

Original Research Article

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Prevalence and Characterization of Extended Spectrum Beta Lactamase Producing *Escherichia coli* from Broilers

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ABSTRACT

In the current study prevalence and characterization of extended spectrum beta-lactamase(ESBL) production of *Escherichia coli* was detected from broilers of West Bengal, India. A total of 248 cloacal swabs samples were examined for *E.coli* by standard bacteriological techniques. *E.coli* isolates were serotyped and also screened for extended spectrum beta-lactamase by double disc diffusion assay and by PCR detection of *bla*_{CTX-M}, *bla*_{SHV}, and *bla*_{TEM} genes. The antimicrobial sensitivity profile of the isolated *E.coli* was demonstrated against 13 antimicrobial agents. One hundred thirty one (52.82%) *E.coli* were identified and the isolates belonged to 12 different serogroups viz. O2, O20, O35, O83, O87, O88, O116, O117, O119, O128, O135, O141 and untyped isolates. Thirty seven (28.24%) of *E.coli* were detected as ESBL-positive by double disc diffusion assay. Among the studied genes, *bla*_{CTX-M} gene was detected in 32 (24.43%), *bla*_{SHV} gene in 3 (2.29 %) isolates and *bla*_{TEM} gene in 2 (1.53%) isolates. Highest antimicrobial susceptibility of *E.coli* isolates was observed to chloramphenicol (69.36 %) and levofloxacin (62.9 %). Highest degree of resistance was observed to cefepime (95.12%) followed by nalidixic acid (88.88%), co-trimoxazole (83.33%), ampicillin (79.03%), cefotaxime (78.46%), tetracycline (72.58%) and ciprofloxacin (62.90%). The present study revealed that broilers were reservoirs of ESBL producing *E.coli* and resistant to many classes of antimicrobials.

Keywords

Antimicrobial sensitivity, Broilers, disc, *E.coli*, ESBL, PCR

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Introduction

Escherichia coli are commensal organism in the gastrointestinal tract of warm-blooded animals and it has been extensively used to

monitor antimicrobial resistance in food animals including poultry. During the past few decades, drug resistance in *E.coli* has increased dramatically worldwide. Diverse class of antimicrobials is used to raise poultry

in most countries, mostly through the oral route, with the aim to prevent and treat diseases, enhance growth and productivity (Page and Gautier, 2012; Landini and Albarelllos, 2015). Resistant poultry pathogens to antimicrobials may cause treatment failure leading to economic losses and were potential reservoir of resistant bacterial genes that may represent a risk to human health (Nhung *et al.*, 2017).

Antimicrobial resistance to penicillins and cephalosporins is mainly due to extended-spectrum beta-lactamase (ESBL) production. ESBL confers resistance to 3rd- and 4th generation cephalosporins but not to cephamycins (cefoxitin) and carbapenems, and is inactivated by clavulanic acid. The three β -lactamases CTX-M (active on cefotaxime, first isolated at Munich), SHV (sulfhydryl reagent variable) and TEM (named after the patient Temoneira) are the most important representatives of ESBL *E. coli* colonizing and infecting poultry (Olsen *et al.*, 2014). Prevalence of ESBL producing *E. coli* has been reported both in human and food animal isolates (Bhoomika *et al.*, 2016). It is uncertain whether ESBL *E. coli* represent a direct threat to poultry production but it certainly represents a major problem to human clinical medicine. However, there was no consensus regarding the zoonotic potential of ESBL *E. coli* (Olsen *et al.*, 2014).

Past research has reported that broiler farms tend to use more antimicrobials and harbor a higher level of resistance than layer farms. In a study of Brower *et al.*, (2017), the prevalence of ESBL-positive strains was reported higher in broiler farms than layer farms. Recently, occurrence of ESBL producing *E. coli* were reported in several Indian states in healthy farmed poultry and backyard poultry (Samanta *et al.*, 2014; Samanta *et al.*, 2015, Shrivastav *et al.*, 2016) and other food producing animals such as pigs

(Lalzampaia *et al.*, 2013). Very little information is currently available that addresses the prevalence of ESBLs in animals for active surveillance which can help understand the epidemiology of ESBL burden in India (Kuralayanapalya *et al.*, 2019; Walia *et al.*, 2019). In view of the above, the present study was performed to detect the prevalence, serotypes and antimicrobial susceptibility, ESBL-production of *Escherichia coli* isolates in broilers of West Bengal, India.

