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Characterization of Avian Pathogenic *Escherichia coli* Associated with Complicated Chronic Respiratory Disease

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A B S T R A C T

Among other factors, infectious diseases are the real threat to the poultry development. Of the various infectious microorganisms *Escherichia coli* and *Mycoplasma* are of prime importance. These microorganisms persist for reasonably long periods in the flocks and there by lead to high economic losses through mortality, loss of weight and production. *E. coli* cannot readily infect the air sacs unless they are previously invaded by *Mycoplasma gallisepticum* alone or in combination of either infectious bronchitis or Newcastle disease virus. The present research was therefore designed to study the susceptibility of *Mycoplasma* infected birds towards *E. coli* as the average mortality rates observed in the occurrence of CRD as an individual disease was 8 per cent, whereas *E. coli* combined outbreaks recorded 15 per cent. In the present study, a total of 255 clinical specimens which included choanal swabs (244) from live and trachea (11) from dead birds affected with Mycoplasmosis were processed. Out of total 68 (12.15%) *E. coli* isolates recovered, 31 (45.58%) were associated with Mycoplasmosis and were recovered from choanal swabs. Recovered *E. coli* isolates subjected to AST by using different antibiotics like ampicillin (AMP), oxytetracycline (O), tylosine (TL), erythromycin (E), enrofloxacin (EX), norfloxacin (NX) and gentamicin (GEN). Multiple drug resistance was observed in all isolates of *E. coli.* Conventional characterization of *E. coli* isolates revealed, 12 (41.37%) isolates haemolytic, 26 (83.87%) invasive *E. coli* and 7 (22.58%) isolates positive for haemagglutination, respectively. In *cvi* gene PCR, 6 (19.32%) isolates yielded an amplification product of ~1181 bp. Out of 20 isolates tested which are resistant to ampicillin, 3 (15%) and 17 (85%) isolates yielded a product of ~403 bp in bla_{TEM} gene specific PCR using chromosomal and plasmid DNA, respectively. Plasmid profiling of the *Escherichia coli* isolates detected four different sizes of plasmids (~700 bp, ~1100bp, ~1650bp and >12kbp) with presence of >12kbp plasmid in all the isolates. The plasmids were distributed at random in the isolated *E. coli* strains. Correlation of presence of bla_{TEM} gene and plasmid profile was observed with resistance to ampicillin seen in AST.

Introduction

Complicated chronic respiratory disease is an infectious disease of birds causing serious economic losses to the poultry industry. CCRD is usually caused by *Escherichia coli* in association with poultry Mycoplasmosis (Nagalakshmi *et al.*, 2013 and Kamble, 2014).

Cultural isolation and identification of *Escherichia coli* associated with Mycoplasmosis is of crucial importance since it provides useful information on epidemiology of the disease and facilitates tracing the origins of pathogen. Severe airsacculitis characterized by appearance of large masses of caseous exudate in the air sacs and egg peritonitis were observed in combined infection with *E. coli*, whereas uncomplicated CRD affected birds showed milder airsacculitis (Sivaseelan and Balasubramaniam, 2013).

Now a day, the molecular techniques include various types of PCR assays targeting different virulence genes are widely employed for detection of pathogenic *Escherichia coli* and plasmid profiling for to know the gene involved in antibiotic resistance among *Escherichia coli*. Inspite of being such an important disease; there is no such work carried out on this aspect of complicated chronic respiratory disease. Therefore, present investigation was designed for characterization of *Escherichia coli* associated with poultry Mycoplasmosis which can help in prevention and control of disease and thereby reducing the economic losses.

Materials and Methods

Materials

Clinical specimens: Choanal swabs and tracheal tissue

Culture media: MacConkey agar and Eosin methylene blue agar (HiMedia) used for isolation of *E. coli*, Congo red agar prepared by Trypticase soy agar supplemented with 0.003% Congo red dye and 0.15% bile salt was used for determination of invasive *E. coli* while blood agar was used for haemolysis assay.

Reference strain: *Escherichia coli* (ATCC® 25922™)

Oligonucleotide primers: *E. coli cvi*-F 5'-TGG-TAG-AAT-GTG-CCA-GAG-C-3', *cvi*-R 5'-AAG-GAG-CTG-TTT-GTA-GCG-AAG-CC-3', Ewers *et al.*, (2005) and bla_{TEM} –F 5'- TTT-CGT-GTC-GCC-CTT-ATT-CC-3', bla_{TEM} –R 5'- ATC-GTT-GTC-AGA-AGT-AAG-TTG-G- 3' Bali *et al.*, (2010).

