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Determination of prevelance and molecular characterization of extended spectrum beta-lactamase (ESBL) producing *Escherichia coli* in poultry from J&K, India

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Abstract
The study was aimed at finding the prevelance of Extended Spectrum beta-lactamase (ESBLs) producing *E. coli* in poultry and to characterize them with respect to *bla*$_{TEM}$, *bla*$_{CTX-M}$ and *bla*$_{SHV}$ gene. Out of total 200 faecal samples collected, 400 presumptive ESBL producing *E. coli* were isolated. Of the 400 isolates, 150 (37.5%) were found to be resistant to cefotaxime and ceftazidime. Resistance to cefotaxime was observed in 60.0% and ceftazidime in 40.0% isolates and 46.6% isolates showed resistance to both. All the 150 isolates were confirmed as ESBL producers by Double disc synergy test. The overall prevelance from this study is 37.5% and is the first report of its kind in Jammu region (J&K). Further, all the ESBL producers were tested for the presence of *bla* gene. Of 150 ESBL isolates, 110 (73.2%) carried the *bla*$_{TEM}$, *bla*$_{CTX-M}$ and *bla*$_{SHV}$ gene/s. Of these 110 isolates, 27.2% carried *bla*$_{TEM}$ gene alone**,** 48.1% carried *bla*$_{CTX-M}$ gene, 1.81% carried *bla*$_{SHV}$ only and 22.72% isolates carried both *bla*$_{TEM}$ /*bla*$_{CTX-M}$ genes.

Keywords: *bla*$_{TEM}$, *bla*$_{CTX-M}$, *bla*$_{SHV.}$, cefotaxime, ceftazidime, ESBLs

Introduction
Escherichia coli (*E. coli*) are Gram-negative, facultative, anaerobic, rod-shaped bacteria, which are present in the intestinal tract of most animal species including man. The bacterium belongs to the family *Enterobacteriaceae* that was described in 1885 by a German paediatrician, Theodor Escherich in the faeces of a child suffering from diarrhoea [7]. In recent years, there has been considerable increase in the incidence of drug resistance in bacteria due to extensive and indiscriminate use of antimicrobial agents for therapy, prophylaxis or growth promotion. Beta Lactam antibiotics are the most common treatment for bacterial infections [9]. Production of beta-lactamases is the main mechanism of bacterial resistance to these classes of antibiotics [11]. The introduction of the third generation cephalosporins into clinical practice in the early 1980s was considered as a major breakthrough in the fight against beta-lactamase mediated bacterial resistance to antibiotics. However, resistance to these new beta–lactam antibiotics due to beta- lactamases emerged quickly [15]. Extended spectrum beta-lactamases are the enzymes that mediate resistance to extended–spectrum (third generation) cephalosporins (e.g., ceftazidime, cefotaxime, and ceftriaxone) and monobactams (e.g., aztreonam) but do not affect cephamycins (e.g., cefoxitin and cefotetan) or carbapenems (e.g., meropenem or imipenem). ESBLs belong to group 2be or group 2d (OXA-type) of Bush-Jacoby-Medeiros functional classification scheme. The major ESBL producers include TEM, CTX-M and SHV and minor being OXA, PER, VEB, SFO, BES, BEL, TLA and GES. These enzymes have been derived either from broad spectrum beta-lactamases following mutations in the beta-lactamase genes or have been derived from chromosomal beta-lactamases of certain environmental bacteria. Due to presence on plasmids and aided by transferable genetic elements, these genes have spread within and across the bacterial species. ESBL producing organisms have been detected all over the world and its presence in clinical infection results in treatment failure. ESBLs have been found in a wide range of Gram-negative rods. However, the vast majority of strains expressing these enzymes belong to the family *Enterobacteriaceae* [1].

Materials and Methods

Sample Collection

A total of 200 faecal samples were collected from symptomatic and asymptomatic poultry irrespective of age from different areas of Jammu region between the period from March 2018 to May 2019. These samples were taken aseptically in swabs from cloaca of live birds as well as intestine of slaughtered birds. These samples were transported to the laboratory on ice, where experimental work was carried out. The samples were processed immediately or stored at 4 ºC to be processed later on.

