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# Phenotypic and Molecular characterization of extended spectrum beta lactamase producing *Escherichia coli* from chicken

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**Abstract**
This study was undertaken to isolate and characterize extended spectrum beta-lactamase (ESBL) producing *Escherichia coli* from chicken of retail meat market of Anand. Out of a total of 150 chicken muscle samples, 92 (61.33%) samples yielded positive isolation of *E. coli*. Cultures identified as *E. coli* were serotyped. All the *E. coli* isolates were screened by antibiotic susceptibility test against different antibiotics of beta-lactam group, which revealed different susceptibility patterns. A total of 67 (72.82%) isolates were found resistant to used antibiotics. Out of the 92 isolates, 25 (27.17%) isolates were found positive as ESBL producers based on the Epsilometer test (E-test). All 92 *E. coli* isolates were subjected for PCR based detection of ESBL genes. 19 (20.65%) isolates were found positive for resistance gene $bla_{CTX\text{-}M\text{-}3}$ and 8 (8.69%) isolates for $bla_{SHV}$ by PCR assay, whereas no isolates were found positive for $bla_{CTX\text{-}M\text{-}9}$and $bla_{TEM}$ *genes.* As a consequence of all the above-mentioned findings, the presence of ESBL producing *E. coli* in chicken indicates consuming such meat may create significant ESBLs resistance to the studied antibiotics in consumers.

**Keywords:** *E. coli*, chicken, ESBL, E-test, PCR

## Introduction
There is growing global awareness that the high level of antibiotic use in food animal production is closely linked to the risk of antimicrobial resistance (AMR) as bacteria mutate and develop traits that become resistant to the commonly used antibiotics. Not only are there concerns over the AMR effects on animal production and productivity, but on the transmission of resistant genes and bacteria between different species. At issue is the frequent and inappropriate use of antibiotics on animals and humans which accelerates the emergence and spread of resistant pathogens. Indeed, many anftibiotics used in animal husbandry are used also in human medicine, thus increasing the risks of cross-over and the emergence of multi-resistant pathogens [8].

ESBLs are β-lactamases capable of conferring bacterial resistance to the penicillins, first-, second-, and third-generation cephalosporins, and aztreonam (but not the cephamycins or carbapenems) by hydrolysis of these antibiotics, and which are inhibited by β-lactamase inhibitors such as clavulanic acid [12].

A close resemblance of strains and ESBL genes were found between ESBL-producing *Escherichia coli* (ESBL-EC) from chicken meat and from humans. This makes chicken meat a plausible source of ESBL-EC in humans. ESBL genes usually are located on plasmids, which may also carry virulence factors [5].

In 2016, poultry meat represented about 36 percent of global meat production. World poultry meat production increased from 120.5 MMT in 2017 to 122.5 MMT in 2018 [8]. India is the fourth-largest chicken producer in the world after China, Brazil and the USA. Poultry is the most organised sector in animal agriculture in India, worth Euro 14,500 million. In India, the per capita consumption of chicken has gone up from 400 gm to 2.5 kg per annum in the last 5 years [15].

There is the unavailability of sufficient guideline on the quality of chicken used for human consumption in Anand city. This piece of work will be useful to safeguard the consumer's health. The present study is contemplated to determine the hygienic quality of chicken of Anand meat market.

## Materials and Methods

### Sample collection

A total of 150 chicken muscle samples were collected from chicken shops in and around Anand under aseptic precautions in sterile screw lid sample collector immediately transferred on the ice at 40C to the laboratory for processing and bacteriological investigation.

### Isolation and identification

A loop full of enriched culture was first streaked on MacConkey agar and incubated at 37˚C for 18-24 h. Lactose fermenting pink coloured colonies from MacConkey agar were subcultured on Eosin Methylene Blue (EMB) agar. The subcultured colonies showing greenish metallic sheen were subjected for the biochemical tests namely Indole production, Methyl red, Voges Proskauer, Simon's citrate agar, TSI test etc. were done for the confirmation of *E. coli* as proposed by [7].

