RESEARCH ARTICLE

Isolation, Antibiogram and Molecular Characterization of *Escherichia coli* in Broiler Flocks

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Abstract

The present study was conducted on 30 broiler flocks (organs of 5 birds per flock homogenized as composite or pooled one sample) for cultural isolation, identification, biochemical characterization and antibiogram profile of *Escherichia coli* (*E. coli*). All the 30 composite samples (100%) revealed colonies with typical morphology and biochemical characteristics of *E. coli*. Antibiogram study was conducted by disc diffusion method and results revealed the highest 100% sensitivity to antibiotic colistin followed by amikacin (83.33%) ceftriaxone (80.00%), chloramphenicol (60.00%), cefotaxime (60.00%) gentamicin (53.33%) and norfloxacin (6.66%), while the organisms were highly resistant to norfloxacin (83.33%) followed by gentamicin (46.66%). In addition, all the 30 *E. coli* isolates were tested for presence of virulence associated genes, *i.e.*, *iss, papC, tsh* and *vat* by PCR using gene specific primers. Result showed the highest prevalence of *iss* gene 26/30 (86.66%) followed by *vat* 24/30 (80.00%), *tsh* 16/30 (53.33%) and *papC* 9/30 (30.00%) in the flocks tested.

Keywords: *Escherichia coli*, Prevalence, Antibiogram, Broiler, Virulence genes.

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Introduction

Escherichia coli is considered as the fundamental cause of morbidity and mortality in birds. Normally, *E. coli* is present in intestinal tract and environment of poultry, but certain strains designated as Avian Pathogenic *Escherichia coli* (APEC) possess specific virulence factors and are able to cause disease. There are various manifestations of *E. coli* infection among which the most severe is colisepticemia, which is characterized by multiple organ lesions like air sacculitis, pericarditis, peritonitis, salpingitis, synovitis, osteomyelitis or yolk sac infection. Now a day, there is increase in incidence and severity of colisepticemia in broilers as well as in layers and thus impose a major threat on the poultry industry. APEC strains impose severe problems on aviculture industries. A number of potential virulence associated genes (*iss, papC, tsh, vat, irp-2, iucD, hlyF, iron*, *cva/cvi*, and *astA*) have been identified in APEC, including their virulence-associated bacterial properties (Dho-Moulin and Fairbrother, 1999). This study was therefore aimed to isolate and study the antibiogram and molecular characterization of *Escherichia coli* in broiler flocks of middle Gujarat.

Materials and Methods

The study was carried out in thirty broiler flocks (5 birds per flock) received for post-mortem diagnosis at Department of Pathology, Veterinary College, Anand. For isolation, identification, antibiogram profile and molecular characterization of *E. coli*, a total of thirty composite samples (consisting of homogenized mixture of heart, liver, lung and spleen from all 5 birds of a flock) were collected during post-mortem examination and stored in cryo-vials at -20°C.

Isolation and Identification of *E. coli*

Thirty composite samples collected from 30 farms were streaked on MacConkey agar plates. After 24 hours of incubation the pink colored colonies were identified and subcultured on Eosin Methylene Blue (EMB) agar. After incubation, bacterial colonies were investigated on the basis of staining, colony morphology, cultural and biochemical characters of pure isolates.

Antibiogram of *E. coli* Isolates

All the thirty isolates of *E. coli* obtained were subjected

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to *in vitro* antimicrobial sensitivity test against seven commonly used antimicrobials, *viz.*, amikacin, ceftriaxone, chloramphenicol, colistin, cefotaxime, norfloxacin, and gentamicin. The test was carried out by disc diffusion technique of Bauer *et al.* (1966). The code and concentrations of antimicrobial discs used are mentioned in Table 1.