Materials and Methods

In the present study, 248 cloacal swabs samples were collected from poultry farms located in different districts across 2 zones of West Bengal, India viz. New alluvial and old alluvial zones. The birds were reared in intensive deep litter system of management. Samples were collected from the age group of 1 week to 5 weeks of broilers. All the collected samples were enriched with peptone water (HiMedia, India) and incubated at 37°C for overnight. The peptone water enriched samples were taken and a loop full of culture was streaked on MacConkey's agar (HiMedia, India) and incubated at 37°C for overnight. Lactose-fermenting, pink single colonies were selected and subcultured on Eosin Methylene Blue agar (HiMedia, India) plates for selective isolation. The characteristic greenish metallic sheen showing colonies were streaked on nutrient agar (HiMedia, India) slants for further morphological and biochemical characterization as per method described by Quinn *et al.*, (1999). *E. coli* isolates were serogrouped at National *Salmonella* and *Escherichia* Centre, Central Research Institute, Kasauli, Himachal Pradesh, India.

Phenotypic detection of ESBLs

For all *E. coli*, Cefotaxime (30 μ g) and ceftazidime disks (30 μ g) with or without

clavulanate (10 µg) [HiMedia, India] were used by diffusion method for phenotypic confirmation of the presence of ESBLs in *E. coli*. A difference of >5mm between the zone diameters of either of the cephalosporin disks and their respective cephalosporin/clavulanate disk was taken for phenotypic confirmation of ESBL production (CLSI, 2017).

PCR screening for ESBL genes

PCR detection of the *bla* genes was performed on all isolates phenotypically positive by combination disk test. Primers (GCC Biotech, India) were used for the specific genes encoding ESBL for CTX-M, SHV and TEM families as described previously (Weill *et al.*, 2004a, b) with little modification. The template deoxyribonucleic acid (DNA) was prepared from freshly cultured bacterial isolates by suspending 2-3 colonies in 500 µl of molecular grade water as described previously (Samanta *et al.*, 2014) with positive and negative controls. Amplification reaction mixture containing 3µl DNA templates, 50pmol each primer set (Table 1), 200mM deoxynucleoside triphosphate, 1U GoTaq DNA polymerase (Promega, USA), 2mM MgCl₂ and 10% DMSO was prepared in a 25µl reaction mixture and subjected to PCR amplification. Amplification was conducted in a thermocycler (Mastercycler personal, Eppendorf, Germany). The cycle condition of the PCR amplification consisted an initial denaturation of 94°C for 10 mins followed by 35 cycles of denaturation at 94°C for 30 secs, annealing at 50°C (53°C for *bla*_{CTX-M}) for 30 secs and elongation at 72°C for 60 secs with a 10 mins final extension period at 72°C. The amplified products were visualized by gel documentation system (UVP, UK) after agarose gel electrophoresis containing 1.5% w/v agarose (SRL, India) and ethidium bromide (0.5µg/ml) (SRL, India).

Antimicrobial susceptibility test

All *E. coli* isolates were tested for their sensitivity and resistant patterns to 13 different antimicrobial agents by the agar diffusion method in Mueller–Hinton agar (HiMedia, India). The antibiotic discs used were as amikacin, ampicillin, gentamicin, levofloxacin, ciprofloxacin, chloramphenicol, co-trimoxazole, nalidixic acid, cefepime, cefotaxime, ceftazidime, piperacillin-tazobactam and tetracycline (HiMedia, India). The total diameter of the zone of inhibition was measured after incubation for 24h at 37⁰C and recorded. The result was interpreted as sensitive, intermediate and resistant as per Clinical and Laboratory Standard Institute (2017). However, intermediate isolates were grouped with sensitive isolates for differing them from resistant. Reference *E. coli* ATCC 25922 was used as quality control strain for antimicrobial susceptibility test.