### Screening of *E. coli* isolates by disk diffusion test

The bacterial isolates were subjected to *in vitro* antibiotic sensitivity test as per the method described by [3]. *In vitro,* antibiotic sensitivity test of the isolates was conducted by paper disc diffusion method using the discs supplied by HiMedia Laboratories Pvt. Ltd., Mumbai (India). Isolates were subjected to antimicrobial sensitivity tests against six antibiotics Ceftriaxone (30 µg), Cefpodoxime (10 µg), Ceftazidime (30 µg), Cefoxitin (30 µg), Ceftazidime + Clavulanate and Aztreonam (30 µg). *E. coli* isolates were grown in Brain Heart Infusion (BHI) broth (HiMedia) for 12-18 hours. The grown cultures were swabbed on Muller-Hinton agar plates (HiMedia) with sterile cotton swabs and left for 30 minutes for pre-diffusion time. Then using sterile forceps different antibiotic discs were placed on the agar surface about two cm apart. The discs were slightly pressed with the forceps to make complete contact with the medium. The plates were incubated at 37°C for 18-24 hours. The diameter of inhibition zones were measured and graded as sensitive, intermediate and resistant according to the guideline provided by the Clinical Laboratory Standard Institute. Results were interpreted as ESBL positive if the difference in the zone of inhibition found ≥5 mm.

### Phenotypic confirmation of *E. coli* isolates for extended-spectrum beta-lactamase using E-test

The E-test ESBL for the detection of ESBL was performed in accordance with the guidelines of the manufacturer. The broth culture incubated at 37˚C until it achieves or exceeds the turbidity of the 0.5 McFarland standard (usually for 2-6 hrs.). Optimally, within 15 minutes after adjusting the turbidity of the inoculum suspension, a sterile swab dipped into the adjusted suspension and the dried surface of sterilized plates of Mueller Hinton Agar were inoculated by streaking the swab over the entire agar surface for two or more times rotating the plate approximately 60˚ each time to ensure an even distribution of inoculum. As a final step, the rim of the agar was swabbed. The antibiotic strip inoculated on agar surface and plates were incubated at 37˚C for overnight. MIC of the zone was measured for ESBL-EC to interpret the result.

### Detection of ESBL encoding genes

The *E. coli* isolates, confirmed phenotypically positive for ESBL production were subjected to molecular detection of certain ESBL encoding genes, *viz., blaTEM, bla CTX-M* and *blaSHV* by Polymerase Chain Reaction using specific primers reported by [10, 16].

### DNA Extraction from *E. coli* isolates

The DNA extraction of *E. coli* isolates was carried out by using the boiling method. A loopful of culture was taken into sterilized microcentrifuge tube which contains 100 µl of DNAse and RNAse free Milli-Q water. Then samples were vortexed and heated at 95˚C for 10 minutes, cell debris was removed by centrifugation and 3 µl of the supernatant was used as a DNA template in PCR reaction mixture.

### Detection of ESBL encoding genes by Polymerase Chain Reaction

The PCR procedure was carried out for the detection of the *blaCTX-M-3, blaCTX-M-9, blaSHV, blaTEM* genes as described by [11, 19, 17]. The reaction mixture was optimized to contain 12.5 µl 2X PCR master mix, 10pmol of each forward and reverse primer, 7.5 µl nuclease-free distilled water and 3 µl of DNA template. Reaction mixture cycled 30 times for *blaCTX-M-3, blaCTX-M-9* genes and 35 times for *blaSHV, blaTEM* genes in a thermal cycler (Applied Biosystem, Sweden) with preheated lid (105 $^0$C) under following cycling conditions. For *blaCTX-M-3* and *blaCTX-M-9* genes, each cycle consisting of 15 minutes of initial denaturation at 95 ˚C, 1 minute of initial denaturation at 95˚C, 1 minute of annealing at 55 ˚C, 1 minute of extension at 72 ˚C and 10 minutes of final extension at 72 ˚C. For *blaSHV* and *blaTEM* genes, each cycle consisting of 15 minutes of initial denaturation at 95 ˚C, 30 seconds of initial denaturation at 94˚C, 30 seconds of annealing at 58 ˚C, 1 minute of extension at 72 ˚C and 10 minutes of final extension at 72 ˚C. On completion of PCR, amplified products were analyzed through 2% agarose gel.