Methods

Collection of material

Success in recovery of *E. coli* after culturing specimens depends on careful collection of suitable specimens, proper transportation and accurate processing of clinical sample. In present study specimens were collected aseptically and placed in sterile leak-proof containers of appropriate size (OIE, 2008). The specimens were packed in stout polystyrene foam containers and transported with care as early as possible.

A total of 255 samples which included, choanal swabs (244) from live birds and trachea (11) from dead birds, suspected for CCRD formed the material for investigation in the present study (Table 1).

Isolation and identification of *E. coli*

Isolation of *E. coli* was carried out on MacConkey agar and Eosin methylene blue agar using suitable incubation conditions. Inoculated agar plates were incubated at 37^{o}C temperature for 24 hrs. The isolates suggestive of *Escherichia coli* were stained with Gram's staining and after observation as Gram negative bacilli, the isolates were further subjected to conventional bacteriological procedures as per Quinn *et al.*, (2002) for confirmation as *Escherichia coli*. The identification of field isolates of

Escherichia coli was done on the basis of various biochemical tests like Indole, Methyl red, Voges proskauer, Nitrate, Citrate, Urease, TSI, Oxidase, Catalase and Motility test.

Antibiotic sensitivity test of *Escherichia coli*

All isolates of *E. coli* were subjected to *In vitro* antibiotic sensitivity test by disc diffusion method described by Bauer *et al.*, (1966) using suitable commercial available antibiotic discs viz., Oxytetracycline, Ampicillin, Tylosine, Erythromycin, Norfloxacin, Enrofloxacin and Gentamicin (Hi Media).

Conventional characterization of *E. coli* isolates

All isolates of *E. coli* were characterized conventionally by using congo red binding assay, haemolysis assay and haemagglutination test for to detect virulence properties of the *E. coli*.

Molecular characterization of *E. coli* isolates

Molecular characterization of *E. coli* isolates was carried out by PCR of *cvi* gene described by Ewers *et al.,* (2005) and bla_{TEM} gene described by Bali *et al.,* (2010) and plasmid profiling. Plasmid DNA and Chromosomal DNA extraction was carried out as per the protocol described by Sambrook and Russell (2008).

Results and Discussion

In cultural isolation from 255 specimens the isolation of *Escherichia coli* was successful from 68 (26.66%) specimens while remaining 187 (73.33%) specimens were proved to be negative for *E. coli*. Out of 68 *E. coli* isolates recovered, 31 (45.58%) isolates were associated with Mycoplasmosis. All *E. coli* isolates were recovered from choanal swabs (Table 2). All 31 field isolates were identified and confirmed as *E. coli* based on results of conventional identification methods (Plate 1, 2, 3, 4, 5, 6, 7, 8 and 9).

All 31 *E. coli* isolates associated with CCRD were tested for susceptibility to seven suitable antibiotics by disc diffusion method. The results of antibiotic susceptibility test indicated maximum resistance of *E. coli* isolates to ampicillin and oxytetracycline (64.51%) followed by tylosine (61.29%) and erythromycin (45.16%). The least resistance was observed to enrofloxacin (38.70%) and norfloxacin (22.58%), whereas all the isolates were sensitive to gentamicin (100%) (Plate 10).

Conventional characterization of 31 *E. coli* isolates revealed 26 (83.87%), 12 (41.37%) and 7 (22.58%) were invasive (Plate 11), haemolytic and positive for haemagglutination test (Plate 12), respectively. Molecular characterization of *E. coli* isolates showed that 6 (19.32%) isolates amplify a product of ~1181bp specific for *cvi* gene (Plate 13).

Out of 20 isolates showed resistant to ampicillin, 3 (15%) and 17 (85%) isolates yielded a product of ~403 bp in bla_{TEM} gene specific PCR using chromosomal and plasmid DNA respectively (Plate 14).

Out of 31 *E. coli*, 16 (51.61%) possessed ~700 bp plasmid, 17 (54.83%) had ~1100 bp plasmid, 6 (19.35%) had ~1650 bp plasmid, while the presence of >12kbp plasmid observed in all the isolates. All the isolates displayed different numbers of plasmids as shown in (Plate 15).

