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

Qazi Nyrah*, Shakil Ahmed Wani, Nazima Nazir, Mir Nadeem Hassan, Shaheen Farooq, Zahid Kashooand Shoiab Kamil**

Division of Veterinary Microbiology and Immunology, Faculty of Veterinary Sciences and Animal Husbandry, Sher-e-Kashmir University of Agricultural Sciences & Technology, Kashmir Shuhama Campus, Srinagar- 190006, INDIA
**Division of Veterinary Pathology, Faculty of Veterinary Sciences and Animal Husbandry, Sher-e-Kashmir University of Agricultural Sciences & Technology, Kashmir Shuhama Campus, Srinagar- 190006, INDIA
*Corresponding author. E-mail: qazinyrah@gmail.com

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Abstract: The present study has determined prevalence, serological diversity, virulence gene profileand *in vitro* antibiogram of *Escherichia coli* (*E. coli*) isolates from respiratory tract of broiler chicken in Kashmir valley along with molecular cloning of *ibe*A gene, an important zoonotic among invasion-associated genes responsible for neonatal meningitis in humans. A total of 224 broilerchickens 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 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%), *iuc*Din (85.46 %), *tsh* in (51.74 %), *cva/cvi* in (33.14 %), *irp*2in (55.23 %), *pap*Cin (33.72 %), *vat* in (31.39 %), *ast*Ain (38.95 %), *fim*Cin (93.60 %) and *ibe*Ain (1.16 %) isolates. The antibiogram screening of *E. coli* isolates revealed 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 amoxacillin-sulbactam (68.60%) and gentamicin (67.44%). The study thus, suggests that the *E. coli* isolates from respiratory tract infection of poultry harbour differential virulence genes along with the genes of zoonotic interest and variable antimicrobial resistance, thus, opening the areas for devising dynamic, emergent and effective treatment line.

Keywords: Antibiogram, *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 loses 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 andis 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 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

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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 swabsand air sacs were collected adopting standard asceptic 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 sampleson MacConkey agar(Hi Media, Mumbai, India) and incubating at 37°C overnight. Rose pink colonies from each sample were picked and subcultured on eosine methylene blue agar (EMB) (Hi Media, Mumbai, India) to observe characteristic metallic sheen. The well separated colonies were subcultured on nutrient agar slants as pure culture and subjected to standard morphological and biochemical tests as described by Buchanan and Gibbon (1994) so as to ascertain their identity as *E. coli.*

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*, *iuc*D, *tsh,* and *cva/cvi* (Dozois *et al*.,1992; Franck *et al.,*1998)andannealing at 60°C for 30 seconds for *irp*2, *pap*C 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 *ast*A (2mM $MgCl_2$ 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), *fim*C (2mM $MgCl_2$ 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 *ibe*A genes

Table 1. Details of primers used in this study.

Pri-mer	Target gene	Primer Sequence 5' - 3'	Primer Conc. (µM)	Product size	Reference
1	*Iss*	F-ATCACATAGGATTCTGCCG R-CAGCGGAGTATAGATGCCA	0.5	309bp	Dozois *et al.* (1992)
2	*iuc*D	F-CGCCGTGGCTGGGGTAAG R-CAGCCGGTTCACCAAGTATCACTG	0.5	714bp	Franck *et al.* (1998)
3	*Tsh*	F-ACTATTCTCTGCAGGAAGTC R-CTTCCGATGTTCTGAACGT	0.5	824bp	Dozois *et al.* (1992)
4.	*cva/cvi*	F-TGGTAGAATGTGCCAGAGCAAG R-GAGCTGTTTGTAGCGAAGCC	0.5	1181bp	Dozois *et al.* (1992)
5.	*irp*2	F-AAGGATTCGCTGTTACCGGAC R-AACTCCTGATACAGGTGGC	0.5	413bp	Dozois *et al.* (1992)
6.	*pap*C	F-TGATATCACGCAGTCAGTAGC R-CCGGCCATATTCACATAA	0.5	501bp	Frank *et al.* (1998)
7.	*Vat*	F-TCCTGGGACATAATGGTCAG R-GTGTCAGAACGGAATTGT	0.5	981bp	Dozois *et al.* (1992)
8.	*ast*A	F-CCATCAACACAGTATATC R-GTCGCGAGTGACGGCTTTGT	0.5	111bp	Yamamoto and Echeverria (1996)
9.	*fim*C	F-GGGTAGAAAATGCCGATGGTG R-CGTCATTTTGGGGGTAAGTGC	0.25	497bp	Janben *et al.* (2001)
10.	*ibe*A	F-TGAACGTTTCGGTTGTTTTG R-TGTTCAAATCCTGGCTGGAA	0.5	813bp	Germon *et al.* (2005)

were screened separately by PCR(1.5mM $MgCl_2$ 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 (0.5µg/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 amplicon (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 *ibe*A gene by PCR using gene specific primers 5'-TGAACGTTTCGGTTGTTTTG-3 and 3'-TGTTCAAATCCTGGCTGGAA-5'with same PCR conditions as used during amplification of *ibe*A (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 (1.5%) 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% foruntypeable.

