RESEARCH ARTICLE

Phenotypic and Molecular Characterization of Extended-Spectrum Beta-Lactamase Producing *Escherichia coli* from Poultry

DB Barad[1]*, JH Purohit[2], BB Javia[3], HH Savsani[4], BS Mathapati[5], SN Ghodasara[6], VA Kalariya[7], JS Patel[8]

Abstract

The present study was undertaken with the objective of phenotypic and molecular characterization of extended-spectrum beta-lactamase (ESBL) producing *E. coli* isolates from poultry. A total of 300 cloacal swabs were collected, i.e., 200 from layer birds and 100 from broiler birds from three different farms in and around Junagadh district of Gujarat state. Out of 300 samples, 126 (42.00%) samples yielded *E. coli*. These isolates belonged to layer 85 (42.50%) and broiler 41 (41.00%) birds. Out of 126 isolates, 27 (21.43%) were confirmed as ESBL producers, i.e., 19 (22.35%) and 8 (19.51%) from layer and broiler birds, respectively, by initial screening of isolates for their susceptibility to any of the third and fourth generation cephalosporins or monobactam antibiotics by disc diffusion method and further confirmation by combination disc method using ESBL identification kits. Out of 27 phenotypically confirmed ESBL *E. coli* isolates *bla*TEM was detected in 21 (77.78%) and blaAmpC was detected in 15 (55.56%) isolates, while all the 27 isolates were found negative for the presence of *bla*SHV and *bla*CTXM genes. All the 27 isolates were found positive for either *bla*TEM or *bla*AmpC gene. Nine (33.33%) out of 27 isolates were found positive for both *bla*TEM and *bla*AmpC genes. The findings warrant the need for more strict regulations for usage of antibiotics in veterinary practices in order to prevent the emergence and dissemination of multidrug resistant *E. coli* pathogens among birds, animals, and humans.

Keywords: Extended-spectrum beta-lactamase, *Escherichia coli*, *bla*TEM, *bla*CTXM, *bla*SHV, *bla*AmpC, Phenotypic characterization.

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Introduction

Escherichia coli (E. coli) are gram-negative bacteria belonging to the *Enterobacteriaceae* family which can be found in diverse environments. The bacteria can also cause disease in poultry popularly known as avian pathogenic *E. coli* (APEC). APEC cause airsacculitis, polyserositis, septicemia and other mainly extra-intestinal diseases in chickens, turkeys and other avian species. Once these bacteria affect poultry, the only treatment is antibiotics (Chansiripornchai, 2009). However, resistance to antimicrobial drugs among bacterial pathogens is an emerging problem. Bacterial resistance to beta-lactam antibiotics has risen dramatically (Medeiros, 1997). Extended-spectrum beta-lactamases (ESBLs) have been defined as plasmid-encoded enzymes found in the *Enterobacteriaceae*, frequently in *Escherichia coli* and *Klebsiella pneumoniae*, that confer resistance to a variety of beta-lactam antibiotics with the ability to hydrolyze and cause resistance to oxyimino 3rd generation cephalosporins (e.g., cefotaxime and ceftazidime) and monobactams (Pitout, 2010).

Within the ESBL family, the most common genes are temoneira (*bla*TEM) and sulfhydryl variable (*bla*SHV) with increasing reports of cefotaximase (*bla*CTX-M) (Sharma *et al.,* 2013). Various subgroups of ESBL genes arise from mutational changes due to point mutations in the genes resulting in subtypes. ESBLs of the *bla*TEM, *bla*SHV, *bla*AmpC, and *bla*CTXM types have frequently been demonstrated in the microbiota of food-producing animals (Smet *et al.,* 2010; EFSA Panel on Biological Hazards, 2011; Agersø *et al.,* 2012).

[1-8]Department of Veterinary Microbiology, College of Veterinary Science and AH, Junagadh Agricultural University, Junagadh, Gujarat, India

Corresponding Author: DB Barad, Department of Veterinary Microbiology, College of Veterinary Science and AH, Junagadh Agricultural University, Junagadh, Gujarat, India, e-mail: dr.dbbarad@rediffmail.com

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This study was aimed at the phenotypic and molecular characterization of extended-spectrum beta-lactamase (ESBL) producing *Escherichia coli* from poultry in and around Junagadh.

Materials and Methods

Total 300 cloacal swabs were collected randomly keeping aseptic precautions by gentle insertion of the sterilized swab into the cloaca of poultry and the swabs were transported in a cool box from place of collection to laboratory. Cloacal swabs were inoculated into MacConkey broth for pre-enrichment and incubated at 37°C for 24 hours. Then incubated inoculum

was streaked on MacConkey agar and incubated at 37°C for 24 hours. Lactose-fermenting pink colonies were further streaked on eosin methylene blue (EMB) agar. The plates were incubated at 37°C for 24 hours and colonies with greenish metallic sheen were observed for confirmation of *E. coli*.