Table.1 Details of specimens used for investigation

No.	Source	Type of bird	Specimen	No. of Samples	Total
1.	Anand (Gujarat)	Breeders	Choanal swabs	50	50
2.	Bangalore (Karnataka)	Breeders	Choanal swabs	45	45
3.	Vijayawada (Andhra Pradesh)	Layers	Choanal swabs	10	10
4.	Aurangabad (Maharashtra)	Breeders	Trachea	11	11
5.	Hyderabad (Andhra Pradesh)	Layers	Choanal swabs	19	40
		Broiler Breeders		21	
6.	Pune (Maharashtra)	Breeders	Choanal swabs	23	70
		Layers		47	
7.	Palghar (Maharashtra)	Broilers	Choanal swabs	15	15
8.	Pen (Maharashtra)	Broiler	Choanal swabs	14	14
Grand Total			Choanal swabs	244	255
			Trachea	11	

Table.2 Results of isolation of *E. coli* from clinical specimens

Sr. No.	Sources	Specimens	No. of Samples	No. of *E. coli* isolates recovered	No. of *E. coli* isolates associated with CRD
1.	Anand (Gujarat)	Choanal swab	50	15	15
2.	Bangalore (Karnataka)		45	20	13
3.	Vijayawada (Andhra Pradesh)		10	01	01
4.	Aurangabad (Maharashtra)	Trachea	11	11	00
5.	Hyderabad (Andhra Pradesh)	Choanal swab	40	10	00
6.	Pune (Maharashtra)		70	07	00
7.	Palghar (Maharashtra)		15	03	00
8.	Pen (Maharashtra)		14	02	02
Total		Choanal swab	244	57	31
		Trachea	11	11	00
Grand total			**255**	**68**	**31**

