

Original Research Article

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Antibiogram and Genotyping of Extended-Spectrum β -lactamase (ESBL) Producing *Escherichia coli* from Poultry

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ABSTRACT

In the present study, total 100 cloacal/intestinal swabs from poultry were collected from the retail chicken meat shop from the city of Junagadh, Gujarat. Of this, total 80 (80%) isolates were identified morphologically and biochemically as *E. coli*. The antibiotic resistance patterns of all these isolates were studied using disc diffusion method and showed 97.5% isolates were resistant to ceftriaxone/salbactam followed by cefotaxime/clavulanate (96.25%), cefoxitin/cloxacillin (92.5%), cefixime (92.5%) and ceftizoxime (92.5%). Total 13 (16.25%) isolates were found phenotypically ESBL producing *E. coli*. Of these, 4 isolates shown difference of ≥ 5 mm zone of inhibition between cefotaxime and cefotaxime + clavulanate discs, 10 isolates shown difference of ≥ 5 mm zone of inhibition between ceftriaxone and ceftriaxone + salbactam discs and 3 isolates shown difference of ≥ 5 mm zone of inhibition between ceftazidime and ceftazidime + clavulanate discs and 28 isolates were found positive for the presence of *bla*_{TEM} gene (445 bp) using molecular based detection, whereas none of the isolates shown desired amplification to *bla*_{SHV} and *bla*_{CTX-M} genes, indicating *bla*_{TEM} gene is the most abundant ESBLs type prevails in this region.

Keywords

Phenotypically,
Chicken meat shop,
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Introduction

Food-borne diseases remain a major public health problem across the world. Moreover, the condition worsens in developing countries like India due to difficulties in maintaining optimal hygienic food handling practices.

Most of the bacterial pathogens associated with human enteric illness originate from animals and can be transmitted directly from animals to humans or indirectly through foods of animal origin, contaminated water, etc. (Newell *et al.*, 2010). Among the genus Enterobacter, *Escherichia coli* is mainly

responsible for causing diarrhea, hemolytic uremic syndrome, and hemorrhagic colitis (HC) (Lanjewar *et al.*, 2010). *E. coli* can contaminate the foods of animal origin, *viz.*, raw milk, meat, and their products and contribute to human food-borne diseases and food spoilage. Several strains of *E. coli* are recognized as important pathogens responsible for causing severe human diseases. Nowadays, curing bacterial infections in human and veterinary medicine is facing numerous problems due to increased antimicrobial resistance (AMR) in bacteria against the most of the antibacterial agents.

The exhaustive use and particularly the misuse of antibiotics have led to the development of resistant bacteria. One of the most important AMR mechanisms in Enterobacteriaceae family is attributed to production of extended-spectrum β -lactamase (ESBL) enzymes.

ESBL enzymes can hydrolyze various antibiotics classified under penicillin and cephalosporin group.

The emergence of ESBL producing *E. coli* in the food-producing animals and in foods of animal origin is a growing problem worldwide (Geser *et al.*, 2012). The three β -lactamases TEM (named after the patient Temoneira), SHV (sulfhydryl reagent variable) and CTX-M (active on cefotaxime, first isolated at Munich) are the most important representatives of ESBLs in relation to *E. coli* colonizing and infecting poultry.

Materials and Methods

Sampling

A total of 100 samples of poultry cloacal/intestinal swab were collected randomly during the year from slaughter house cum retail meat shops as per the guidelines of the International Commission on

Microbiological Specifications for Foods (ICMSF, 1978). All the samples were collected in sterile swab and transported to laboratory by maintaining proper cold chain. Isolation, Identification and biochemical characterization of *E. coli* was carried out as per the standard procedure described by Pamela, (2007). All samples were incubated for at least 6 hrs at 37°C and were cultured on media.

Isolation and identification of *E. coli*

The samples of poultry cloacal/intestinal swab were cultured onto MacConkey's agar and Eosin Methylene Blue (EMB) agar (Himedia Pvt. Ltd., India) and both the media incubated at 37°C for 24 h.