For initial phenotypic confirmation of ESBL production, all the confirmed *E. coli* isolates were screened for their susceptibility to third and fourth generation cephalosporins and monobactam by disc diffusion method using ceftazidime (30 mcg), cefpodoxime (10 mcg), cefepime (30 mcg) and aztreonam (30 mcg) discs. The isolates which exhibited reduce susceptibility to any antimicrobials of initial screening were further subjected to combination disc method (Zali *et al*., 2000). For combination disc methods ESBL identification two kits (HIMEDIA®) were used, *viz*., Kit-1 [Ceftazidime (30 mcg) and Ceftazidime/Clavulanic acid (30/10 mcg)] and kit-2 [Cefotaxime (30 mcg) and Cefotaxime/Clavulanic acid (30/10 mcg)].

The molecular detection of ESBL *E. coli* genes was carried out by PCR. Bacterial genomic DNA was extracted using the Nucleo-pore gDNA Fungal/Bacterial Mini Kit (Genetix Asia Biotech Pvt. Ltd). Here 12.5 µL of 2X PCR mastermix (Fermentas), 1 µL of each primer (10 pmol/uL), 3 µL (1 µg) of genomic DNA template in a total reaction volume of 25 µL was used. *E. coli* (ATCC 25922) strain was used as negative control and ESBL isolates found in the pilot study were used as a positive control for the study. PCR cycling conditions and primary sequences for all four target genes were used as per Tables 1 to 8.

Results and discussion

Out of 300 cloacal swabs collected from apparently healthy chickens, 126 (42.00%) samples yielded *E. coli*. Out of 200 samples collected from layer birds 85 (42.50%) *E. coli* isolates were obtained, and out of 100 samples collected from broilers 41 (41.00%) samples yielded *E. coli*. These findings were nearer to Patel (2013), who reported 25% and 30% of *E. coli* from cloacal swabs from two different poultry farms. While Hossain *et al.* (2008) reported a higher prevalence of *E. coli* 63.6% and 56.4% in broiler and layer birds, respectively. Failure to isolate *E. coli* from all the cloacal samples examined may be attributed to the fact that increased multiplication of other enterobacteria in the intestinal tract of animal under certain circumstances may suppress the growth of *E. coli* or oral administration of broad-spectrum antibacterial agents cause suppression of sensitive member of the intestinal flora, so that the number of the viable bacteria in the faeces reduced almost to zero (Burdon and William, 1968).

Out of 126 *E. coli* isolates**,** a total of 27 isolates exhibited reduced susceptibility to one or more antimicrobials of initial screening (Fig. 1). These isolates were further subjected to combination disc method by using ESBL identification kits. All 27 isolates were confirmed ESBL producer by observing the difference in the zone of inhibition of ≥5 mm of cephalosporin discs alone and their cephalosporin plus clavulanic acid combination discs (Fig. 2). Out of 85 *E. coli* isolates from layer birds 19 (22.35%) isolates and out of 41 *E. coli* isolates from broiler birds 8 (19.51%) isolates were phenotypically confirmed as ESBL producers. The findings were in accordance with Basaiawmoit (2016), and Del Carmen *et al.* (2004) who reported 21.42% and 25% *E. coli* isolates as ESBL producers, respectively while comparatively higher prevalence of ESBL *E. coli* was reported by Blaak *et al.* (2015) and Li *et al.* (2016), i.e., 65% and 88%, respectively.

Out of 27 phenotypically confirmed ESBL *E. coli* isolates *bla*TEM was detected in 21 (77.78%) and *bla*AmpC was detected in 15 (55.56%) (Figs 3 and 4), while all the 27

Table 1: ESBL *bla*TEM gene specific primers

	Primer sequence (5′-3′)	*Target gene*	*Product size*	*Reference*
Forward	ATGAGTATTCAACATTTCCG	*blaTEM*	867	Bhattacharjee *et al*. (2007)
Reverse	CTGACAGTTACCAATGCTTA			

Table 2: PCR cycling conditions for amplification of ESBL *bla*TEM gene

Cycling conditions				
Initial denaturation	*Denaturation*	*Annealing*	*Extension*	*Final extension*
95°C for 5 min	95°C for 1 min	55°C for 1 min	72°C for 1 min	72°C for 10 min
	Repeated for 35 cycles			

Table 3: ESBL *bla*AmpC gene specific primers

	Primer sequence (5′-3′)	*Target gene*	*Product size*	*Reference*
Forward	CCCCGCTTATAGAGCAACAA	*bla*AmpC	631	Shahid *et al*. (2012)
Reverse	TCAATGGTCGACTTCACACC			

Table 4: PCR cycling conditions for amplification of ESBL *bla*AmpC gene

Cycling conditions				
Initial denaturation	*Denaturation*	*Annealing*	*Extension*	*Final extension*
94°C for 5 min	94°C for 30 sec.	57°C for 30 sec.	72°C for 30 sec.	72°C for 10 min
	Repeated for 30 cycles			