Plat.1 *E. coli* on MacConkey agar

Plate.2 *E. coli* on Eosin methylene blue medium

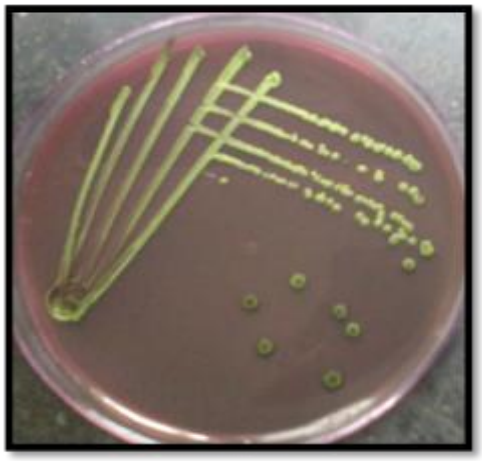


Plate.3 *E. coli* in Gram stained Smear

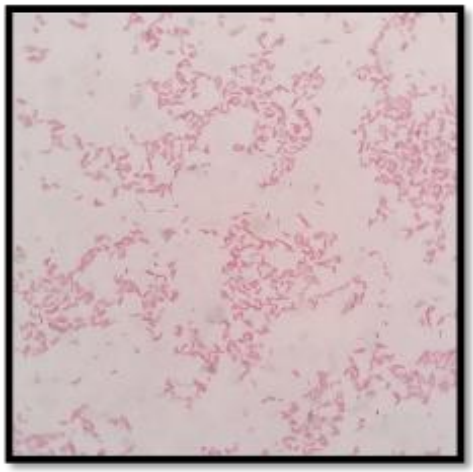


Plate.4 *E. coli* showing Indole positive reaction

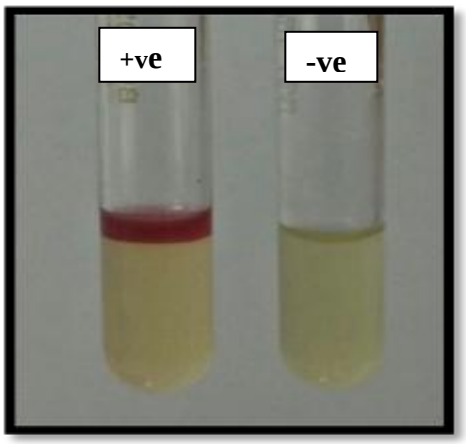


Plate.5 *E. coli* showing MR positive reaction

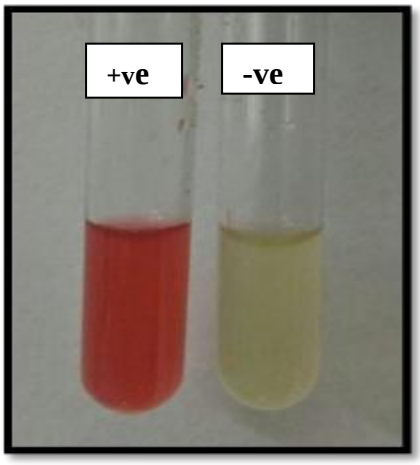


Plate.6 *E. coli* showing VP negative reaction

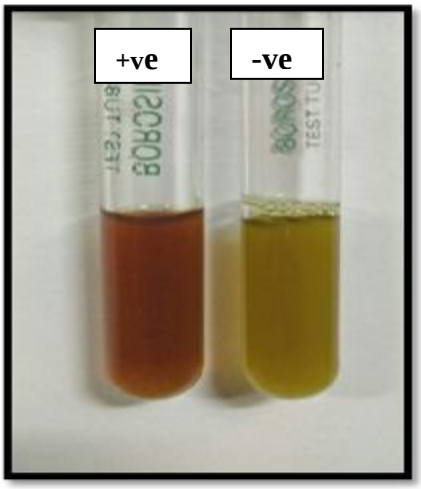


Plate.7 *E. coli* showing citrate negative reaction

