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Identification and Characterization of *Escherichia coli* from Bronchial Plug of Broiler Chicken with Respiratory Distress

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A B S T R A C T

A Twenty-five bronchial plug samples from dead broiler chicken with signs of respiratory distress collected for identification of avian pathogenic *E. coli* (APEC). Samples comprised of one pooled sample each from 25 poultry farms located in and around Junagadh city. Isolation, culture and colony characteristics used for primary detection followed by PCR assay targeting four virulence genes (*iss, papC, tsh* and *vat*)for confirmatory diagnosis. All bronchial plugs were found to be positive for *E. coli* infection by isolation. Out of 25 flock sampled, the highest number of *E. coli* isolates were found positive for the presence of *iss* gene (22) followed by *tsh* gene (15), *vat* gene (13) and *papC* gene (8)by PCR. On antibiogram, overall *E. coli* was highly sensitive to colistin (Methane sulphonate) and meropenem(100%) followed by levofloxacin (76%), ceftriaxone (64%), gentamicin (60%), amikacin (60%), co-trimoxazole (40%), amoxycilin-clavunic acid (8%) and imepenem (8%). These results indicated the high prevalence of the *E. coli* with variable antibiotic resistance in broiler chicken.

Introduction

Poultry sector is confronted with infectious diseases and respiratory tract pathogens are of major concern because they incurred heavy economic losses both in terms of production and cost of treatment (Ali and Reynolds, 2000). The etiology of respiratory disease is complex often involving multiple pathogens simultaneously (Yashpal *et al.,* 2004) including avian pneumovirus (APV), avian influenza virus (AIV), infectious bronchitis

virus (IBV), Newcastle disease virus (NDV), *Mycoplasma gallisepticum* (MG), *Mycoplasma synoviae* (MS), *Escherichia coli* (*E. coli*) and *Av. paragallinarum*. Infected birds expressed respiratory lesions including cough, respiratory distress, poor growth and production leading to high economic losses (Pang *et al.,* 2002).

Respiratory colibacillosis is a respiratory disease caused by secondary infection with pathogenic *E. coli.* Its virulence genes enable to live an extra-intestinal life (Kwon *et al.,* 2008) as recognize avian Pathogenic *E. coli* (APEC). The initial insult may be viral (IBV, NDVor AIV) or bacterial (*P. multocida*) or it may be an environmental such as elevated ammonia levels. It leads to the development of some diseases like septicaemia, egg yolk infection, peritonitis, chronic cutaneous infection, osteomyelitis and swollen head syndrome. Therefore, the present study carried out to investigate the incidence of *E. coli* in selected farms in and around Junagadh city of Gujarat State.

Materials and Methods

The present study carried out in broiler chicken with the history of respiratory distress from twenty-five different broiler flocks brought for post mortem diagnosis at the Department of Pathology, Veterinary College, Junagadh. A total of twenty-five samples of bronchi plug belonged to one pooled sample from each flock used for isolation and identification of *E. coli*. These birds showed caseous bronchitis and tracheitis on gross post-mortem examination.

E. coli was isolated from pooled samples of caseous plugs from each farm by streaking the samples on MacConkey agar. Plates showing pink colonies on MacConkey agar transferred to Eosin Methylene Blue (EMB) agar. Colonies with greenish metallic sheen tentatively identified as *E. coli* organisms and used for further antimicrobial sensitivity testing.

The disk diffusion method (Bauer *et al.,* 1966) used to determine the susceptibility of *E. coli* isolates to several antibiotics of veterinary significance on Mueller Hinton agar. Co-trimoxazole, Gentamicin, Ceftriaxone, Colistin (Methane sulphonate), Levofloxacin, Amoxyclav (Amoxycillin/Clavulanic acid), Amikacin, Imipenem and Meropenem were used in this study. Zones of inhibition were measured to the nearest millimeter using a ruler and reported either as sensitive (S), intermediate resistant (I) or resistant (R) on the basis of criteria set by the Clinical Laboratory Standard Institute (CLSI), 2012.