All the 172 *E. coli* isolates were screened for ten virulence genes.The representative gene profile is depicted in Figures 1to5.The prevalence rate of invasion-associated genes was found highest with *fim*C (93.60%) followed by *iss*(89.53%), *iuc*D(85.46%), *irp*2 (55.23%), *tsh* (51.74%), *ast*A (38.95%), *pap*C (33.72%), *vat* (31.39%), *cva/cvi*(33.14%),and *ibe*A (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%), cephalexin (79.67%), co-trimoxozole (67.74%), enrofloxacine (62.11%). amoxycillin (59.88%), and streptomycin (59.63%). A relatively higher sensitivity was recorded against amikacin (74.41%), amoxacillin-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

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

RTI = Respiratory tract infection

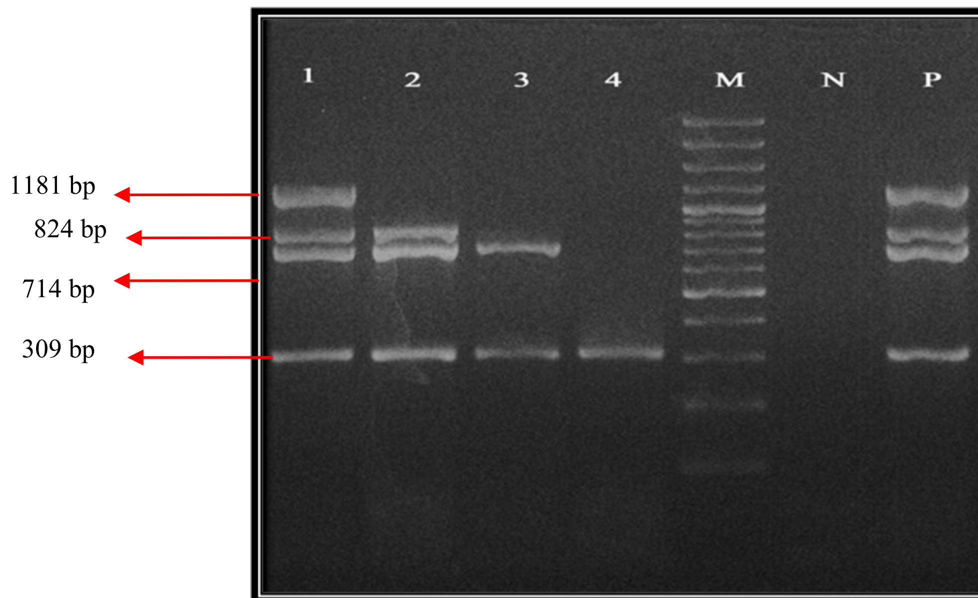

Fig. 1. *Agarose gel electrophoresis of mPCR amplicons of iss, iucD, tsh and cva virulence genes. Lane 1 : Sample +ve for iss, iucD, tsh and cva genes. Lane 2 : Sample +ve for iss, iucD, tsh genes.Lane 3 : Sample +ve for iss, iucD genes. Lane 4 : Sample +ve for iss gene. Lane M : Molecular weight marker. Lane N : Negative control. Lane P : Positive control.*