Molecular Characterization of *E. coli* Isolates

The DNA extraction was done from all the thirty isolates of *E. coli* obtained during the study. The suspension of the organisms from each isolate was prepared by picking up a typical colony in a 200 µl PCR tube in 100 µl of Milli-Q water. The prepared suspension was heated at 95°C for 15 minutes and all the cell debris were removed by centrifugation at 10,000 rpm for 1 minute and 3 µl of the supernatant was used as a template DNA. For the molecular characterization of *E. coli,* extracted DNA from each isolate was subjected to PCR amplification of *E. coli* virulence associated genes. The PCR amplification of virulence associated genes was carried out using primers as in Table 2. The steps and conditions of thermal cycling for different primers of virulence genes used are given in Table 3.

Results and Discussion

Isolation and Identification of *E. coli*

Cultural isolation and identification of *E. coli* was done on MacConkey agar (MCA) plate. Lactose fermentation by bacteria on MCA plates resulted in appearance of pink coloured colonies (Fig. 1). These colonies were subcultured on Eosin Methylene Blue (EMB) agar plate. Colonies with metallic greenish sheen on EMB agar plates were tentatively considered to be positive for *E. coli* (Fig. 2). For confirmation, a loopful colony was selected for Gram staining which revealed pink coloured Gram negative bacilli (Fig. 3). Based on cultural characteristics all the thirty pooled/composite samples were found positive for *E. coli*.

Biochemical Characterization of *E. coli* Isolates

All the thirty *E. coli* isolates obtained were characterized by biochemical tests, *i.e.,* indol production, methyl red (MR), Voges-Proskaur (VP) and citrate utilization test and the results were interpreted according to the Bacteriological Analytical Manual (US FDA: BAM, 2002). All the isolates revealed similar IMViC pattern of + + - - (Fig. 4).

Table 1: Details of antimicrobial agents and concentration of discs used for antimicrobial susceptibility of *E. coli*

				Diameter of zone of inhibition (mm)		
Sr. No.	*Name of the antimicrobial disc*	*Code*	*Conc. (in mcg)*	*Resistant*	*Intermediate*	*Sensitive*
1	Amikacin	AK	30	10	11-15	16
2	Gentamicin	GEN	10	12	13-14	16
3	Norfloxacin	NK	10	12	13-16	17
4	Chloramphenicol	C	30	12	13-17	18
5	Ceftriaxone	CTR	30	19	20-22	23
6	Colistin	CL	10	10	-	11
7	Cefotaxime	CTX	30	22	23-25	26

Table 2: Primer sequence used for the detection of virulence associated genes in *E. coli* isolates

Name of Primers		*Sequences (5'-3')*	*Expected product size (bp)*	*References*
iss	Forward	CCCCAATTGGACAGAGAAAA	174	Ewers *et al.* (2004)
	Reverse	ATCGATGGGCCTATTGTGAG		
papC	Forward	AATAAAAACGTGGCGGACTG	201	
	Reverse	ACGCAGGTAAGCAGAATCGT		
tsh	Forward	TCTCAATGCGTCGTAACAGC	153	
	Reverse	CCTTCAGATGAACGTCAGCA		
vat	Forward	CACGCTACTGAATGCCTGAA	168	
	Reverse	TGGCAGGTTAATGGTGTGAA		

Table 3: Steps and conditions of thermal cycling for detection of virulence associated genes of *E. coli*

Target genes	*Initial Denaturation*	*Denaturation*	*Annealing*	*Extension*	*Final Extension*
iss, papC	94°C, 5 min	94°C, 30 sec 35 cycles	56°C, 30 sec	72°C, 30 sec	72°C, 5 min
tsh. vat	94°C, 3 min	94°C, 30 sec 35 cycles	58°C, 30 sec	72°C, 30 sec	