The colonies with pink color on MacConkey's agar and Dark-centered and flat colonies with metallic sheen on EMB agar were considered as *E. coli*. In addition, it is known that some *E. coli* show purple color with or without metallic sheen on EMB.

The morphological identification of organisms were carried out using colony morphology, revealed small 2–3 mm diameter, circular in shape, regular margin, flat, smooth, lactose fermenting, and translucent.

On Gram staining, the organism revealed Gram-negative rods, uniformly stained with no particular arrangement, non-spore, non-capsulated and motile in hanging drop method. The isolated organisms were further characterized and identified using various biochemical reactions. The isolated organisms were inoculated in a nutrient broth at 37°C for 2–3 hours and performed various biochemical tests *viz.*, Oxidase, catalase, motility, nitrite test, indole, methyl red, Voges-Proskauer, citrate, lactose fermenter, triple sugar iron agar test as per the guideline described by Pamela, (2007).

Phenotypic detection of ESBL producing *E.coli*

Antimicrobial susceptibility testing

The morphologically and biochemically identified *E. coli* isolates were tested for *in vitro* antibiotic sensitivity test using disc diffusion method for their antimicrobial drug susceptibility pattern and phenotypically identification of ESBL *E. coli* using disc diffusion method on Mueller-Hinton agar (MHA) (Himedia Pvt. Ltd., India) (CLSI, 2012). The commercially available antibiotic disks (Himedia Pvt. Ltd., India) were used for antimicrobial susceptibility testing. The antibiotic disc used for the study were cefoxitim/cloxacillin (CXX30/200), cefotaxime (CTX 30), cefotaxime + clavulanate (CEC 30/10), cefixime (CFM5), ceftriaxone (CTR 10), ceftriaxone + salbactam (CIS 30/15), ceftizoxime (ZX30), ceftazidime (CAZ 30) and ceftazidime + clavulanate (CAC 30/10) for phenotypic confirmation of the presence of ESBL. A lawn culture of *E.coli* was made on the MHA plate and disc was placed at an appropriate distance from each other and incubated aerobically overnight at 37°C. A difference in zone of inhibition of ≥ 5 mm of either of antibiotic disc alone and their clavulanate/salbactam containing disc indicate production of ESBL (Table 1).

Resistance criteria as per the CLSI guidelines (CLSI, 2014) for standard isolate (ATCC No 25922) of *E. coli*.

Molecular characterization of ESBL-encoding genes from *E. coli*

Isolation of Genomic DNA

The genomic DNA of all *E. coli* isolates was extracted by boiling method (Khoramrooz *et al.*, 2016). Briefly, four colonies of the overnight culture of *E. coli* were suspended in

300 μ l distilled water, after 10min boiling, centrifuged at 12,000 rpm for 5 min; then, 200 μ l of the supernatant was stored at -20°C until polymerase chain reaction (PCR) amplification. The isolated DNA samples were checked for purity and concentration of DNA were detected by 0.8% agarose gel electrophoresis and stored at -20°C till it use.

All the isolates presumptive of *E.coli* were screened for the detection of *bla*_{TEM}, *bla*_{SHV}, and *bla*_{CTX-M} genes by polymerase chain reaction (PCR) as described by Monstein *et al.*, (2007) with some modifications. The oligonucleotide primers specific for the *bla*_{SHV} (Paterson *et al.*, 2003), *bla*_{TEM} (Monstein *et al.*, 2007), and *bla*_{CTX-M} (Boyd *et al.*, 2004) genes used in the PCR assay and desired amplicon sizes are given in Table 2.

The primers used in this study were procured from the Genetix Biotech Asia Pvt. Ltd. The each PCR reaction was carried out in 25 μ l volume containing 12.5 μ l of master mix (Genetix Biotech Asia Pvt. Ltd), 10 pmol of each primer, and 3 μ l of DNA template.