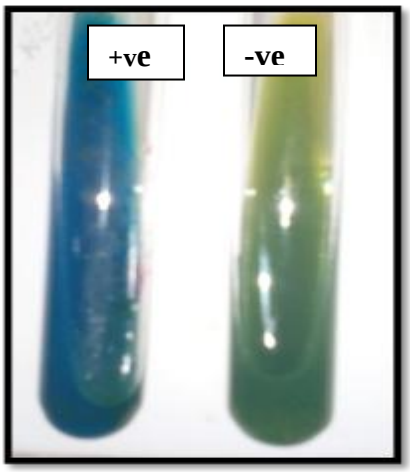


Plate.8 *E. coli* showing urease negative reaction

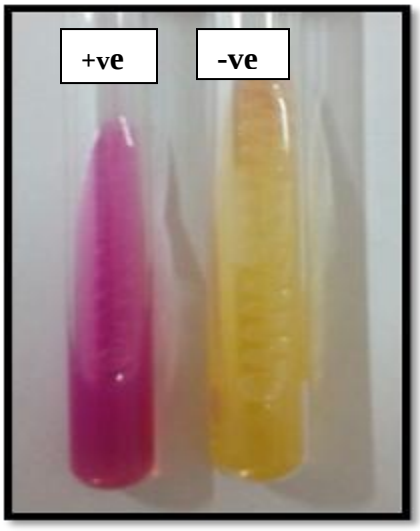


Plate.9 *E. coli* showing oxidase negative reaction

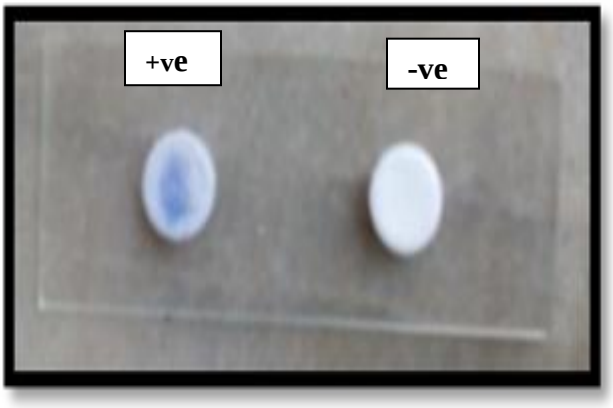


Plate.10 *E. coli* showing catalase positive reaction

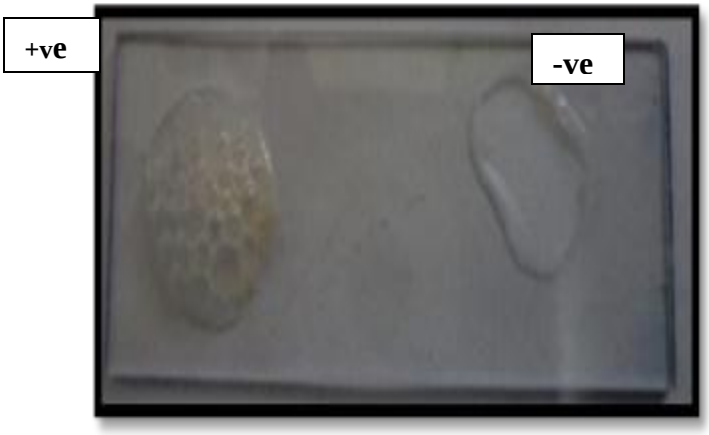


Plate.11 *E. coli* showing TSI negative reaction

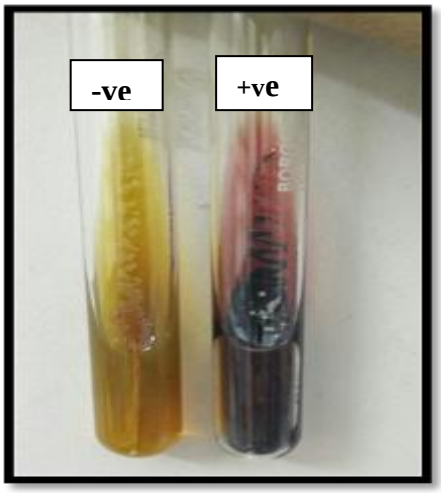


Plate.12 Antibiogram of *Escherichia coli* isolates showing multi drug resistance