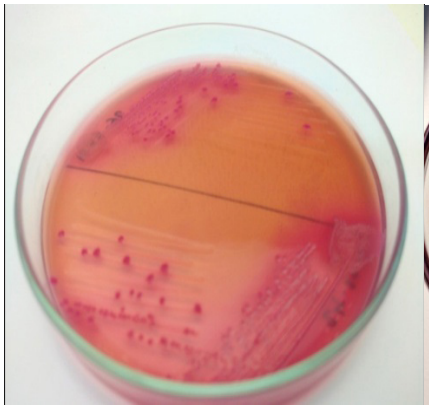

Fig. 1: Lactose fermenting pink colour colonies of *E. coli* on MacConkey agar

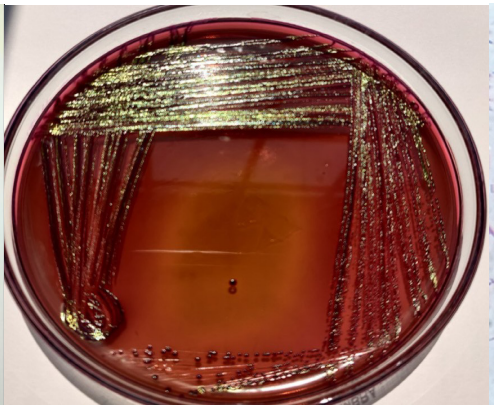

Fig. 2: Greenish metallic sheen produced by *E. coli* on EMB agar

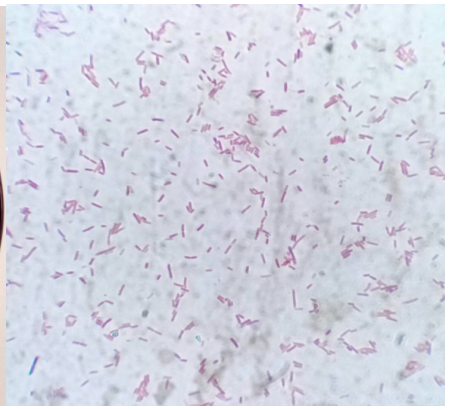

Fig. 3: Micrograph revealed pink coloured gram negative bacilli (*E. coli*) (1000X).

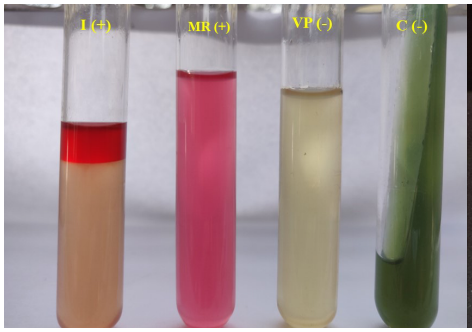

Fig. 4: Photograph showing result of IMViC test for *E. coli* (++--)

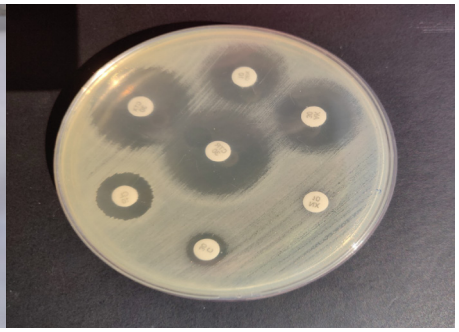

Fig. 5: Image showing *in vitro* antibiotic susceptibility pattern of *E. coli* by disc diffusion method.

Antibiogram of *E. coli* Isolates

The *in vitro* antimicrobial drug sensitivity pattern of all the thirty *E. coli* isolates tested against seven commonly used antimicrobials (Fig. 5) revealed that the *E. coli* isolates were highly sensitive to antibiotic colistin, *i.e.*, methane sulphonate 30/30 (100.00%) followed by amikacin 25/30 (83.33%), ceftriaxone 24/30 (80.00%), chloramphenicol 18/30 (60.00%), cefotaxime 18/30 (60.00%) gentamicin 16/30 (53.33%) and norfloxacin 2/30 (6.66%), whereas they were highly resistant to norfloxacin 25/30 (83.33%) followed by gentamicin 14/30 (46.66%) and chloramphenicol 11/30 (36.66%) as shown in Fig. 6.

Chaudhari (2017) and Halfaoui *et al.* (2017) also showed highest sensitivity of *E. coli* to colistin (100 and 93.46 %). On the contrary, Mohamed *et al*. (2014), Jahantigh and Dizaji (2015), Subedi *et al.* (2018) and Awad *et al*. (2020) showed lower sensitivity.