The oligonucleotides primers and their expected size of PCR products are presented in Table 2. The PCR condition was performed using thermal cycler (Verity, Applied Biosystem by life technology, Singapore). The cycling condition for each ESBL gene producing primers were performed with initial denaturation of 95°C for 10 min; 30 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 s, extension at 72°C for 2 min followed by a final extension step at 72°C for 10 min. The PCR products were electrophoresed on 1.5% agarose gel after completion of PCR cycle and stained with 0.5 μ g/ml ethidium bromide and visualize under UV transilluminator and recorded using a Gel Documentation System (Bio-Print ST4, Vilberlourmet).

Results and Discussion

Isolation of *E. coli* from poultry

In the present study, total 100 cloacal/intestinal swabs from poultry were collected from the retail chicken shop from the city of Junagadh, Out of this, total 80 (80%) isolates were identified morphologically and biochemically as *E. coli*. Morphologically these isolates were Gram-negative rods, uniformly stained with no particular arrangement, non-spore forming, non-capsulated and motile in hanging drop method. Biochemically they are oxidase negative, catalase positive, reduces nitrates to nitrites, indole positive, methyl red positive, Voges–Proskauer negative, citrate not utilized, lactose fermenter, triple sugar iron agar showed both butt and slant yellow with gas production.

During the current study, the occurrence of *E. coli* from cloacal/intestinal swabs were recorded 80% using morphological and biochemical test. As compare to the present study, nearer percentage of recovery of *E. coli* were reported by Lalzampaia *et al.*, (2014), they have reported 76.12% (102/134) isolates were *E.coli* recovered from the fecal sample of poultry. Patyal *et al.*, (2012), reported 68% prevalence rate in Jaipur, Rajasthan and 70% prevalence in chicken meat in Mathura city of Uttar Pradesh (Sharma, 2010)

As compare to present study, the lower prevalence of *E. coli* were observed by different researcher, 66.32% prevalence were observed by Bhoomika *et al.*, (2016) in chicken meat samples, Younis *et al.*, (2017) identified 36.5% (73/200) of *E. coli* isolates from poultry of different origin based on morphological and biochemical characteristics. The 40% prevalence was reported by Rashid *et al.*, (2013) in Jammu. Whereas, Kilic *et al.*, (2007) recorded

recovery rate of 48% of *E. coli* out a total of 100 chicken samples examined. In Egypt, 20% (49/242) *E. coli* strains were isolated (Ammar *et al.*, 2015), thus the recovery of highest number of *E. coli* from cloacal or intestinal swab attributed to normal commensals of this organisms to GIT of poultry (Table 3).

Phenotypic detection of ESBLs production

A total 100 cloacal/intestinal swab samples were collected from the poultry. Of these, 80 bacterial isolates were identified as *E. coli* based on morphological and biochemical test. The antibiotic resistance patterns of all these isolates were carried out using *in vitro* antibiotic sensitivity test using disc diffusion method. The antibiotic sensitivity test showed that, 97.5% isolates were resistant to ceftriaxone/salbactam (CIS 30/15) followed by cefotaxime/clavulanate (CEC 30/10) (96.25%), ceftazidime/clavulanate (CAZ 30/10) (92.5%), cefixime (CFM 5) (92.5%) and ceftizoxime (CZX 30) (92.5%) described in Table 4.

Overall 13 (16.25%) isolates were found phenotypically ESBL producing *E. coli* out of total 80 presumably identified as *E. coli*. whereas, 67 isolates were non-ESBL producing *E. coli*. Out of these 13 ESBL *E. coli* isolates, 4 isolates shown difference of ≥ 5 mm zone of inhibition between cefotaxime (CTX 30) and cefotaxime + clavulanate (CEC 30/10) discs, 10 isolates shown difference of ≥ 5 mm zone of inhibition between ceftriaxone (CTR 10) and ceftriaxone + salbactam (CIS 30/15) discs and 3 isolates shown difference of ≥ 5 mm zone of inhibition between ceftazidime (CAZ 30) and ceftazidime + clavulanate (CAC 30/10) discs (Table 5). Overall 13 isolates were positive in phenotypic detection of ESBL producer isolates (Table 2). The extensive use of β -lactam antibiotics in poultry, increases the ESBL-mediated resistance in Gram-negative

bacilli and narrow down the therapeutic window of this group of antibiotics (Hu *et al.*, 2006). Unfortunately the emergence of antibiotic resistance bacteria is threatening the effectiveness of many antimicrobial agents.