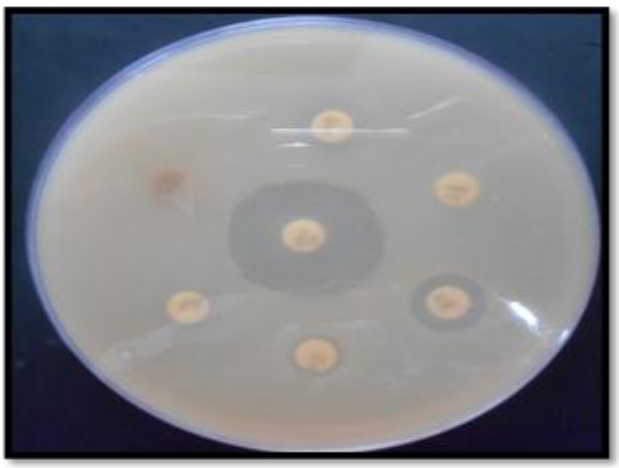


Plate.13 Invasive and non invasive *E. coli* on Congo red agar medium

Plate.14 Haemagglutination test *of E. coli* field isolates

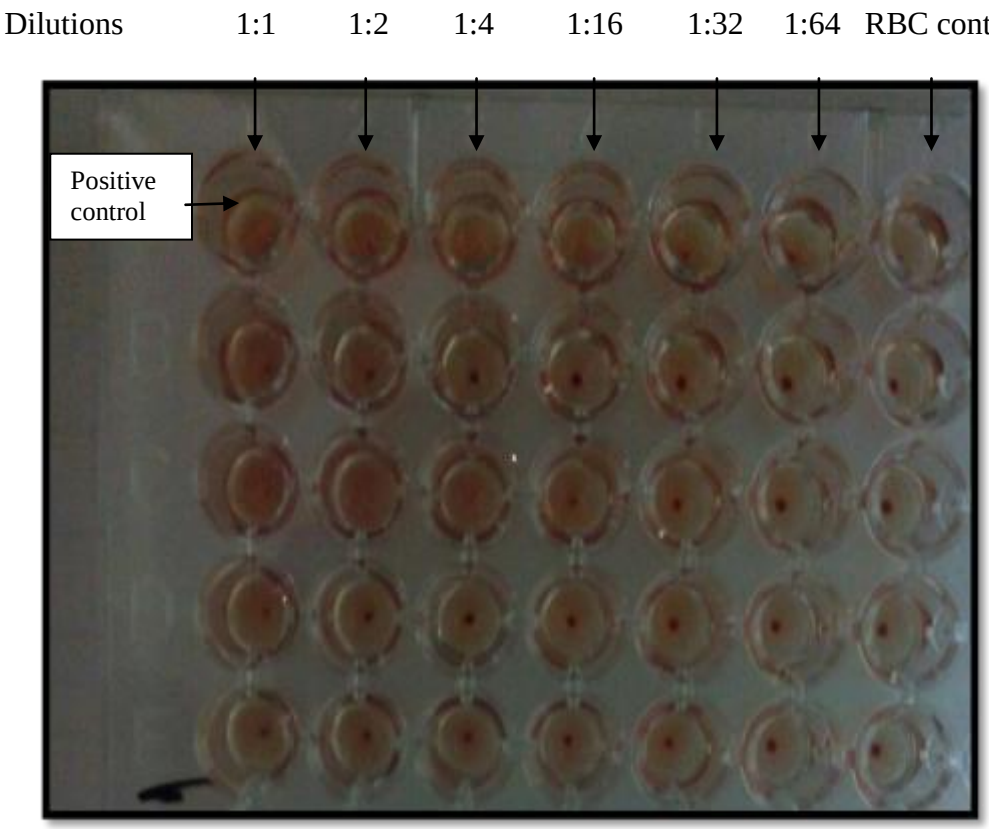
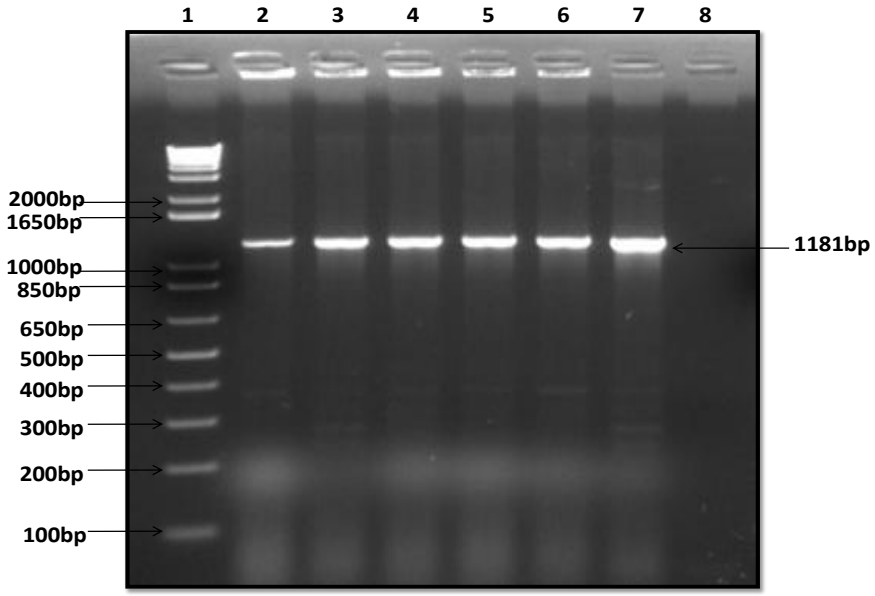
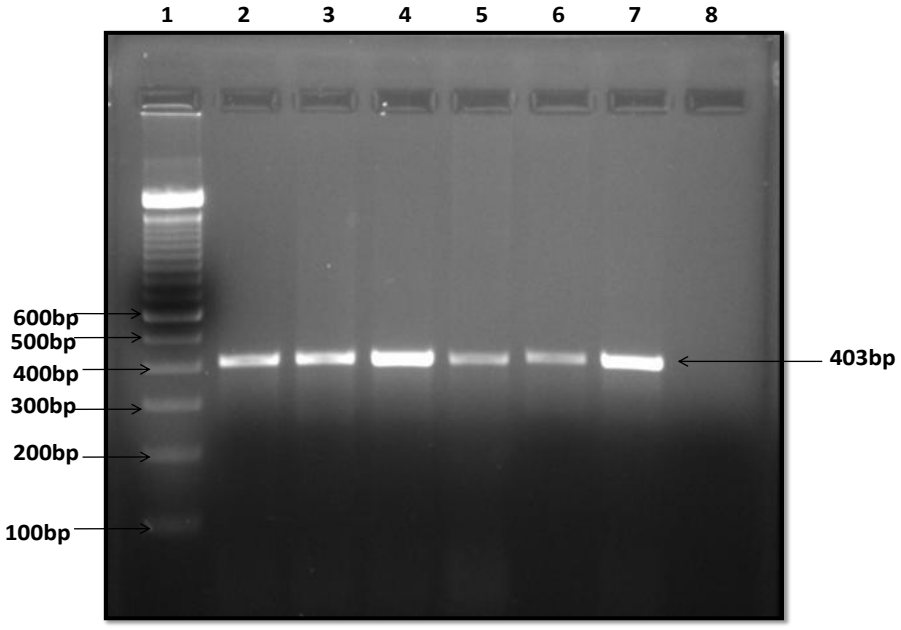


