



## Molecular characterization of multidrug-resistant avian pathogenic *Escherichia coli* isolated from poultry and poultry products

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### ABSTRACT

Overall occurrence of *Escherichia coli* in the current study was 7.55% and corresponding group wise occurrence was 13%, 2.66%, 10% and 7% in a faecal and cloacal samples, environmental samples, Retailer's shop's samples, and restaurant samples respectively. Amongst the different type of samples processed, highest recovery was from samples collected at poultry farms (13%) followed by Retailer's shop (10%) suggesting heavy infection and contamination occurring at the initial points of poultry chain. In this study, four isolates failed to exhibit pathogenic potential of *E. coli* by using *in vitro* pathogenic assays viz. Congo red dye binding assay and haemolysis by using 5% sheep blood agar, also turned out to be negative on virulent marker based PCR, suggesting proficient efficacy of targeted genes viz. *ecp* and *uidA* for precise and time saving determination of pathogenic potential of *E. coli* isolates. Besides, one isolate recovered from raw chicken meat sample showed resistance against all 15 antibiotics and other two isolates recovered from litter samples showed resistance against 14 antibiotics suggesting development of multi-drug resistance in common food pathogens, against the most widely used and effective drug of choices in animal as well as human medicine.

**Keywords:** *E. coli*, *ecp*, Food borne, Multidrug resistance, PCR, *uidA*

Food-borne infections are mostly linked to unsanitary activities, such as the use of contaminated devices and materials in food processing (Wilfred *et al.* 2012, Akbar and Anal 2011). Pathogens such as *Salmonella*, *Escherichia coli* and *Campylobacter* can be transmitted through poultry meat, red meat, sweets, and eggs (Hughes *et al.* 2007). The pathogen and food vehicle responsible for the majority of food-borne infections are difficult to identify. *E. coli* organisms are the faecal contamination indicators, and the presence of *E. coli* isolates in food implies the presence of possible enteric pathogens. This bacterium can spread to humans via contaminated food, water, direct contact with animals, human-to-human transfer, and can cause serious illnesses (Chinen *et al.* 2009, Karmali *et al.* 2010, Baker *et al.* 2016).

Antibiotic resistance among bacteria is one of the world's most serious health problems. Their indiscriminate use and misuse has resulted in the development of resistance, affecting the effectiveness of bacterial infection treatment and prevention (Fish and Ohlinger 2006). Owing to the extensive distribution of antibiotic resistance determinants, infections with various antimicrobial resistant bacteria have been growing at an alarming rate (O'Brien, 2002, Marshall

*et al.* 2009). The polymerase chain reaction (PCR) is a vital technology used in clinical and research laboratories for a wide range of applications, including pathogen detection for foodborne disease diagnosis. The principle for PCR distinguishes non-pathogenic organisms from pathogenic ones, allowing for rapid and highly specific detection of infectious disorders, including those caused by bacteria or viruses (Cai *et al.* 2014).

Considering the growing awareness about food safety, especially the rapid detection, characterization and antimicrobial resistance among food-borne pathogens, the present study was undertaken aiming the food pathogen *E. coli*, with the objectives to isolate, identify and confirm pathogenic *E. coli* species from faecal samples of broiler poultry birds and environmental samples from organized broiler poultry farms, retail market chicken meat shops, hotels and small chicken meat product vendors in and around Udgir city of Maharashtra. Moreover, the correlation between *in vitro* pathogenic assays and molecular assay i.e. PCR employing *ecp* and *uidA* genes was also studied for judging the efficacy of these genes as a pathogenic marker for precise and time saving determination of pathogenic potential of *E. coli* isolates. The isolates were also evaluated for antimicrobial resistance pattern.

### MATERIALS AND METHODS

*Samples collection and transportation:* Samples (450)

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comprising of poultry shed, poultry shed environment, retailer shops, and restaurant chicken meat samples were randomly collected from poultry chain during 2021. The details of sampling are given in Table 1. The poultry shed environmental samples, raw chicken meat samples from retailer shops and restaurant samples were collected in sterile sample containers (HiMedia, India). The cloacal, faecal swabs and chopping board/ knives swabs were collected by using sterile cotton swabs (HiMedia, India). All samples were labelled and immediately transported to the laboratory by maintaining cold chain for isolation and identification of *E. coli*.