In the present study, an attempt was made to understand the prevalence of ESBL producing *E. coli* from the poultry slaughtered at different meat shop as poultry chicken consumptions remains higher as compare to other meat in local market.

Little lower rate of prevalence in comparison to present finding were also reported by Kar *et al.*, (2015) identified 16 (6.35%) isolates were identified as ESBL *E. coli* out of 252 isolates of *E. coli* obtained from the poultry by using combination disc method and ESBL E-test.

Similarly, Hu *et al.*, (2006), they have reported 12.9% (4/31) of clinical Enterobacteriaceae isolates were ESBL producers and Bhoomika *et al.*, (2016) reported 10.99% (21/191) isolates as presumptive ESBL producers using phenotypic method. In contrary to present finding, Kumar *et al.*, (2014) reported 55.55% (100) *E. coli* isolates were ESBL producers and 44.45% (80) isolates were non-ESBL producers.

Roshene *et al.*, (2015) reported 100% (20/20) isolates were found to be extended spectrum beta lactamase producers using Ceftazidime (30µg), ceftazidime/clavulanic acid (30/10µg) and 11/20 (55%) of isolates were shown to be positive for ESBL using cefotaxime (30µg), cefotaxime/clavulanic acid (30/10µg), whereas in present finding higher percentage of ESBL *E. coli* identified by ceftriaxone (CTR 10) and ceftriaxone + salbactam (CIS 30/15) disc.

Overdevest *et al.*, (2011) reported 42.74% (112) isolates of *E. coli* were producing ESBL enzymes based on phenotypic screening from fresh meat samples including poultry meat.

Molecular characterization of ESBL-encoding genes of *E. coli*

The ESBL genes are located on plasmids that can be easily transferred between and within bacterial species. Some ESBL genes are mutant derivatives of established plasmid-mediated β -lactamases (e.g., *bla*_{TEM}/*SHV*), and others are mobilized from environmental bacteria (e.g., *bla*_{CTX-M}). During the present study, all the presumptive 80 *E. coli* isolates were screened for the presence of *bla*_{TEM}, *bla*_{SHV} and *bla*_{CTX-M} genes by polymerase chain reaction (PCR) as described by Monstein *et al.*, (2007) in Table 2. Of these 80 isolates, 28 isolates were found positive for the presence of *bla*_{TEM} gene (445 bp) (Plate 1), whereas none of the isolates shown desired amplification to *bla*_{SHV} and *bla*_{CTX-M} genes, indicating *bla*_{TEM} gene is the most abundant ESBLs type in prevails in this region (Table 6).

In comparison to phenotypic detection of ESBLs production (13 isolates), the molecular based characterization of ESBL-encoding gene shown higher sensitivity (28 isolates), considering molecular method is highly suitable and truthful for the identification of ESBL *E. coli*. TEM is the most common ESBL found worldwide, and therefore its occurrence in chicken isolates was not surprising. Although the TEM genes are not classified as ESBL but they have been noted to confer ESBL properties (Paterson *et al.*, 2005).

Similar to present finding Bhoomika *et al.*, (2016) reported overall prevalence of *bla*_{TEM} and *bla*_{CTX-M} genes among *E. coli* isolate was 3.66% and 2.09%, respectively, whereas none of the isolates expressed the *bla*_{SHV} gene. Similarly, Apaka *et al.*, (2010) also reported a higher prevalence of *bla*_{TEM} gene (100%) than *bla*_{CTX-M} (37.5%) and *bla*_{SHV} (4.1%) gene. Younis *et al.*, (2017)

carried out Multiplex PCR for detection of antibiotic resistance genes including *bla*_{TEM}, *bla*_{SHV}, *aadA*, *tetA*, and *sul1* revealed the recovery rate of these antibiotic resistance genes was 78%, 23%, 54%, 60%, and 87%, respectively.