Results and Discussion

In the present study, one hundred thirty one (131/248, 52.82%) bacterial isolates were identified as *E. coli* (Table.2) on the basis of staining property, colony characteristics, and standard biochemical reaction. Prevalence rate (52.82%) of *E. coli* in the present study was in agreement with previous studies (Joshi *et al.*, 2012). *E. coli* prevalence rate of 52.63% has been reported in their study. While higher prevalence rates of 65% was detected in another study (Samanta *et al.*, 2014). Samples that gave negative bacterial culture may be collected from farms that may have used antibiotic treatment prior to sample collection.

E. coli is considered as a member of the normal microflora of the poultry intestine, but certain strains (APEC) can spread into various internal organs and cause colibacillosis characterized by systemic fatal disease. In the

present study, *E.coli* isolates belonged to 12 different serogroups viz. O2, O20, O35, O83, O87, O88, O116, O117, O119, O128, O135, O141 and untyped isolates. Avian colibacillosis were implicated with diverse O serogroups including those isolated in the present study viz. O2, O35, O88 O87, O119 and O141 (Dho-Moulin and Fairbrother, 1999; Kunert Filho *et al.*, 2015). Other serogroups O83, O116, O117, O128, and O135 in the present study may be commensals. In a study of Shiva Shankar *et al.*, (2010), major serotypes recorded were O78, O75, O2, O6 and O111 from heart blood samples (n=120) of colisepticemic birds. In another study, 10 serogroups were reported from poultry and except O119 all serogroups O60, O80, O84, O95, O102, O110, O114,, O120 and O132 were different from present study (Joshi *et al.*, 2012). Mostly different serogroups O17, O20, O22, O102, O114 of *E.coli* were detected except O119 from poultry cloacal swabs and poultry farm environment samples in a previous study of West Bengal (Samanta *et al.*, 2015). The serogroups O2 and O149 were also encountered from human origin in India (Thakur *et al.*, 2016).

Out of 131 *E.coli*, 37 (28.24%) were detected ESBL-positive by double disc assay (Figure 1). Among the three *bla* genes, 32 (24.43%)

E.coli isolates were found to possess *bla*CTX_M gene (Figure 2), 2 (1.53%) isolates *bla*TEM gene and 3 (2.29 %) isolates *bla*SHV gene. In partial agreement with the current study, 29.4% of *E. coli* isolates from farmed poultry of West Bengal were found to possess ESBL genes with predominance of *bla*CTX_M gene (43.4%) followed by *bla*SHV (34.7%) and *bla*TEM (21.7%) genes (Samanta *et al.*, 2014). This variations may happen as there are various subgroups of ESBL genes arising from point mutations in the genes resulting in multiple subtypes. Kar *et al.*, (2015) found much lower prevalence (16/252, 6.35%) multidrug resistant ESBL-producing *E. coli* from fecal samples of poultry from Odisha. High frequency of *bla*CTX_M ESBL-producing *E. coli* were reported from poultry in several European countries viz. from Poland (Wasył *et al.*, 2012), Germany (Laube *et al.*, 2014), UK (Randall *et al.*, 2011). Widespread prevalence of ESBL producing *E. coli* in broiler farms as well as emission in its surrounds was well established with 100% similar PFGE pattern (Laube *et al.*, 2014). Despite reduced use of antimicrobials in poultry production, high prevalence of ESBL *E. coli* in retail chicken meat samples was noticed in France (Casella *et al.*, 2017). However, different sampling protocols and methodologies used make comparison of data between countries incompatible.