Following agarose gel electrophoresis, PCR amplicons were visualized under UV trans-illumination in a Gel Documentation system (SynGene, Gene Genius Bio-Imaging System, UK) and data were recorded photographically.

## Results and Discussion

Out of total 150 meat samples collected from chicken, 92 (61.33%) isolates were found positive for *E. coli*. All the *E. coli* isolates were subjected for screening for ESBL production by antibiotic susceptibility test against five different antibiotics of beta-lactam group, which revealed different susceptibility patterns. A total of 67 (72.82%) isolates were found resistant to used antibiotics of which 17 (18.47%) isolates were resistant to all used antibiotics whereas 31 (33.69%) isolates were variably resistant to used antibiotics.

27 (29.34%) isolates found resistant to cefoxitin, 44 (47.82%) isolates to aztreonam, 33 (35.86%) isolates to ceftriaxone, 42 (45.65%) isolates to ceftazidime, 61 (66.30%) isolates to cefpodoxime. *E. coli* isolates were confirmed as ESBL producer by Epsilometer test (E-test). In the present study, out of the 92 isolates, 25 (27.17%) isolates were confirmed as ESBL producers based on the Epsilometer test (E-test).

The *E. coli* isolates, confirmed to be phenotypically positive for ESBL production were subjected to molecular detection of certain ESBL encoding genes, *viz., blaTEM, blaCTX-M-3, blaCTX-M-9* and *blaSHV* by Polymerase Chain Reaction (PCR). Out of the 25 ESBL producing isolates confirmed by E-test, 19 (20.65%) isolates were found positive for resistance gene *bla CTX-M-3* (780 bp) (Fig. 1) and 8 (8.69%) isolates

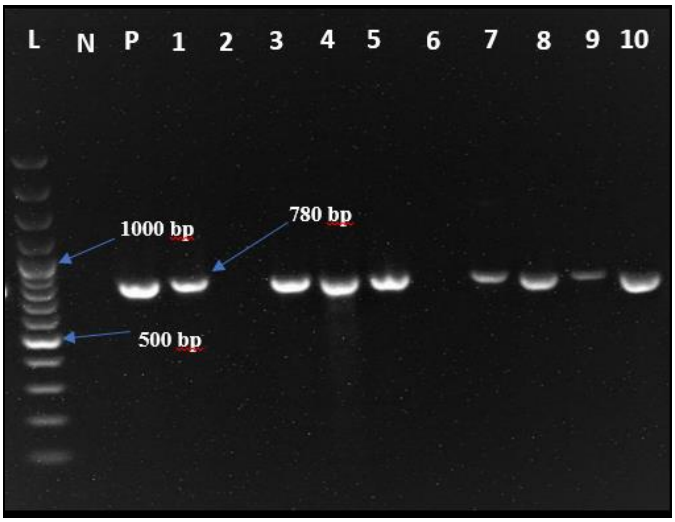
for *blaSHV* (768 bp) (Fig.2) by PCR assay, whereas no isolates were found positive for *bla CTX-M-9* (876 bp) and *blaTEM* (698 bp) genes.

In the present study findings out of 150 samples, 92 (61.33%) samples yielded *E. coli*. Nearly similar findings were reported earlier by other scientists; 63.10% by [18], 65.20% by [13]. While some earlier studies indicated the lower prevalence of *E. coli* as 1.5% by [6] and 38.7% by [12], which was indicative of good hygienic practices prevailing in the area covered under previous studies.