Phenotypic tests for the detection of ESBLs

Isolation of Presumptive ESBL producing *Escherichia coli*

The faecal samples were inoculated into nutrient broth and incubated at 37 ºC until the suspension matching 0.5McFarland standard (1.5×10^8 CFU/mL) was obtained and 10 µl of this suspension was then spread on ESBL ChromoSelect Agar (Sigma-Aldrich) plates using sterile spreader. Two pink colonies were selected from each plate and streaked on the nutrient agar slant separately, incubated overnight and stored at 4 ºC for further screening.

Screening of Presumptive ESBL producing *E. coli* for resistance to ceftazidime and cefotaxime by disk diffusion test

The ESBL isolates were subjected to screening for resistance to cefotaxime and ceftazidime by disk diffusion test as recommended by CLSI. A suspension of each isolates matching 0.5 McFarland standard (1.5×10^8 CFU/mL) was made in nutrient broth. Using sterile cotton swab, the bacteria were spread on Mueller Hinton agar to obtain a lawn culture. After allowing the plate to dry, the cefotaxime and ceftazidime antibiotic disks were placed on the surface and the plates were incubated at 37 ºC for 18-24 hours. Following growth, the diameter of the zone of inhibition around the disks were measured and recorded. The disk potency and inhibition zone diameters used for inferring resistance is displayed in Table 1. Isolates showing resistance to at least one of the antibiotics were considered for further processing.

Table 1: Disk potency and zone diameters for inferring resistance in the screening test

Antibiotic disk	Resistant, if zone diameter was
cefotaxime (30 µg)	≤ 18 mm
ceftazidime (30 µg)	≤ 22 mm

Confirmation of ESBL producing *E. coli* by cephalosporin/clavulanate combination disks

Isolates of *E. coli* that were resistant to cefotaxime and/or ceftazidime were subjected to the phenotypic confirmatory test for ESBL production by using Double Disks Synergy Test as recommended in 2010 by CLSI guidelines which advocates use of ceftazidime (30µg) (CAZ), ceftazidime + clavulanic acid (30/10 µg) (CAC), cefotaxime (30µg) (CTX), cefotaxime + clavulanic acid (30/10µg) (CEC) discs. An increase in the zone diameter by ≥5 mm around the disks containing cephalosporin with clavulanic over the disks containing cephalosporin alone confirmed ESBL production.

Molecular characterization of ESBL producing *E. coli* isolates

Extraction of bacterial DNA

All the ESBL producers were subjected to the molecular characterization. The DNA was isolated by snap and chill method. which includes boiling of colonies suspended in distilled water for 10 min to release DNA, cooled on ice for 10 min and centrifuged at 10,000×g in a for 1 min. About 2 µL of the supernatant was used as the template for polymerase chain reaction.

Detection of ESBL producing *E. coli* isolates

All the isolates found to be positive for ESBLs production phenotypically, were tested for the presence of $bla_{CTX\text{-}M}$, bla_{SHV} (MNI&MNII) and bla_{TEM} genes by PCR assay as per using specific primers (Table 2).

Table 2: List of primer sequences and predicted amplicon length

Primer	Sequence (5'-3')	Target gene	Amplicon size (bp)	Reference
CTX-M/F CTX-M/R	TTTGCGATGTGCAGTACCAGTAA CGATATCGTTGGTGGTGCCATA	$bla_{CTX\text{-}M}$	544	[6]
MNI MNII	CGCCGGGTTATTCTTATTTGTCGC TCTTTCCGATGCCGCCGCCAGTCA	bla_{SHV}	1016	[13]
TEMF TEMR	ATAAAATTCTTGAAGACGAAA GACAGTTACCAATGCTTAATC	bla_{TEM}	1080	[17]

Electrophoresis

About 10 microlitres of PCR product was electrophoresed in a 1% (w/v) agarose gel for 1 hr at 5 V/cm with a Standard molecular weight marker.

Results and Discussion

Isolation of Presumptive ESBL producing *Escherichia coli*

A total of 400 presumptive ESBL producing *E. coli* isolates (2 from each sample) were obtained from 200 faecal samples. They were identified as pink or purple coloured colonies as depicted in Plate- 1.