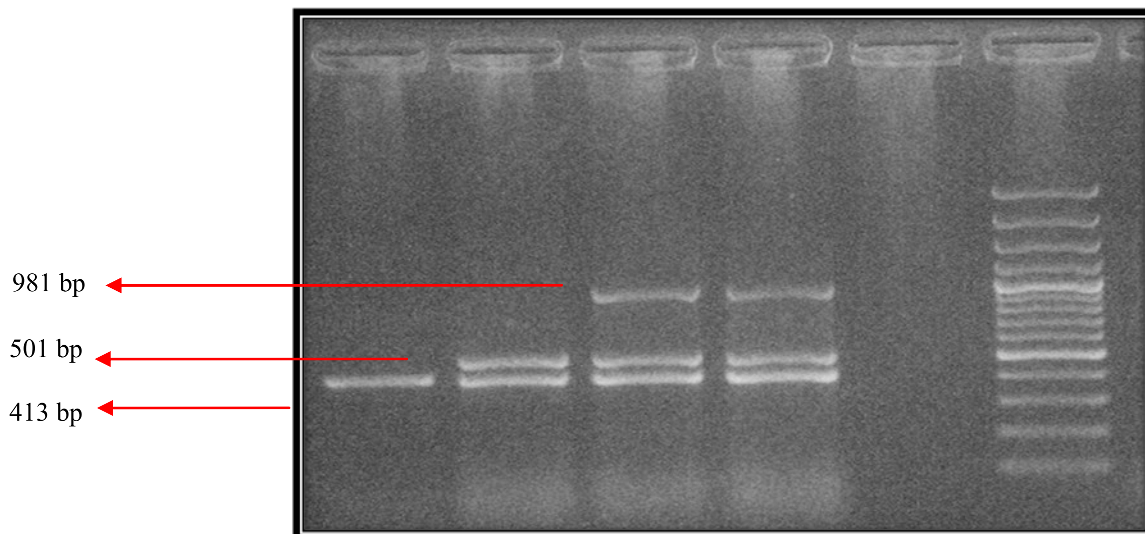

Fig. 2. *Agarose gel electrophoresis of mPCR amplicons of irp2, papC, and vat virulence genes. Lane 1 : Sample +ve for irp2 gene. Lane 2 : Sample +ve for irp2, and papC genes. Lane 3 : Sample +ve for irp2, papC and vat genes. Lane M : Molecular weight marker. Lane N : Negative, control. Lane P : Positive control.*

The recombinant plasmids subjected to DNA sequencing, to obtain the nucleotide sequence of *ibe*A were subjected to homology assessment byBasic Local Alignment Search Tool (BLAST), NCBIGenBank database resulted in 99%homology to invasion protein complete cds *ibe*A genesof 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-

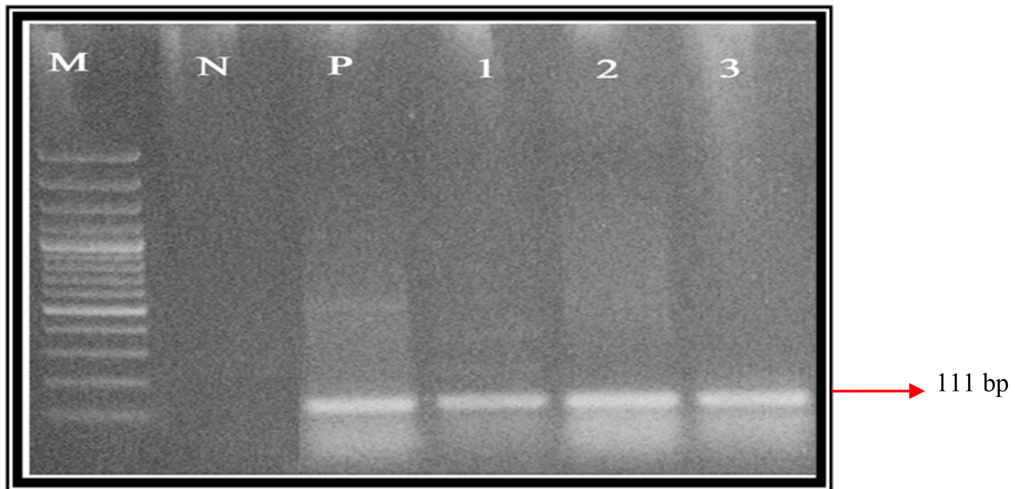

Fig. 3. *Agarose gel electrophoresis of amplicon of astA virulence gene. Lane 1, 2, 3 : Sample +ve for astA gene. Lane M : Molecular weight marker, Lane N: Negative control, Lane P: Positive control.*