Table.1 Oligonucleotides used in PCR for ESBL genes

Sl. No.	Target Genes	Primer sequences (5'-3')	Annealing temp (°C)	Reference
1	<i>bla</i> CTX _M consensus	Forward- AATGTGCAGCACCAAGTAA Reverse-CGCGATATATCGTTGGTGGTGGTG	53	Weill <i>et al.</i> , 2004a
2	<i>bla</i> SHV	Forward-TTATCTCCCTGTTAGCACC Reverse-GATTTGCTGATTTGCTCGG	50	Weill <i>et al.</i> , 2004b
3	<i>bla</i> TEM	Forward- ATAAAATTCTTGAAGACGAAA Reverse- GACAGTTACCAATGCTTAATC	50	Weill <i>et al.</i> , 2004b

Table.2 Prevalence and distribution of extended spectrum betalactamase (ESBL) genes of *E.coli* isolates of broilers

Agro climatic Zones (sample no.)	No. of <i>E. coli</i> isolates (%)	β lactamase positive(no.)		
		<i>bla</i> _{CTX-M}	<i>bla</i> _{SHV}	<i>bla</i> _{TEM}
New alluvial zone (162)	89 (54.94)	21	2	2
Old alluvial zone(86)	42 (48.83)	11	1	0
Total (248)	131 (52.82)	32(24.43%)	3(2.29%)	2(1.53%)

Table.3 Antimicrobial susceptibility profile of *E.coli* isolates of broilers

Sl. No.	Class of antimicrobials	Antimicrobials (Conc. in µg)	No. of isolates		
			Sensitive (%)	Intermediate (%)	Resistant (%)
1	Aminoglycosides	Amikacin (30)	55 (41.94)	36 (27.42)	40 (30.65)
2.		Gentamicin (10)	34 (25.81)	42 (32.26)	55 (41.94)
3.	Beta lactams	Ampicillin (10)	6 (4.84)	19 (14.52)	103 (79.03)
4.		Piperacillin-Tazobactam(100/10)	14 (11.11)	87 (66.67)	29 (22.22)
5.		Cefepime (30)	0 (0)	6 (4.88)	125 (95.12)
6.		Cefotaxime (30)	2 (1.54)	24(18.46)	103 (78.46)
7.		Ceftazidime (30)	20 (15.38)	81 (61.54)	30 (23.07)
8.	Phenicols	Chloramphenicol (30)	63 (48.39)	27 (20.97)	40 (30.65)
9.	Quinolones and fluoroquinolones	Nalidixic acid (30)	7 (5.41)	11 (8.11)	116 (88.88)
10.		Ciprofloxacin (5)	10 (8.06)	38 (29.03)	82 (62.90)
11.		Levofloxacin (5)	57 (43.55)	25 (19.35)	49 (37.09)
12.	Sulphonamides	Co-trimoxazole (25)	18 (13.88)	2 (1.61)	116 (88.88)
13.	Tetracycline	Tetracycline (30)	23 (17.74)	13 (9.68)	95 (72.58)



Figure.1 Combination disc diffusion test of representative isolate of *E. coli*

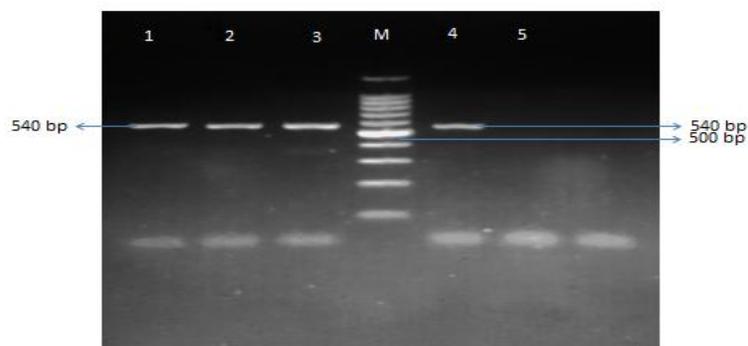


Figure 2: PCR Detection of *blaCTX-M* gene from ESBL *E.coli* isolates

Lane 1 - 3, representative samples showing *blaCTX-M* gene positive, lane M 100bp DNA ladder, Lane 4 positive control, Lane 5-negative control(PBS)