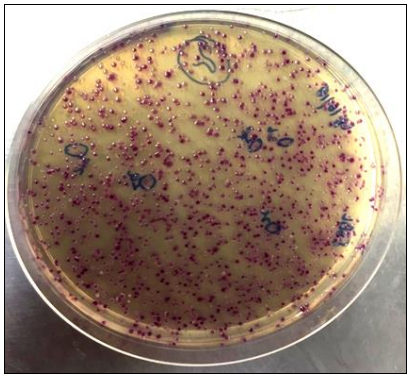

Plate 1: Isolation of ESBL producing *E. coli* on Chromogenic agar

Screening of Presumptive ESBL producing *E. coli* for resistance to Ceftazidime and Cefotaxime by disk diffusion test

A total of 150 (37.5%) isolates were found to be resistant. Resistance to cefotaxime and ceftazidime was observed in 90 isolates (60.0%) and 60 isolates (40.0%), respectively and 70 (46.6%) isolates showed resistance to both. Resistance and sensitivity to cefotaxime and ceftazidime as depicted in Plate-2.

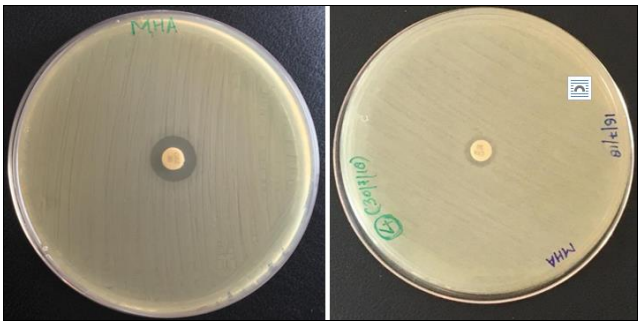

Plate 2: Screening of *E. coli* isolates for resistance to ceftazidime (CAZ) (Left) and cefotaxime (CTX) (Right) by disk diffusion method.

Present study revealed that the total prevelance of ESBL producing *E. coli* in poultry of Jammu region is 37.5% and is reported for the first time in Jammu region. This study revealed higher prevelance when compared to other studies, where it is recorded as 3.73% from Mizoram [10], 9.41% from Odisha [8] and 33.5% from Jabalpur [16]. Our findings stimulate with the result obtained by Shrivastav *et al.* (2016). As analysed from the different reports, the prevelance rate of ESBL in poultry has increased systematically from 3.73% in 2014 to 37.5% in the present time in India. When the Indian scenario is compared with the worldwide scenario, the prevelance reported was 11% from Turkey [12], 40% from Portugal [3], 80% from Dutch[5] and 85.7% from Zambia [2]. The reason for the higher rate of prevelance recorded in Jammu region in the present study could be indiscriminate use of 3 generation cephalosporins as the source of growth promoters and disease prevention in poultry and other reason being the Plasmid- mediated horizontal transfer of *bla* gene.

Screening for ESBL production by Double Disks Synergy Test

All the 150 isolates were confirmed as ESBL producers based on the Double Disks Synergy Test as these isolates showed a difference of ≥ 5 mm between the zone diameters of either of the cephalosporin disks used and their respective cephalosporin/ clavulanate disk as shown in plate-3 and plate-4.

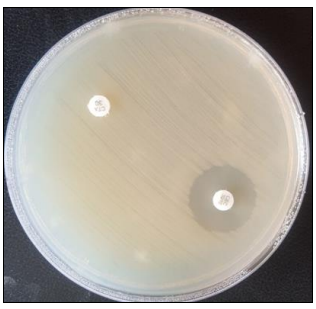

Plate 3: Phenotypic confirmation of ESBLs production in *E. coli* isolates by disk diffusion method using cefotaxime (CTX) and cefotaxime+clavulanic (CEC) acid disks.

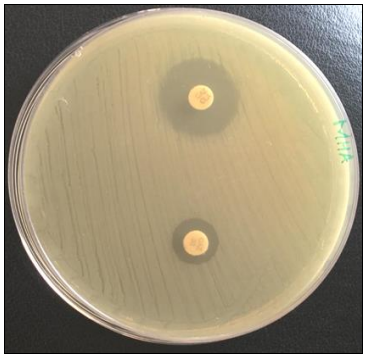

Plate 4: Phenotypic confirmation of ESBLs production in *E. coli* isolates by disk diffusion method using ceftazidime (CAZ) and ceftazidime+clavulanic (CEC) acid disks.