For PCR, DNA extracted from approximately 50 mg of each thawed 25 bronchial plug samples belonged to one pooled sample from each farm by phenol-chloroform method. The quality of extracted DNA assessed by submarine agarose gel electrophoresis using 0.8% agarose in 0.5X tris-borate EDTA (TBE) buffer (pH 8.0) with ethidium bromide (Sambrook and Russel, 2001). The DNA was visualized under UV light and documented by gel documentation system (VilberLourmat UV Transilluminator, Bio-print-st4, 900/20m xpress). The quantity and purity of extracted DNA samples checked using nano-drop sprectophotometer ND 200c(Thermo Scientific) at 260/280 nm wavelength. Extracted DNA samples were stored at -20 ^{o}C till used as template for PCR targeting *iss, papC, tsh* and *vat* genes. PCR carriedout for APEC virulance marker genes using primers (Ewers *et al.,* 2004) synthesized by Eurofins Genomics India Pvt. Ltd. as presented in Table 1.

The PCR was standardized and performed in 25 µl reaction mixture containing 12.5 µl 2X Taq PCR master mix (Applied Biosystem)

having Taq DNA polymerase, PCR buffer containing 1.5 mm $MgCl_2$ and dNTPs. Each forward and reverse primer at 1.5 µl of 10 pmol/µl,3 µl of DNA template and 6.5 µl of nuclease free water added. The PCR amplification of all genes performed with an initial denaturation at 94 ^oC for 5 min. followed by 35 cycles of denaturation for 30 sec at 94 ^oC, anneling at 58 ^oC except for iss gene (56 ^oC) for 30 sec, extention at 72 ^oC for 30 sec and final extention at 72 ^oC for 7 min. For visualization, PCR products (6 µl) mixed with 1 µlgel loading dye was electrophoresed along with 100 bp DNA molecular weight ladder (New England Biolabsinc.) on 1.8% agarose gel containing ethidium bromide (1% solution @ of 8 µl /100 mL) at constant 80 V for 80 min in 1.0X TBE buffer.

Results and Discussion

Tissue samples were streaked on MacConkey (MCA) agar plate and pink colonies were considered as gram negative lactose fermenting isolates (Fig. 1). The pure colonies transferred to Eosin Methylene Blue agar (EMB) agar plate and colonies with greenish metallic sheen considered as *E. coli* isolates (Fig.2).All bronchial plugs were found to be positive for *E. coli* infection by isolation.

The *E. coli* isolates tested against nine commonly used antimicrobials and these isolates were highly sensitive to colistin (Methane sulphonate) and meropenem followed by levofloxacin, ceftriaxone, gentamicin, amikacin, co-trimoxazole, amoxicillin & clavunic acid and imipenem. However these isolates were resistant to imipenem followed by amoxicillin & clavunic acid, co-trimoxazole, ceftriaxone, amikacin, gentamicin and least to levofloxacin (Fig. 3, Table. 2). The detection of virulence genes of *E. coli* carried out directly from caseous plugs by PCR. Out of 25 pooled positive samples, the highest frequency of *iss* gene (22, 88%) (Fig.4) followed by *tsh* gene (15, 60%), *vat* gene (13, 52%) and *papC* gene (8, 32%) was recorded.

Among the 25 pooled caseous plug samples, 14 isolates were carrying two virulence genes (iss,tsh-5 samples, iss, vat-5 samples, iss, papC-3 samples and tsh, papC in 1 sample) followed by 5 samples had the simultaneous presence of three genes (iss, vat, tsh-4 samples and vat, tsh, papC-1 samples) while, three isolates each were having four genes and single gene either as tsh (1) or iss (2) in samples.

The *E. coli* isolates tested against nine commonly used antimicrobials and these isolates were highly sensitive to colistin (Methane sulphonate) and meropenem. Similarly, Chaudhari *et al.,* (2017) also recorded sensitivity of colistin (100%) followed by ceftriaxone (93.33%), gentamicin (80%), levofloxacin (76.67%), co-trimoxazole (63.33%) and amoxyclav (10%), however Sahoo *et al.,* (2012) reported sensitivity of amikacin as 60 percent. Contrary to our findings Jamoh *et al.,* (2018) recorded resistant of *E. coli* isolates against amoxicillin (87.23%) followed by minimum resistance against imipenem (1.22%).