Highest antimicrobial susceptibility of *E.coli* isolates was observed to chloramphenicol (48.39%) followed by levofloxacin (43.55%) with additional (20.97%) and (19.35%) isolates, respectively showed intermediate sensitivity (Table 3). High degree of resistance was observed to beta lactams viz. cefepime (95.12%), ampicillin (79.03%), and cefotaxime (78.46%). Most isolates were also resistant to 3 or more classes of antimicrobials like nalidixic acid (88.88%), ciprofloxacin (62.90%), co-trimoxazole (83.33%) and tetracycline (72.58%).

Resistance of *E.coli* isolates to β -lactam antibiotics like cefepime (95.12%), ampicillin (79.03%), cetazidime (61.54%), cefotaxime (78.46%) in the present study was mostly because of ESBL-production. Similarly, Lazampuia *et al.* (2014) also recorded higher resistance to β -lactam antibiotics in 134 *E.coli* isolates of poultry in the state of Mizoram, India. Resistance of *E. coli* to potentiated sulphonamides (co-trimoxazole) was also reported earlier by several workers (Joshi *et al.*, 2012; Sahoo *et al.*, 2012; Ibrahim *et al.*, 2019). In the present study, increasing resistance of *E. coli* to quinolones and fluoroquinolones like nalidixic acid (88.88%), ciprofloxacin (62.90%) was

detected. In disagree to this study, *E.coli* was reported highly sensitive to ciprofloxacin, enrofloxacin, pefloxacin and norfloxacin from Bangalore, India (Sharada *et al.*, 2010). This rise in resistance may be due to extensive use of fluoroquinolones in intensively reared poultry production to check early chick mortality and enteritis. However, most of the *E.coli* isolates were sensitive to levofloxacin (62.9%) which may be due to the fact that this drug was introduced for past few years in poultry production in India. Resistance of *E.coli* to tetracycline was reported in many previous studies (Sharada *et al.*, 2010; Mane *et al.*, 2012). The observations were similar in the present study. Susceptibility to aminoglycoside, gentamicin was also declined (58.07%) in the present study. In contrast Sahoo *et al.*, (2012) recorded a higher susceptibility of *E.coli* to gentamicin (85.72%). Aminoglycosides like amikacin showed high sensitivity (78.95%) in one study by Joshi *et al.*, (2012). Highest susceptibility of *E.coli* was recorded with chloramphenicol (69.36%) may be due to the negligible use of this drug in poultry. Previously Joshi *et al.*, (2012) found 100% sensitivity of *E.coli* to chloramphenicol in their study. Most of the ESBL-producing *E.coli* were found resistant to other class of antimicrobials like

aminoglycosides and sulphonamides in the present study. Similar findings of multidrug resistant ESBL-producing *E.coli* were also recorded by other workers (Kar *et al.*, 2015). Bhave *et al.*, (2019) reported high degree of resistance to nalidixic acid (95.89%), tetracycline (95.89%), trimethoprim (89.04%), colistin (82.88%), and ciprofloxacin (54.11%), including β -lactam antimicrobials ampicillin (84.93%) and amoxicillin/clavulanic acid (81.51%) by *E. coli* isolates. β -lactam antimicrobials, especially the third-generation cephalosporins, are the most common antimicrobials used for livestock and human infections. Resistance to these drugs causes occasional treatment failure and use of most expensive last resort carbapenem antimicrobials like imipenem that are commonly used for human therapy. Plasmids mediated ESBLs may also carry multiple resistance genes for non- β -lactam and their indiscriminate use can lead to coselection and/or coresistance in bacteria populations (Ewers *et al.*, 2012).

In conclusion, the present study revealed occurrence of multidrug resistant ESBL-producing *E. coli* in broilers of West Bengal, India with a predominance of blaCTX-M gene. Hence, poultry farms or meat products might be an important reservoir for ESBL-producing bacteria leading to farm contamination and persistent threat of spreading in humans.

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