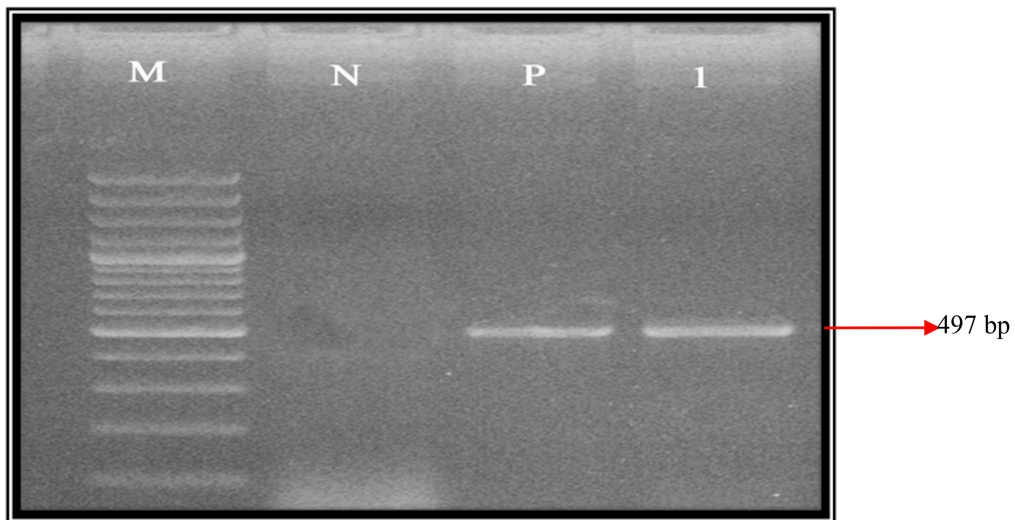

Fig. 4. *Agarose gel electrophoresis of amplicon of fimC virulence gene. Lane 1 : Sample +ve for fimC gene. Lane M : Molecular weight marker, Lane N: Negative control, Lane P: Positive control.*

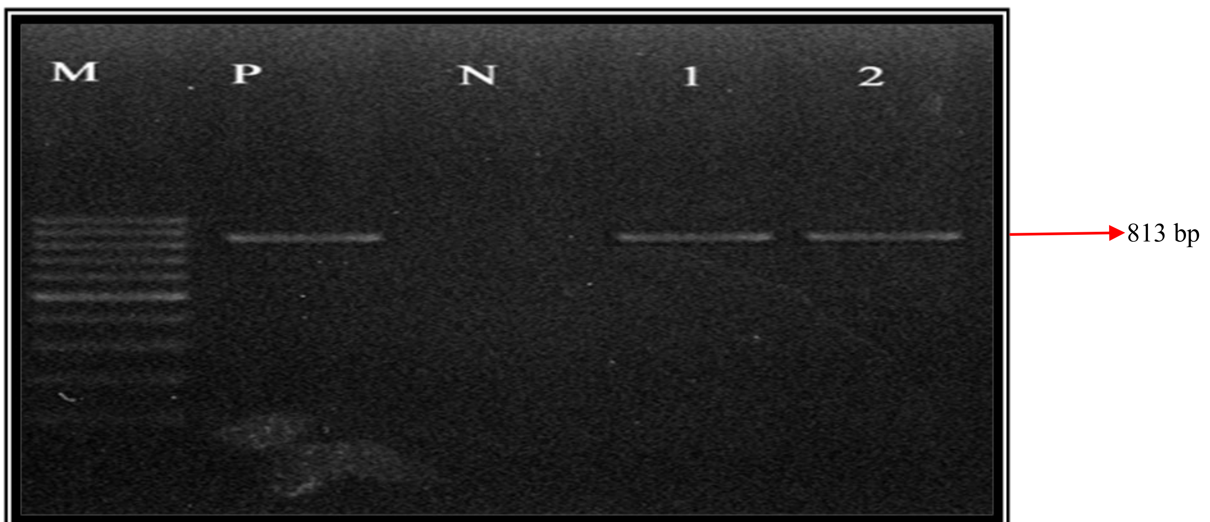

Fig. 5. *Agarose gel electrophoresis of PCR amplicons of ibeA virulence gene. Lane 1 and 2 : Sample +ve for ibeAgene, Lane M : Molecular weight marker, Lane N: Negative control, Lane P : Positive control.*