Antimicrobials have distinct advantages in management of infection and growth in commercial broiler chicks. However, indiscriminate use of antimicrobials may lead to antimicrobial resistance due to selection pressure. It also promotes the proliferation of drug resistant strains of *E. coli*.

Table.1 Detail of primers used for confirmation of Avian Pathogenic *E. coli*by PCR

Target gene	Primer sequence		Product Size
iss	F	5'-CCCCAATTGGACAGAGAAAA-3'	174 bp
	R	5'-ATCGATGGGCCTATTGTGAG-3'	
papC	F	5'-AATAAAAACGTGGCGGACTG-3'	201 bp
	R	5'-ACGCAGGTAAGCAGAATCGT-3'	
tsh	F	5'-TCTCAATGCGTCGTAACAGC-3'	153 bp
	R	5'-CCTTCAGATGAACGTCAGCA-3'	
vat	F	5'-CACGCTACTGAATGCCTGAA-3'	168 bp
	R	**5'-TGGCAGGTTAATGGTGTGAA-3'**	

Table.2 *In-vitro* antibiotic sensitivity pattern against Avian Pathogenic *Escherichia coli* isolates

Sr. No.	Antibiotics	Sensitivity	Intermediates	Resistance
1	Co-trimoxazole	(10/25) 40%	(01/25) 4%	(14/25) 56%
2	Gentamicin	(15/25) 60%	(03/25) 12%	(07/25) 28%
3	Ceftriaxone	(16/25) 64%	-	(9/25) 36%
4	Colistin (Methane sulphonate)	(25/25) 100%	-	-
5	Levofloxacin	(19/25)76%	(0125) 4%	(05/25) 20%
6	Amoxicillin and clavunic acid	(02/25) 8 %	(03/25) 12%	(20/25) 80%
7	Amikacin	(15/25) 60%	(2/25) 8%	(8/25) 32%
8	Imipenem	(2/25) 8%	-	(23/25) 92%
9	Meropenem	(25/25) 100 %	-	-

Fig.1 Isolation of *E. coli* on MacConkey (MAC) agar plate showing pink colony.

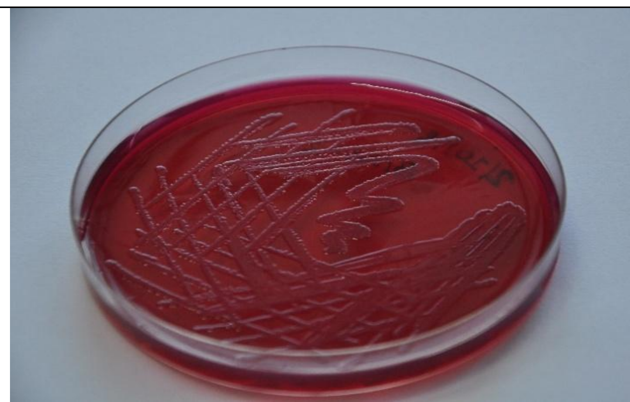


Fig.2 *E. coli* colonies on Eosin Methylene Blue (EMB) agar showing characteristic greenish metallic sheen.

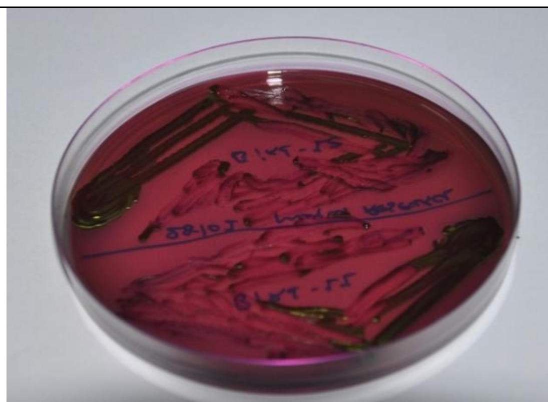


Fig.3 *In vitro* antibiotic sensitivity test against the Avian pathogenic *E.coli* isolates.