Table 1. Samples collected from different sources for the screening of *E. coli*

Type of sample	Total
<i>Faecal samples / cloacal samples</i>	
Faecal samples	50
Cloacal samples	50
<i>Environmental samples</i>	
Water	50
Feed	50
Litter	50
<i>Retailer shops samples</i>	
Raw chicken meat sample	50
Swabs of chopping board/ knives	50
<i>Restaurant samples</i>	
Chicken products from restaurants	50
Chicken products from small chicken meat vendors	50
Total	450

*Isolation, identification and phenotypic characterization of E. coli:* Cultural isolation and identification of *E. coli* was carried out as per the standard protocol given by Feng *et al.* (2002). Briefly, cloacal swabs, faecal swabs, poultry shed environmental swabs, butchers knife swab etc. were taken directly into the pre-sterile MacConkey's enrichment broth in the ratio of 1:9 while solid samples *i.e.* restaurant chicken meat pieces, chicken meat samples from retailer's shop were triturated using pestle and mortar and was added to the sterile MacConkey's broth as per ratio of 1:10 and incubated at 37°C for 24-48 h. The presumptive grown cultures were streaked on MacConkey's agar plate by taking a loopful of inoculum and plates were incubated at 37°C for 24 h. Further, the MacConkey's agar plates were observed for development of characteristic pink colonies of *E. coli*. A single isolated characteristic colony was picked up from MacConkey's agar and streaked on an Eosin Methylene Blue (EMB) agar plate and incubated for 18-24 h at 37°C. EMB agar plates were observed for

the development of characteristic green metallic sheen. Pure cultures of *E. coli* isolates were taken on Brain Heart Infusion (BHI) agar slants. These slant cultures were stored at 4-8°C for biochemical and molecular characterization.

*Gram's staining:* Gram staining of the presumptive *E. coli* isolates was performed as per the method of Preston and Morel (1962) and pink coloured Gram negative rods were observed under binocular microscope (Olympus, Japan).

*Biochemical characterization:* Biochemical characterization was carried out as per the method described by Quinn *et al.* (1994). The battery of biochemical tests performed for identification of *E. coli* was IMViC (Indole, Methyl red, Voges Proskauer and Citrate) tests.

For determining the virulence potential of the presumptive isolates of *E. coli*, Congo red dye binding and haemolysis assays (5% defibrinated sheep blood agar) were performed.

*Congo red dye binding assay:* The test was carried out as per the protocol given by Berkhoff and Vinal (1986). On a Congo red agar plate, a freshly grown colony of *E. coli* from BHI agar was streaked and kept incubated for 24 h at 37°C. The reaction was kept under observation for two days and observed on 18<sup>th</sup>, 24<sup>th</sup>, 48<sup>th</sup> h. The presence of red colonies indicated positive colonies while dye did not bind to the negative colonies and remained white or grey in colour, even after incubation of 72 h.

*Haemolysis assay:* For haemolysis assay, freshly grown *E. coli* isolates were streaked and incubated at 37°C for 24-48 h on 5% Sheep blood agar as per the protocol of Beutin *et al.* (1989). The production of zone of haemolysis was recorded.

*Molecular characterization of E. coli:* The *E. coli* isolates were screened for molecular characterization by using Polymerase chain reaction (PCR) targeting species specific (*uidA*) and virulence marker (*ecp*) genes.

*Extraction of DNA:* The *E. coli* colonies were subjected for DNA extraction as per the manufacturer's protocol (HiPurA™ Multi sample DNA Purification kit, HiMedia, India).

*Polymerase chain reaction for E. coli.* The PCR reaction was set in 20 µl using specific primers presented in Table 2. PCR reaction comprised of 10 µl of 2× Master mix (Thermo Fisher Scientific, India), 25 pmol of each primer, 100 ng of total DNA and 7 µl nucleus free water. Amplification was carried in thermal cycler (Prima 96 PCR machine, Himedia, India) employing the optimized cyclic conditions as shown in Table 3.