The present sensitivity pattern of *E. coli* isolates to Amikacin (83.33%) was well supported by Sahoo *et al*. (2012) Subedi *et al*. (2018) and Sarba *et al.* (2019), while Soufi *et al*. (2009) reported 100 % sensitive of *E. coli* isolates to amikacin. Similarly, sensitivity to ceftriaxone (80.00%) observed during the present study was also well supported by Sahoo *et al*. (2012) and Chaudhari (2017).

The sensitivity of *E. coli* to chloramphenicol (60.00%), cefotaxime (60.00%) and Gentamicin (53.33%) observed during the present study was well in accordance with the reports of Soufi *et al*. (2009), Sahoo *et al*. (2012), Halfaoui *et al.* (2017), Subedi *et al.* (2018) and Awad *et al*. (2020).

Further, the highest resistance (83.33%) of *E. coli* isolates was observed against norfloxacin in the present study. Mohamed *et al*. (2014) and Jahantigh and Dizaji (2015) also showed comparable 96.00 and 88.00 % resistance to norfloxacin.

The pattern of antibiotic sensitivity using different antibiotic discs against *E. coli* isolates in poultry has been reported time to time by previous workers. The sensitivity/ resistance pattern of a specific antibiotic was found to be variable by different workers. The antibiotic sensitivity pattern of the present study showed colistin to be most sensitive antibiotic followed by amikacin, ceftriaxone, cefotaxime and chloramphenicol, while gentamicin and norfloxacin were found less sensitive. It should be noted that, the antibiotic resistance to *E. coli* in poultry is becoming a major issue and unnecessary use of antibiotics should be avoided. When necessary, specific antibiotics should be used after the sensitivity test.

Molecular Characterization of *E. coli* Isolates

During the present study, PCR based identification of all thirty *E. coli* samples for the *iss, papC, tsh* and *vat* genes was targeted by using gene specific primers, which revealed the highest (26/30 ,86.66%) prevalence of *iss* gene followed by *vat (*

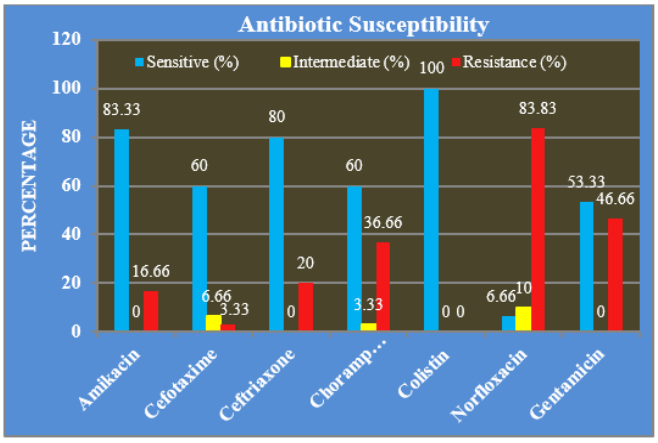

Fig. 6: Chart showing *in vitro* antibiotic sensitivity results of *E. coli* isolates against various antibiotics.

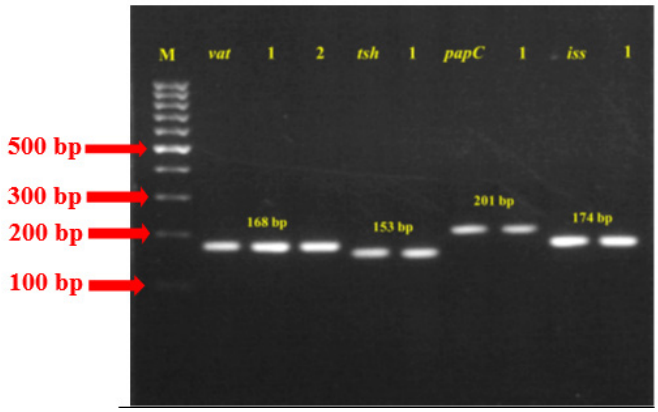

Fig. 7: Agarose gel showing amplified product of *E.coli* virulence gene.
M: DNA ladder-100 bp 1 and 2: test sample
vat: +ve control for *vat*, *papC*: +ve control for *papC*
tsh : +ve control for *tsh*, *iss*: +ve control for *iss*