In another study in Assam and Meghalaya, out of 182 isolates, 81 (44.50%) isolates were found positive for ESBL production of which 38 (20.87%) were from Assam and 43 (23.62%) were from Meghalaya. The number of isolates which showed resistance to amoxicillin, ampicillin, cefixime, cefotaxime, ceftazidime, ceftriaxone and piperacillin respectively was 90-100% [2] which is nearly similar to present study findings.

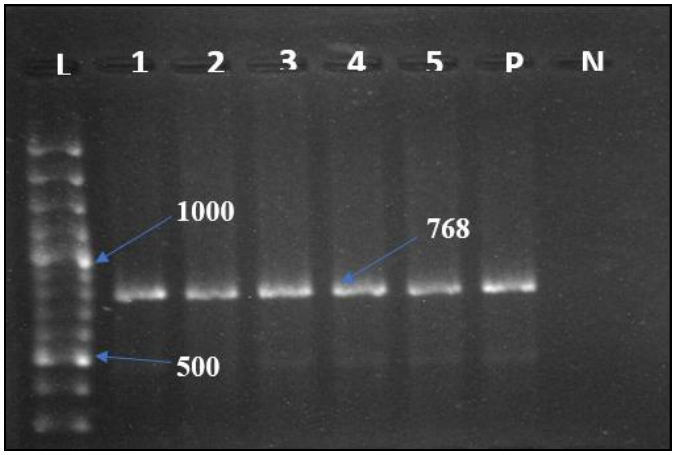
[14] found the highest percentages of drug resistance in isolates of *E. coli* were detected from raw chicken (23.3%) but the present research finding shows higher resistance compared to the study of [14]. Comparatively higher ceftazidime resistance found in the present study than [4]. This may be due to more frequent use of ceftazidime in the field under present study.

In present study out of the 92 isolates, 25 (27.17%) samples were phenotypically confirmed as ESBL producers in agreement 21.42% prevalence reported by [2] from Assam and Meghalaya. In comparison to present finding a high incidence of *blaSHV* (29%), *blaTEM* (6.34%) and *blaCTX-M* (29%) reported among *E. coli* isolates by [11]. The higher rate of prevalence of *blaCTX-M* over *blaTEM* gene was recorded in this study, which is in agreement with the studies conducted by [16] as well as [9]. In india, data on ESBL-producing *Escherichia coli* from poultry and livestock are scarce. Most common ESBL gene detected in poultry *E.coli* is *blaCTX-M* [10]. *blaCTX-M* may be increased due to the wide use of third-generation cephalosporins, especially ceftriaxone and cefotaxime or may be associated with high mobilization of the encoding genes [1]. In the present study, no isolates were found to harbour *bla*CTX-M-9 gene which is in agreement with the study of [11].




(LANE L = 100bp DNA ladder; LANE N = Negative control; LANE P = Positive control; LANE 3,4,5,7,8,9 and 10 = Positive Samples; LANE 6= Negative Sample)

**Fig 1:** Agarose gel showing PCR amplified product (780bp) for $bla_{CTX\text{-}M\text{-}3}$ gene in *E. coli* isolates




(LANE 1 = 100bp DNA ladder; LANE 2-6 = Positive Samples; LANE 6 = Positive control; LANE 7 = Negative control)

**Fig 2:** Agarose gel showing PCR amplified product (768bp) for $bla_{SHV}$ gene in *E. coli* isolates

## Conclusion

The isolation results showed the presence of *E. coli* which are of public health importance. Consumption of chicken having ESBL producing *E. coli* within, create significant ESBL mediated resistance to antibiotics studied in consumers. Though raw meat is not commonly consumed in India, it is a tradition in tribes and people are consuming half-cooked meat as fashion. ESBL producing *E. coli* are capable to spread of multidrug-resistance responsible for treatment failure.

Unhygienic handling and improper cooking of chicken meat may contribute to the spread of ESBL resistance. Appropriate surveillance methods are needed to keep watch on the levels of such resistance. Regional guidelines and rules should be framed to prevent the spread of ESBLs and indiscriminate use of antibiotics.

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