*Agarose gel electrophoresis.* The PCR products were electrophoresed in 1% molecular biology grade agarose

Table 2. Primers used for amplification of genes

Target gene	Primer sequence (5'-3')	Product Size (bp)	References
<i>uidA</i>	F:TGGTAATTACCGACGAAAACGGC R:ACGCGTGGTTACAGTCTTGCG	162	Alqahtani <i>et al.</i> 2015
<i>ecp</i>	F:TGAAAAAAAAGGTTTTCTGGCAATAGC R:CGCTGATGATGGAGAAAGTGAA	500	Avelina <i>et al.</i> 2010

Table 3. Cycling condition for amplification of *uidA* and *ecp* genes of *E. coli*

Processing stage	Temperature and Time	<i>uidA</i>		<i>ecp</i>	
		Cycles	Temperature and Time	Cycles	Temperature and Time
Step I	Initial denaturation	94°C for 5 min	1 cycle	95°C for 5 min	1 cycle
Step II	Denaturation	94°C for 40 sec	35 cycles	95°C for 45 sec	40 cycles
	Annealing	55°C for 1 min		60°C for 1 min	
	Extension	72°C for 50 sec		72°C for 2 min	
Step III	Final extension	72°C for 7 min	1 cycle	72°C for 10 min	1 cycle
Step IV	Hold	4°C		4°C	

along with 100 bp DNA ladder. The bands were observed by using ultraviolet transilluminator and photographed in gel documentation system (Gel-Pro Analyzer, Syngene, USA).

**Antimicrobial susceptibility testing:** Antimicrobial susceptibility testing was performed by disc diffusion method as per Bauer *et al.* (1966) against 15 antibiotics, each belonging to distinct classes/groups (Supplementary Table 1). The most widely used antibiotics in poultry and human health were selected for the present research work. The zones of inhibition were measured after 18 h and after 48 h of incubation. The isolates were classified as sensitive, intermediate sensitive or resistant based on the diameter of the zone of growth inhibition (CLSI 2020).

## RESULTS AND DISCUSSION

**Occurrence of *E. coli* isolates:** In present investigation, on screening 450 samples collected from different sources of poultry chain, retailers and restaurants, altogether 34 samples were found positive for *E. coli* suggesting the overall occurrence of *E. coli* to the tune of 7.55% (Table 4).

**Congo red dye binding assay:** Congo red dye binding assay carried out to determine the pathogenicity of the isolates, revealed positive results in 30 presumptive

*E. coli* isolates. The positive isolates showed development of intense brick red colonies on Congo red agar. Other isolates, one from litter material and three from raw chicken meat samples turned out negative (Table 4).

**Haemolysis assay:** Haemolysis was shown by the same 30 presumptive isolates who showed Congo red binding, while remaining four isolates did not reveal haemolysis (Table 4).

**Polymerase chain reaction:** In present study, all 34 *E. coli* isolates were subjected for assessment of pathogenicity by employing PCR, targeting a species specific (*uidA*) and a virulence associated (*ecp*) genes, with the product size of 162 bp and 500 bp, respectively (Fig. 1). The results showed 100% positivity for *E. coli* species specific- *uidA* gene for all 34 isolates. While, 30 *E. coli* isolates showed presence of virulence associated (*ecp*) gene except by an isolate recovered from litter material sample, and three isolates from raw chicken meat samples (Table 4).

**Antibiogram study of recovered *E. coli* isolates:** The findings revealed that, the majority of the isolates exhibited multidrug resistance patterns, an isolate recovered from raw chicken meat sample demonstrated resistance against all 15 antibiotics while, two other isolates recovered from

Table 4. Overall positivity for isolation, Congo red assay, haemolysis assay and PCR of *E. coli* isolates

Source	Type of sample	No. of samples	Isolates	% Positivity	Congo red dye binding	Haemolysis	<i>uidA</i> Gene	<i>ecp</i> Gene
Cloacal samples/ faecal samples	Cloacal samples	50	09	18%	09	09	09	09
	Faecal samples	50	04	08%	04	04	04	04
	Total	100	13	13%	13	13	13	13
Environmental samples	Water	50	01	02%	01	01	01	01
	Feed	50	01	02%	01	01	01	01
	Litter	50	02	04%	01	01	02	01
	Total	150	04	02.66%	03	03	04	03
Retailer's shop's samples	Retailer's shop's samples	50	06	12%	03	03	06	03
	Swabs of chopping board/ knives	50	04	08%	04	04	04	04
	Total	100	10	10%	07	07	10	07
Restaurant samples	Chicken products from restaurants	50	02	04%	02	02	02	02
	Chicken products from small chicken meat vendors	50	05	10%	05	05	05	05
	Total	100	07	07%	07	07	07	07
Total		450	34	07.55%	30	30	34	30
% Positivity		-	-	-	88.23%	88.23%	100%	88.23%