Table.1 Zone diameter interpretative criteria for *E. coli*

| Name of Antibiotic | Resistant | Intermediate | Sensitive |
|--|-----------|--------------|-----------|
| Cefoxitim/Cloxacillin(CXX30/200) | 25 | 26-34 | 35 |
| Cefotaxime/Clavulanic acid (CEC30/10) | 29 | 30-37 | 38 |
| Cefixime (CFM5) | 15 | 16-18 | 19 |
| Ceftriaxone/Sulbactam (CIS30/15) | 30 | 31-37 | 38 |
| Ceftizoxime (ZX30) | 21 | 22-24 | 25 |
| Cefotaxime (CTX30) | 22 | 23-25 | 26 |
| Ceftriaxone (CTR10) | 19 | 20-22 | 23 |
| Ceftazidime (CAZ30) | 17 | 18-20 | 21 |
| Ceftazidime/Clavulanic acid (CAC30/10) | 26 | 27-34 | 35 |

Table.2 Oligonucleotide primer pairs for genotyping of ESBL *E. coli* using three different gene

| Target gene | Primer sequence | Amplicon size (bp) |
|-----------------------------|--|--------------------|
| <i>bla</i> _{SHV} | ATG CGT TAT ATT CGC CTG TG TGC TTT GTT ATT CGG GCC AA | 747 |
| <i>bla</i> _{TEM} | TCG CCG CAT ACA CTA TTC TCA GAA TGA ACG CTC ACC GGC TCC AGA TTT AT | 445 |
| <i>bla</i> _{CTX-M} | ATG TGC AGY ACC AGT AAR GTK ATG GC TGG GTR AAR TAR GTS ACC AGA AYC AGC GG | 593 |

Table.3 Occurrence of ESBL *E. coli* and *bla*_{TEM}, *bla*_{CTX-M}, and *bla*_{SHV} gene from poultry isolates

| Types of sample | Number of samples collected | Number of positive Samples of <i>E. coli</i> (%) | ESBL production (%) | | | |
|--------------------------|-----------------------------|--|---------------------|---------------------------|-----------------------------|---------------------------|
| | | | Phenotypic method | <i>bla</i> _{TEM} | <i>bla</i> _{CTX-M} | <i>bla</i> _{SHV} |
| Cloacal/Intestinal swabs | 100 | 80 (80) | 13 (16.25) | 28 (35) | 0 (0) | 0 (0) |

Table.4 Percentage of isolates found resistance to different antibiotics by disc diffusion method (N=80)