Detection of *bla* genes by PCR

Out of 150 ESBL isolates only 110 (73.2%) isolates carried the gene/s screened for bla_{TEM}, bla_{CTX-M} and bla_{SHV}. Out of total 110 isolates, only 30 (27.2%) isolates carried bla_{TEM} gene alone, 53 (48.1%) isolates carried bla_{CTX-M} gene alone, 2 (1.81%) isolates carried bla_{SHV} only and 25 (22.72%) isolates carried both bla_{TEM} /bla_{CTX-M} genes. Isolates carrying bla_{TEM} gene produced an amplicon of 1080 bp, those carrying bla_{SHV} showed an amplicon of 1016 bp and the presence of bla_{CTX-M} gene was detected by the amplification of 544 bp product as depicted in plate 5 and plate 6.

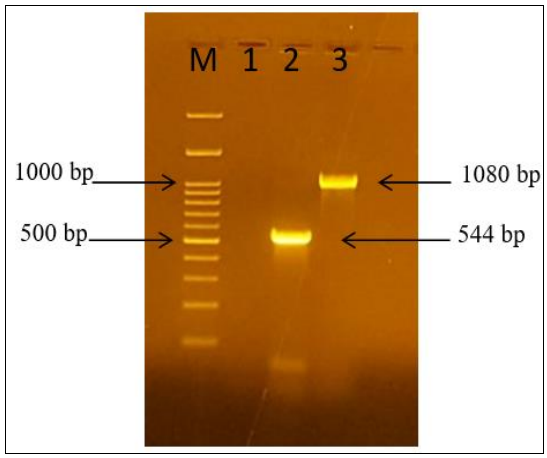

Plate 5: PCR assay for detection of bla_{CTX-M} and bla_{TEM} genes from phenotypically positive isolates. Lane M, 100bp DNA ladder. Lane 1, negative control. Lane 2, bla_{CTX-M} positive. Lane 3, bla_{TEM} positive.

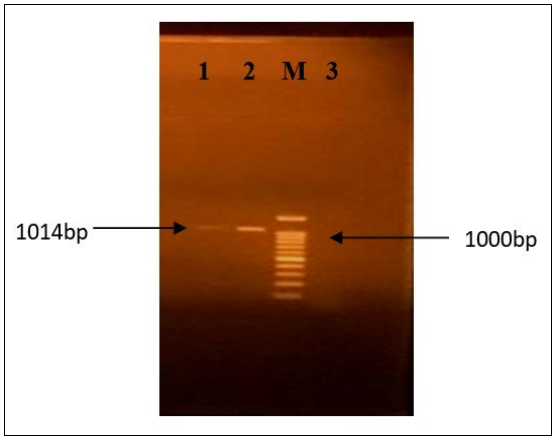

Plate 6: PCR assay for detection of bla_{SHV} gene from phenotypically positive isolates. Lane 1 and 2, bla_{SHV} positive. Lane M, 100bp DNA ladder. Lane 3, negative control

In our study, 110 isolates carried the gene/s screened for bla_{TEM}, bla_{CTX-M} and bla_{SHV}. Of which only 30 isolates carried bla_{TEM} gene alone, 53 isolates carried bla_{CTX-M} gene alone, 25 isolates carried both bla_{TEM} /bla_{CTX-M} genes where as 2 isolates carried bla_{SHV} only. In contrast to this study, from Mizoram [12] reported the $bla_{CTX-M-1}$ and/or bla_{TEM} type ESBLs in poultry, While from Odisha [8] reported bla_{SHV}, bla_{CTX-M} and bla_{TEM} genes in poultry. bla_{TEM-52}, $bla_{CTX-M-14}$ and $bla_{CTX-M-32}$ have been reported from Portugal [3] and from France [4] reported bla_{TEM}, bla_{SHV}, bla_{CTX-M}, bla_{OXA}, bla_{CMY} in poultry. The present study reveals that bla_{CTX-M} is the most abundant ESBLs type in poultry of Jammu region with *E. coli* being the major ESBL producer. The increased rate may be due to wide use of third generation cephalosporins especially ceftriaxone and cefotaxime or may be associated with high mobilization of the encoding genes.

Conclusion

This study concluded that ESBLs limit the efficacy of extended spectrum cephalosporins and are also associated with high morbidity and mortality. Moreover their indiscriminate use may be responsible for future therapeutic problems. Thus there is an urgent need to focus on the antibiotic selection and to reduce the spread of these increasingly resistant pathogens.

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Competing Interests

The authors declare that they have no competing interests.

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