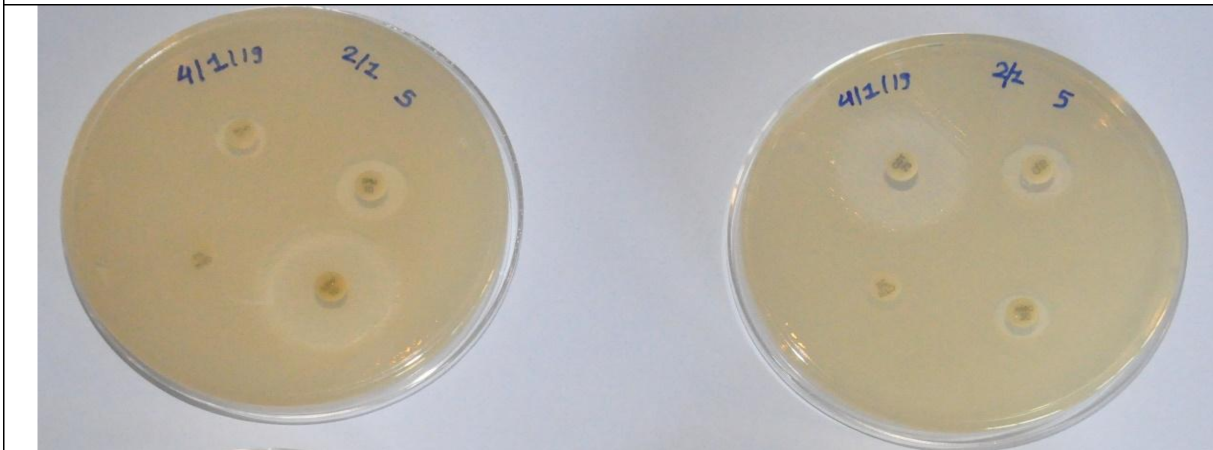
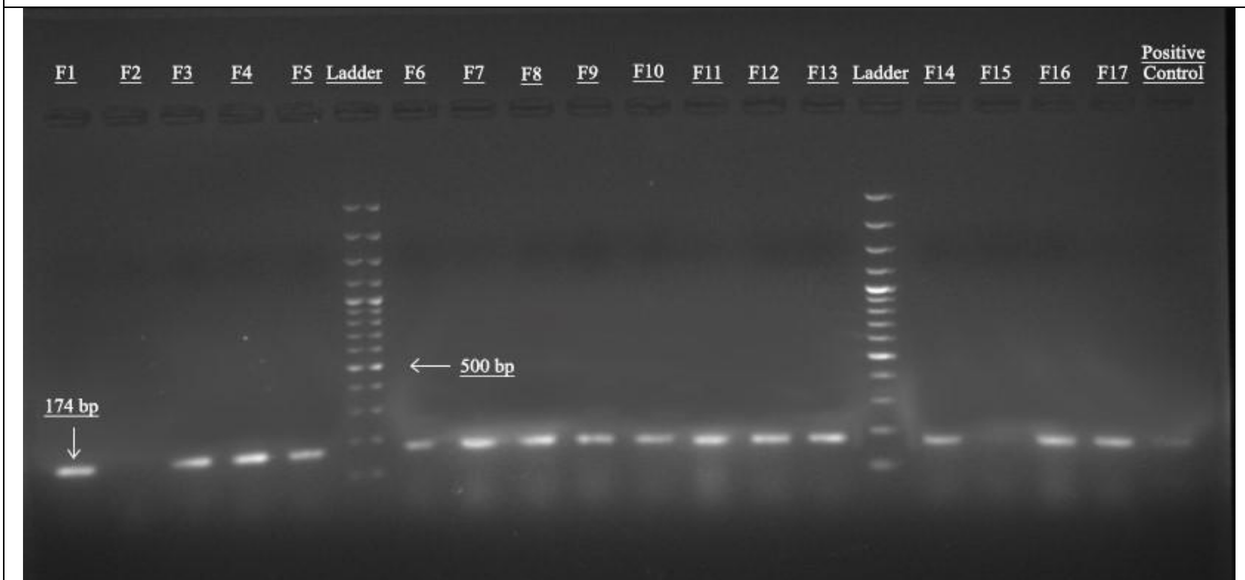