Plate.15 PCR of *cvi* gene of *E. coli* isolates



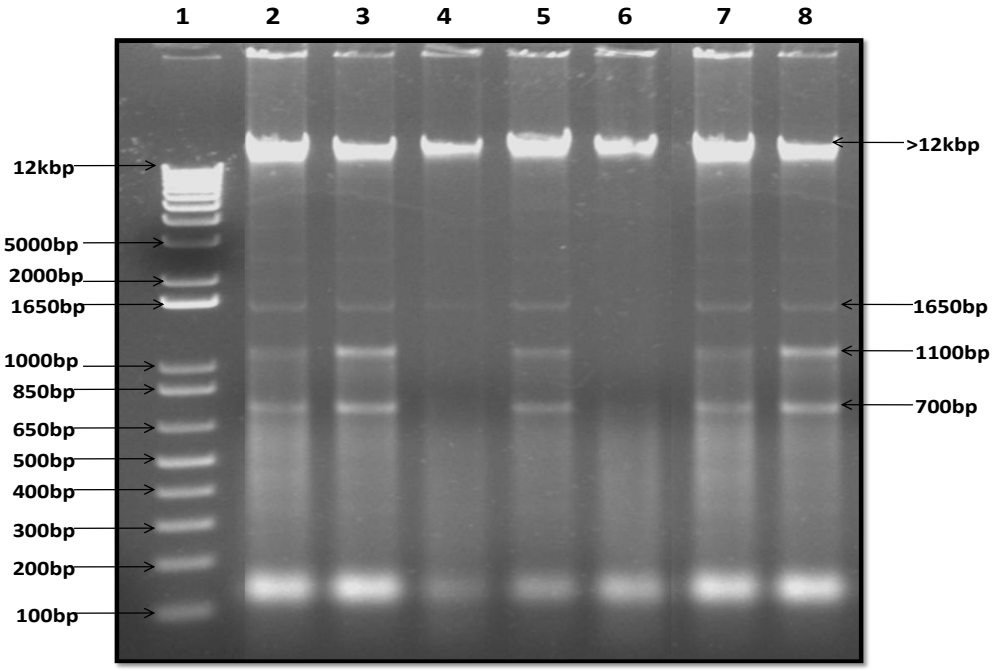
100 bp standard DNA ladder, Lane-2 to Lane-6: positive *E. coli* isolates, Lane-7: Positive control and Lane-8: Negative control)

Plate.16 PCR of bla$_{TEM}$ gene of *E. coli* isolates



(Lane-1: 100 bp standard DNA ladder, Lane-2 to Lane-6: positive *E. coli* isolates, Lane-7: Positive control and Lane-8: Negative control)

Plate.17 Plasmid profiling of *Escherichia coli* isolates



Lane-1: One kb plus standard DNA ladder, Lane-2 to Lane-8: plasmid bands of different sizes of *E. coli* isolates

In the present investigation, isolation of *Escherichia coli* was successful only from 31 (45.58%) isolates. Several researchers (Radwan *et al.,* 2014, Ramasamy *et al.,* 2008, Syuhada *et al.,* 2013 and Fatma *et al.,* 2008) throughout the world have reported isolation of *Escherichia coli* from different clinical specimens with varying degrees of success (17% to 60%). Since number of factors are likely to influence the successful isolation of *Escherichia coli* from clinical specimens; especially the availability of appropriate material at the appropriate stage of the disease, season of sample collection, transportation conditions and processing time.

All 31 *E. coli* isolates associated with CCRD were tested for susceptibility to seven suitable antibiotics by disc diffusion method. The *E. coli* isolates in the present study showed multi drug resistance towards six out of seven antibiotics used. The source of the resistance may be assumed from the poultry feeds consumed, since antibiotics are used as feed additives to improve feed efficiency and weight gain. Many antibiotics are also used in feed and water to control the diseases. Indiscriminate use of antibiotics has provided selective pressure for the emergence of drug resistance resulting in a larger proportion of *E. coli* resistance as reported by (Atere *et al.,* 2015).

In the present study, conventional characterization of *E. coli* isolates was carried out using Congo Red (CR) binding assay, haemolytic activity and haemagglutination test. The results of present study showed that, out of 31 *E. coli* isolates, 26 (83.87%) were CR positive. The similar findings for Congo red binding activity were observed by (Aziz *et al.,* 1995 and Seifi *et al.,* 2015) in the range of (82.6% to 88.75%) CR positive *E. coli* strains, respectively.