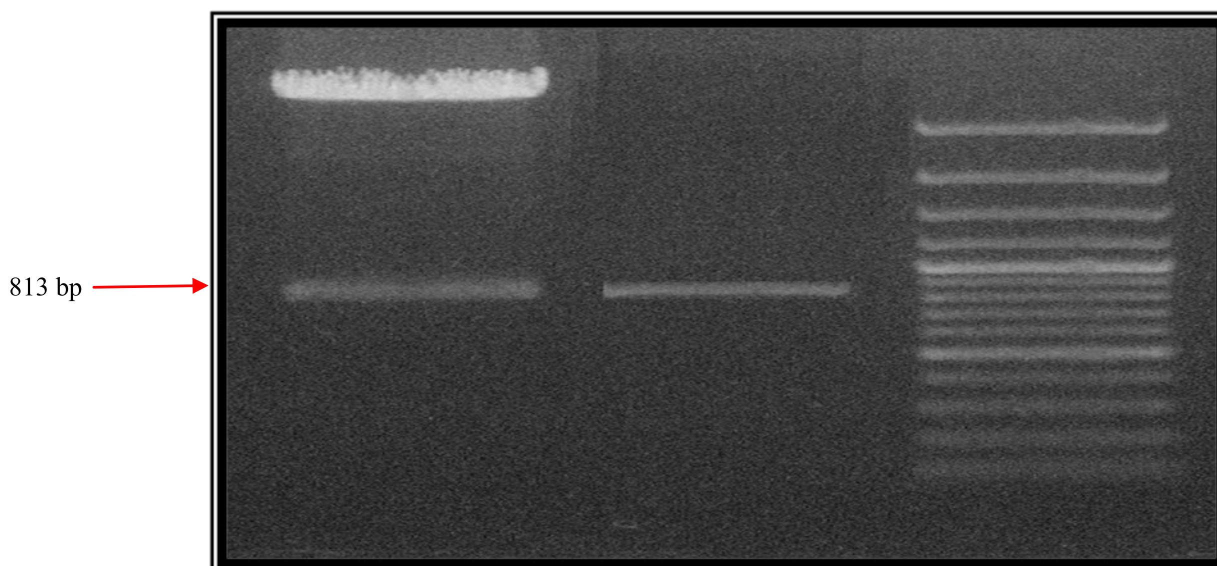

Fig. 6. *Lane 1: Release of ibeA gene product from P drive cloning vector by restriction digestion with EcoRI. Lane 2 : PCR product of ibeA gene. Lane M : Molecular marker.*

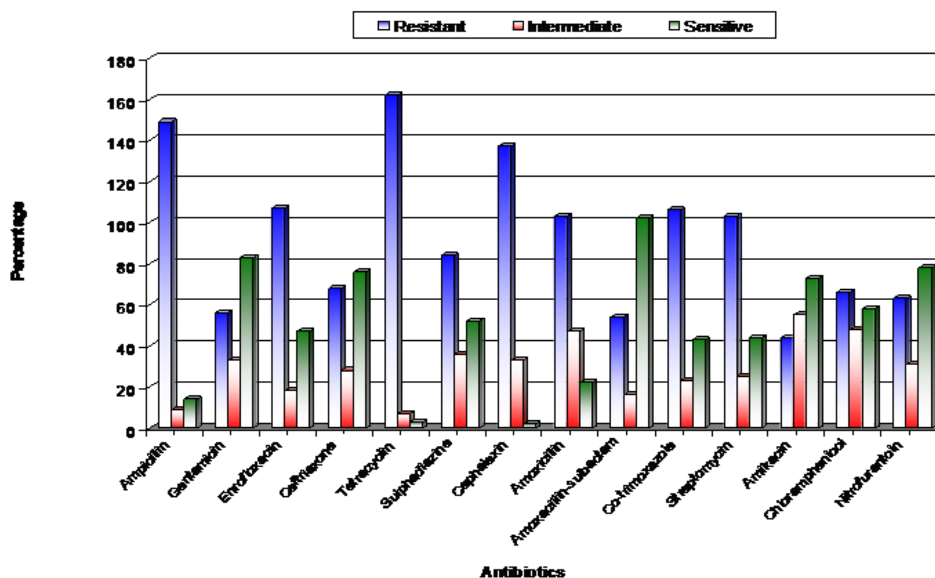

Fig. 7. *Graphical representation of antibiotic sensitivity/resistance pattern of E. coli isolates .*

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, respectively 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 amoxacillin-sulbactam (31.4%) and gentamicin (32.5%). These findings corroborate with the findings of Salehiand Bonab (2006) from Iran but disagree with

Omer *et al.* (2010) from Sudan who found high number of *E. coli* isolates were resistant to amoxacillin-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, iuc*D*, tsh, cva/cvi, irp*2*, pap*C*, vat, ast*A*, fim*C and *ibe*A 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 *ibe*A 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 cephalaxin (79.67%). A relatively higher sensitivity was recorded against amikacin (74.41%), amoxacillin-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.

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