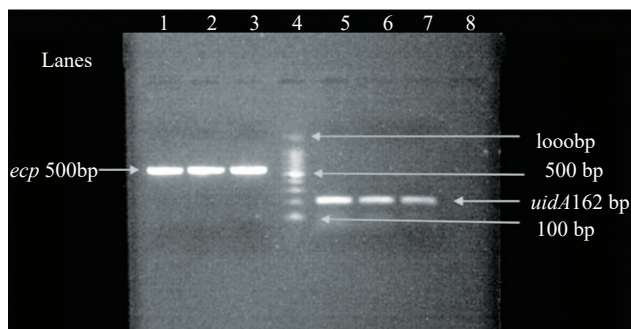


Fig. 1. PCR of *uidA* and *ecp* for *E. coli*. Lane 1: Positive control for *uidA* gene; Lane 2 and 3: Isolates of *E. coli* recovered showing *uidA* gene; Lane 4: 100 bp DNA ladder (Takara Bio, India); Lane 5: Positive control for *ecp* gene; Lane 6 and 7: Isolates of *E. coli* recovered showing *ecp* gene; Lane 8: Non template control.

litter samples showed resistance against 14 antibiotics (Supplementary Table 3).

The isolates of current study showed the highest sensitivity to Gentamicin (88.23%) followed by Cefotaxime (82.35%), Trimethoprim (58.82%) and other antibiotics with less than 50% sensitivity. The resistance was observed against Enrofloxacin (94.11%), Tetracycline and Lincomycin (85.29% each), Vancomycin and Nalidixic acid (82.35% each), Cefalexin (70.58%), Ofloxacin (67.64%), and Amoxicilline-clavulanic acid (61.76%) as presented in Supplementary Table 1.

High percentage of multidrug resistance was noted in almost all isolates, recovered from different points of poultry chain (Supplementary Table 2), poses an enormous risk to public health. The majority of these isolates were found to be resistant to antibiotic classes based on the data obtained are Fluoroquinolones (94.11%), Tetracyclines and Lincosamides (85.29% each), Quinolones and Glycopeptides (82.35% each), first generation cephalosporins (70.58%) and  $\beta$ -lactams (61.76%) (Supplementary Table 1).

In developing countries, bacterial food-borne microbe, like *Escherichia coli*, enforce a significant burden on health care systems and can noticeably reduce the economic productivity of the countries. In present study, overall occurrence of *E. coli* at different points of poultry chain was observed to the tune of 7.55%. The presence of *E. coli* (13%) in faecal and cloacal samples can be corroborated with results of Morabito *et al.* (2001) and Raji *et al.* (2006) who reported 10.8% and 9.6% positivity of *E. coli* in faecal and cloacal samples of feral pigeon and chicken. The results of present study contradicted with the results of Wani *et al.* (2004) and Doregirae *et al.* (2016) who showed very high percentage of *E. coli* (80.2% and 88.8%, respectively) in chicken samples. Positivity of *E. coli* on lower side may be attributed to the method of sampling and the healthy status of the birds at a time of sample collection. The presence of *E. coli* (2.66%) in environmental samples observed in present investigation, can be corroborated with results of Timur *et al.* (2009) who reported 14.58% and 15% positivity of *E. coli* in water and feed collected

from poultry farms. While the results of present study contradicted with the results of Chowdhury *et al.* (2011) who reported 57.14% occurrence of *E. coli* in poultry feed and that of Draz *et al.* (1996) showing 36.80% positivity in water samples. Low positivity of *E. coli* may be attributed to the good managerial practices adopted at farm level comprising hygienic farm environment and supply of clean drinking water to the birds.