Production of haemolysin is usually associated with pathogenicity of *E. coli* especially responsible for more severe forms

of infection (Johnson, 1991). The result of haemolysin production i.e., 41.37% (12/31) of present study laid between the range reported by different authors. Varying results of haemolytic activity were observed by different workers viz., higher rate of *E. coli* strains positive for haemolytic activity observed in 73%, 56.47%, 59%, 92.9% and 84.2% by Aziz *et al.,* (1995), Fatma *et al.,* (2008), Bashar *et al.,* (2011), Al-Arfaj *et al.,* (2015), Hassan and Bakeet (2014), respectively. Whereas very low haemolytic activity of 1.52%, 10% and 37.03% was observed by Shankar *et al.,* (2010), Raji *et al.,* (2003) and Ruaa *et al.,* (2014) respectively.

The adhesion ability of an organism is assessed by determining the haemagglutination, because the erythrocyte membrane is believed to possess the homologous of the mucosal substances involved in bacterial adherence to epithelial cells (Hager *et al.,* 2010). Haemagglutination assay in the present study demonstrated 7 (22.58%) isolates positive. The findings of present research range between the Ruaa *et al.,* (2014) and Aziz *et al.,* (1995) stated that 3.70% and 87.5 % of *E. coli* isolates were able to induce hemagglutinin respectively.

In the present study, molecular characterization of 31 *E. coli* isolates was carried out for detection of *cvi* and bla$_{TEM}$ gene by PCR and antibiotic resistance by plasmid profiling.

Out of 31 *E. coli* subjected to PCR, targeting *cvi* gene, 6 (19.32%) *E. coli* isolates were found positive for *cvi* gene. All *cvi* gene PCR positive isolates yielded an amplification product of ~1181 bp. The results of PCR of *cvi* correlated with results of Ewers *et al.,* (2005) and Arabi *et al.,* (2013) where they reported an amplification product of ~1181 bp specific for *cvi* gene of *E. coli,* respectively.

Out of 31 *E. coli* isolates, 20 isolates which showed resistance to ampicillin phenotypically were subjected to PCR by using chromosomal DNA and plasmid DNA for amplification of bla$_{TEM}$ gene. Out of 20 isolates, 3 (15%) and 17 (85%) isolates yielded a product of ~403 bp in bla$_{TEM}$ gene specific PCR using chromosomal and plasmid DNA, respectively. The results of PCR of bla$_{TEM}$ gene correlated with result of Bali *et al.,* (2010), who reported an amplification product of ~403 bp specific for beta lactam resistant gene of *E. coli*. The higher percentage of bla$_{TEM}$ gene was observed in plasmid DNA (85%) than chromosomal DNA (15%) i.e., suggesting plasmid borne resistance to ampicillin. The results of bla$_{TEM}$ gene correlates with the findings of the Wang *et al.,* (2013) and Ammar *et al.,* (2015) detected the bla$_{TEM}$ gene by using plasmid DNA in 65.9% and 100% *E. coli* isolates, respectively and reported the resistance to beta lactam antibiotic.

In plasmid profiling of 31 *E. coli* isolates, four different sizes of plasmids were estimated viz., ~700 bp, ~1100 bp, ~1650 bp and >12 kbp. Nsofor and Iroegbu (2013) reported that isolates with high multi drug resistance profile possessed multiple plasmids with large sizes in the range of 1 to 120 Kb.

In conclusion from the present research it is concluded that association of *Escherichia coli* with chronic respiratory disease was observed in 45.58% cases with overall incidence of 12.15% in respiratory tract infection. *Escherichia coli* isolates associated with chronic respiratory disease could be recovered from choanal swabs. Gentamicin was found to be most effective antibiotic followed for treatment of *Escherichia coli* infection in CCRD. Most of *Escherichia coli* isolates (83.87%) were found to be pathogenic based on Congo red binding activity and showed association in different virulence factors. All

isolates positive for *cvi* gene in molecular characterization of virulence were also positive for virulence factor tested phenotypically. Correlation of presence of bla_{TEM} gene and plasmid profile was observed with resistance to ampicillin seen in AST. Further studies focussing on evaluation of PCR analysis of various suitable genes associated with the virulence and antibiotic resistance on a larger numbers of clinical isolates will produce valuable data useful in molecular epidemiology. Studies on synergistic effect of different combinations of antibiotics for effective treatment against both *Mycoplasma* spp. and *E. coli* infection are recommended.

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