24/30, 80.00%), *tsh* (16/30, 53.33%) and *papC (*9/30, 30.00%) (Fig. 7; Fig. 8). Similar findings for presence of virulence gene *iss* of *E. coli* in broiler chickens have been reporteed by Ewers *et al.* (2004), Mohamed *et al.* (2014), Deshmukh (2016) and Chaudhari (2017). Similarly, Subedi *et al.* (2018) and Awad *et al.* (2020) also identified the presence of *iss* gene in the highest number of *E. coli* isolates . In contrast, Delicato *et al.* (2003) and Won *et al.* (2009) reported lower frequency of *iss* gene in *E. coli* isolates.

Nine (30.00%) isolates yielded an amplified product of 201 bp of *papC* gene (Fig. 7). Similarly, Delicato *et al.* (2003), Ewers *et al.* (2004), Won *et al.* (2009), Deshmukh (2016) and Chaudhari (2017) detected lower frequencies of *papC* at the rate of 18.50, 22.70, 14.40, 27.50 and 33.33%, respectively, whereas, Roussan *et al.* (2014) and Mohamed *et al.* (2014) found this gene at the rate of 50.00 and 44.40%, respectively, on *E. coli* isolates in poultry.

Sixteen (53.33%) *E. coli* isolates yielded an amplified product of 153 bp of *tsh* gene (Fig. 7). This was in line with observations of Ewers *et al.* (2004), Won *et al.* (2009), Deshmukh (2016) and Chaudhari (2017), whereas, Roussan

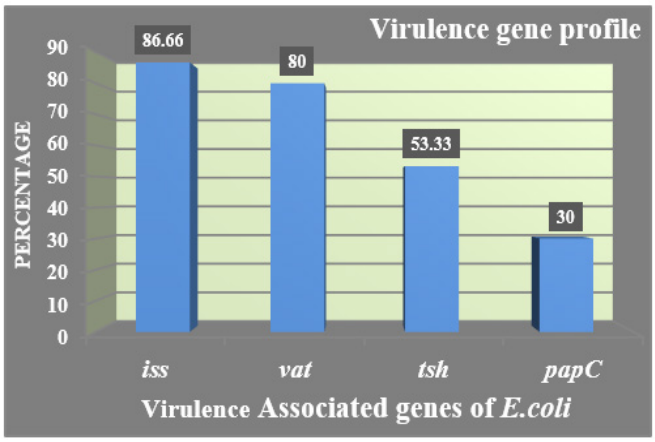

Fig 8: Chart showing virulence genes profile of thirty broiler flocks positive for Avian Pathogenic *E. coli*

et al. (2014) and Subedi *et al.*(2018) found higher occurrence of this gene at the rate of 66.00 and 62.20 %, respectively. In contrast, Delicato *et al.* (2003) reported lower occurrence of *tsh* gene in strains of *E. coli* isolated from chickens with colibacillosis.

Twenty four (80.00%) *E. coli* isolates yielded an amplified product of 168 bp of *vat* gene (Fig. 7), which concurred well with the reports of Roussan *et al.* (2014) and Chaudhari (2017), respectively. Whereas, Won *et al.* (2009) and Deshmukh (2016) found lower prevalence of this gene.

On the basis of results obtained during the present study, it can be concluded that a combination of more than one virulence associated genes may be responsible for pathogenicity of *E. coli* organism and are capable of causing colisepticemia. All the thirty *E. coli* isolates revealed presence of multiple virulence associated genes; 19 were found carrying two out of four virulence associated genes. Seven samples were positive for three genes, while four had all the four virulence associated genes in *E. coli* genome.

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