.39, 38.95, 93.60 and 1.16 per cent, respectively. None of the isolates possessed all the ten virulence genes targeted in this study. The *ibeA* gene, one of the important invasion-associated genes responsible for neonatal meningitis in humans was detected in two *E. coli* isolates. *E. coli* isolates showed high resistance against tetracycline (94.71%), ampicillin (92.67%) and cephalexin (79.67%). A relatively higher sensitivity was recorded against amikacin (74.41%), amoxicillin-sulbactame (68.60%) and gentamicin (67.44%). The present study leaves scope for similar investigations in humans with neonatal meningitis to precisely elucidate the zoonotic significance of APEC in this part of globe.

**REFERENCES**