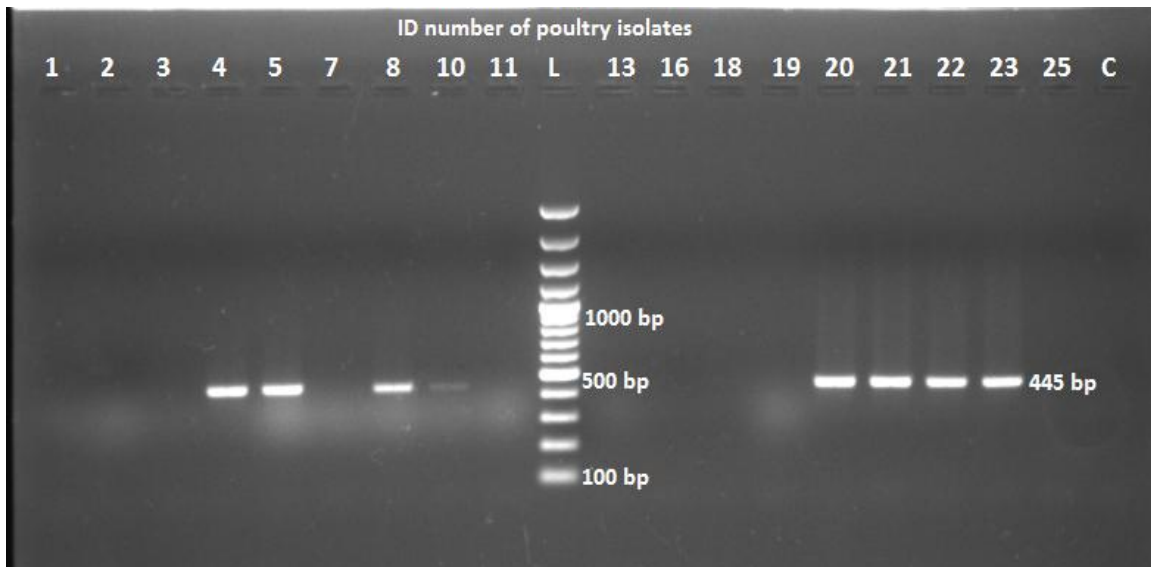
| Antibiotic | No. of isolates resistant | Percentage of Isolates resistant |
|---------------------------------------|---------------------------|----------------------------------|
| Cefoxitim/Cloxacillin (CXX30/200) | 74 | 92.5 % |
| Cefotaxime/Clavulanic acid (CEC30/10) | 77 | 96.25 % |
| Cefixime (CFM5) | 74 | 92.5 % |
| Ceftriaxone/Sulbactam (CIS30/15) | 78 | 97.5 % |
| Ceftizoxime (CZX30) | 74 | 92.5 % |

Table.5 Phenotypic detection of ESBL (N=80)

| Antibiotic used for ESBL | No. of isolates found resistant No (%) |
|------------------------------|--|
| Cefotaxime + Clavulanic Acid | 4 (5) |
| Cefotaxime | |
| Ceftriaxone + Salbactum | 10 (12.5) |
| Ceftriaxone | |
| Ceftazidime+ Clavulanic Acid | 3 (3.75) |
| Ceftazidime | |

* As per the guidelines of CLSI, 2010 for consideration criteria of ESBL in *E. coli*

Plate.1 PCR amplicons of ESBL producing *E. coli* PCR (*bla_{TEM}* gene = 445 bp) from poultry isolates.



1 to 25 = Clinical samples from poultry isolates
 C = Control ESBL negative (*E. coli* ATCC no. 25922)
 L = 100bp ladder

Table.6 Molecular characterization of *bla* genes among ESBL-producing *E. coli* isolates (n=80)

| <i>bla</i> genes | No. of isolates positive |
|-----------------------------|--------------------------|
| <i>bla</i> _{SHV} | 0 |
| <i>bla</i> _{TEM} | 28 |
| <i>bla</i> _{CTX-M} | 0 |

Indicating higher percentage of prevalence of *bla*_{TEM} gene similar to present finding. Ammar *et al.*, (2015) who found that *bla*_{TEM} and *sulI* genes had the highest prevalence among the tested antibiotic resist genes which have being amplified in all tested isolates (100%). In addition, *bla*_{TEM} was detected in most of β -lactams resistance strains. In contrast to present finding, a relatively higher prevalence of *bla*_{SHV} was recorded in 88% in Spain (Colom *et al.*, 2003), 79% in Egypt (Eid and Erfan, 2013) and 79.8% by Overdevest *et al.*, (2011) in chicken.

During the study, the PCR was used for detection of two or more ESBL producing genes in a single isolate. This method provided an efficient, rapid differentiation of ESBL producing genes among *E. coli* and provided a rapid screening of large number of isolates. Thus, this could be used as a rapid tool for epidemiological studies among ESBL isolates. ESBLs producing *E. coli* was recorded from poultry birds in Junagadh area, Gujarat. The PCR analysis confirmed 35% isolates harboring *bla*_{TEM} gene. Thus, transmission of such organisms to humans via the food chain cannot be ignore. Therefore strict antibiotic regimes are needed to prevent emergence and dissemination of such resistant genes harboring organisms among humans, animals and poultry.

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