Berkhoff and Vinal (1986) and Roy *et al.* (2006) reported recovery of 100% Congo red binding isolates in their investigation and recommended use of the Congo red binding assay as a phenotypic marker to distinguish between invasive and non-invasive isolates. Berkhoff and Vinal (1986) also stated that there is direct correlation between Congo red dye binding ability of *E. coli* and septicaemia causing ability of *E. coli*. Our results confirm these findings as 30 out of 34 isolates showed Congo red binding and haemolysis.

The PCR amplification of *uidA* gene and *ecp* virulence marker gene are very specific in species confirmation and virulence determination of *E. coli* (Bekal *et al.* 2003). PCR amplifications of *ecp* and *uidA* genes in present study are in agreement with Godambe *et al.* (2017) and Munhoz *et al.* (2018) who used similar markers for screening of pathogenic *E. coli*. This finding establishes the proficient efficacy of targeted genes viz. *ecp* and *uidA* for time saving determination of pathogenic potential of *E. coli* isolates. Munhoz *et al.* (2018) investigated the location of main subunit fimbrial genes known to be key adhesion factors produced by numerous *E. coli* pathotypes in 72 aEPEC strains. He found that *ecpA*, *fimA*, *hcpA*, and *lpfA* fimbrial genes were present in a high percentage (94-100%) of aEPEC strains, which is consistent with our findings. Godambe *et al.* (2017) investigated the use of two *E. coli* specific molecular markers (*uidA* and flanking area of *uspA*) in conjunction with a microbiological technique for confirmation of diagnosis. The *ecpA* gene is particularly frequent among APEC isolates that its expression is variably regulated in these strains, and that *ecp* contributes to APEC pathogenicity. Stacy *et al.* (2014) investigated that knocking down the *ecpA* and *ecpD* genes stopped *ecp* production and expression, as well as biofilm development and motility *in vitro* and pathogenicity *in vivo*.

The present study reported four *E. coli* isolates failing to exhibit pathogenic potential by using *in vitro* pathogenicity assays also turned out to be negative on virulent marker based PCR.

The most widely used antibiotics in poultry and human health were selected for the present research work. The findings of this investigation revealed that, the majority of the isolates exhibited multidrug resistance pattern; it was observed that all 34 isolates were resistant to at least four antibiotics tested against. In present study, high percentage of antibiotic resistance observed amongst all recovered *E. coli* isolates in the restaurant group and small chicken meat vendor's product group. It is an alarming situation to observe highly antibiotic resistant bacteria in ready to

eat food products. The majority of these isolates were found to be resistant to antibiotic classes based on the data obtained are fluoroquinolones, tetracyclines, lincosamides, quinolones, glycopeptides, cephalosporins followed by  $\beta$ -Lactams. Similar kind of findings was noted by Shekh *et al.* (2013) wherein sensitivity to gentamicin and resistance to a tetracycline and enrofloxacin was shown by *E. coli* isolates. Shecho *et al.* (2017) and Dutta *et al.* (2011) also observed a high resistance to tetracycline in cloacal swabs of healthy chickens in their investigation which coincides with the results obtained in present investigation. Sayah *et al.* (2004) reported cephalosporin and tetracycline resistant *E. coli* from animal sources and humans. Bryan *et al.* (2004) and Chakravarty *et al.* (2015) acknowledged the high resistance shown to tetracycline which resembles with our results.

We observed that all *E. coli* isolates were resistant to at least four antibiotics. Similarly, in a study conducted in Bangladesh, a high prevalence rate of multi-drug resistance (MDR) *E. coli* strain (75.06%) from chicken meat samples collected from retail meat shops had been reported (Rahman *et al.* 2020). Even higher MDR *E. coli* prevalence rates of 80% in isolates from chicken meat samples in Bharatpur, Metropolitan city of Nepal (Brower *et al.* 2017) and 94% in poultry chicken of Punjab state of India (Shrestha *et al.* 2017) were also reported.

To summarise, the current study found that the *in vitro* pathogenicity assays and molecular assay established a confident correlation affirming skilled efficacy of targeted genes viz. *ecp* and *uidA* for the time saving determination of pathogenic potential of *E. coli* isolates. Moreover, growing public health concerns of multidrug antimicrobial resistance demands awareness among the personnel involved in managerial practices of poultry birds. Antibiotic indiscriminate application in poultry husbandry may be the cause of emerging multi-drug resistance in food pathogens against drugs of choice in human and veterinary treatment.

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