Fig.4 Agarose gel showing PCR amplified product of iss gene (174 bp) of APEC from caseous plug samples (17 farms), Lane 1-5, 7-14 and 16-19 (17 farms samples), Lane 6 and 15 : Ladder (100 bp DNA) and Lane 20 : Positive control.



When it is coupled with poor environmental sanitation and personal hygiene, which can be a potential threat to the public health and poultry industry. The resistance of these organisms to the commonly used antibiotics might be attributed to large scale and haphazard use of these antibiotics over a long period of time (Arora *et al.,* 2000; Mohanty *et al.,* 1992). Rational use of antibiotics is the key approach to improve the antibiotic performance and tackling antimicrobial resistance. The efficacy of antimicrobial activities are influenced by many factors like bacterial status (susceptibility and resistance, tolerance, persistence, biofilm), antimicrobial concentrations and host factors like impact on gut micro-biota (Li *et al.,* 2017).

The detection of virulence genes of *E. coli* were carried out by various workers have

found the frequency of *iss* gene ranged from 73.8% to 96.4% among *E. coli* isolates (Arabi *et al.,* 2013, Rocha *et al.,* 2008). However, contrary to the present findings Won *et al.,* (2009) reported only 41.5% isolates carrying *iss* gene during the study.

For *tsh* gene, findings of present study were in accordance with Ewers *et al.,* (2004), Roussan *et al.,* (2014) and Chaudhari *et al.,* (2017) who reported frequency of *tsh* gene in APEC as 53.3%, 66% and 50% respectively. In contrary, Arabi *et al.,* (2013) reported the highest occurrence of *tsh* gene (96.4%) among eight APEC genes targeted.

During the present study, *E. coli* from 13 tissue samples (52%) showed amplification by PCR for *vat* gene. Higher occurrence of vat gene was reported by Arabi *et al.,* (2013), Chaudhari *et al.,* (2017) and Roussan *et al.,* (2014) as 85.7%, 83.33% and 70% respectively. Whereas Ewers *et al.,* (2004) and Deshmukh (2016) found complimentary results of *vat* gene as 48.7% and 42.5% respectively.

Won *et al.,* (2009) and Chaudhari *et al.,* (2017) recorded low frequency of papC gene (14.4% to 33.33%) in APEC. In contrast to the present findings Roussan *et al.,* (2014) and Arabi *et al.,* (2013) found higher occurrence of papC gene at 50.00 and 82.10 percent, respectively.

Avian pathogenic *E.coli* (APEC) with virulence genes can live extra-intestinal life. The bacterial adhesion to the epithelial cells is considered to be an important step in the established of colibacillosis. The adhesion factor is *P fimbria* coded by *papC* operon, which is located in bacterial chromosome and prevents phagocytosis. *iss* gene contributes to increase survival of APEC in serum whereas temperature-sensitive haemagglutinin (*tsh*) gene responsible for bacterial adhesion to host cell during membrane binding process. This gene helps in fibrin precipitation in air sacs, increase colonization and induce ulcers. *vat* gene responsible for vacuolating and transferring of cytotoxic produced by APEC.

Molecular detection of more than one virulence genes associated with *E. coli* during the present study reflected the combined effects of virulence genes, which may be responsible for pathogenicity and antibiotic resistance of *E. coli* organisms. Among the four different *E. coli* associated genes, iss and tsh gene were found more commonly associated though vat and papC genes were also present.

Conflicts of interest/Competing interests

Authors declare no conflict of interest/ competing interests.

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