Table 5: ESBL *bla*CTXM gene specific primers

	Primer sequence (5'-3')	*Target gene*	*Product size*	*Reference*
Forward	CAATGTGCAGCACCAAGTAA	*bla*CTXM	540	Datta *et al*. (2013)
Reverse	CGCGATATCGTTGGTGGTC			

Table 6: PCR cycling conditions for amplification of ESBL *bla*CTXM gene

Cycling Conditions				
Initial denaturation	*Denaturation*	*Annealing*	*Extension*	*Final extension*
95°C for 5 min	95°C for 30 sec.	65°C for 30 sec.	72°C for 30 sec.	72°C for 10 min
	Repeated for 5 cycles			
	95°C for 30 sec.	62°C for 30 sec.	72°C for 30 sec.	
	Repeated for 10 cycles			
	95°C for 30 sec.	60°C for 30 sec.	72°C for 30 sec.	
	Repeated for 15 cycles			
	95°C for 30 sec.	58°C for 30 sec.	72°C for 30 sec.	
	Repeated for 15 cycles			

Table 7: ESBL *bla*SHV gene specific primers

	Primer sequence (5'-3')	*Target gene*	*Product size*	*Reference*
Forward	AGGATTGACTGCCTTTTTG	*bla*SHV	393	Bhattacharjee *et al*. (2007)
Reverse	ATTTGCTGATTTCGCTCG			

Table 8: PCR cycling conditions for amplification of ESBL *bla*SHV gene

Cycling conditions				
Initial denaturation	*Denaturation*	*Annealing*	*Extension*	*Final extension*
94°C for 5 min.	94°C for 30 sec.	60°C for 30 sec.	72°C for 30 sec.	72°C for 10 min
	Repeated for 20 cycles			
	94°C for 30 sec.	58°C for 30 sec.	72°C for 30 sec.	
	Repeated for 10 cycles			

isolates were found negative for the presence of *bla*SHV and *bla*CTXM genes. All the 27 isolates were found positive for either *bla*TEM or *bla*AmpC gene. Nine out of 27 isolates were found positive for both *bla*TEM and *bla*AmpC genes. Similarly, high prevalence of *bla*TEM gene was observed by Yuan *et al*. (2009) who reported 29 isolates harbored *bla*TEM gene out of 31 isolates. Borah *et al*. (2014) also reported *bla*TEM in 25/28 isolates, *bla*AmpC in 27/28 isolates, while lower prevalence was observed for *bla*SHV in 6/28 isolates and *bla*CTX-M in 8/28 isolates. In contrast to that Kar *et al*. (2015) reported *bla*CTXM gene in 13/18 ESBL *E. coli* isolates.

Conclusion

The findings of the present study warrant the need for more strict regulations for usage of antibiotics in veterinary practices in order to prevent the emergence

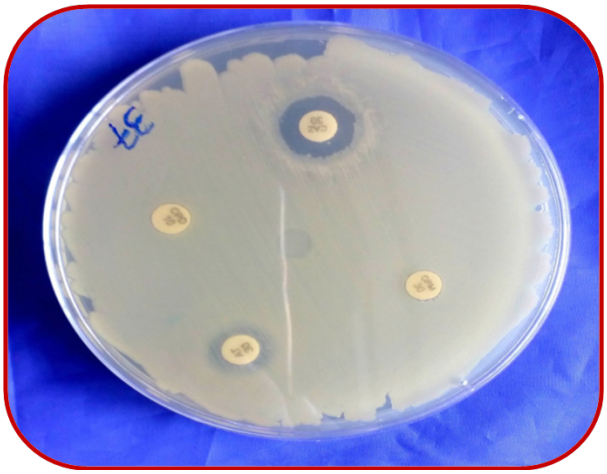

Fig.1: Initial ESBL screening with Ceftazidime (CAZ 30), Cefpodoxime (CPD 10), Cefepime (CPM 30) and Aztreonam (AT 30) showing resistance to all antibiotic discs

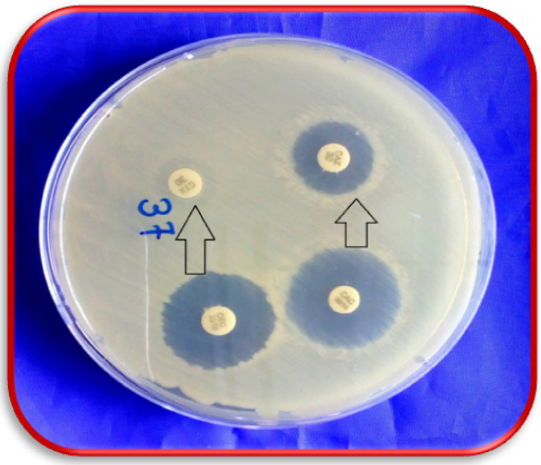

Fig. 2: Combination disc method by Ceftazidime (CAZ 30), Ceftazidime/ Clavulanic acid (CAC 30/10) and Cefotaxime (CTX 30), Cefotaxime/ Clavulanic acid (CEC 30/10) showing >5 mm zone diameter difference between plain and Clavulanic acid combination discs

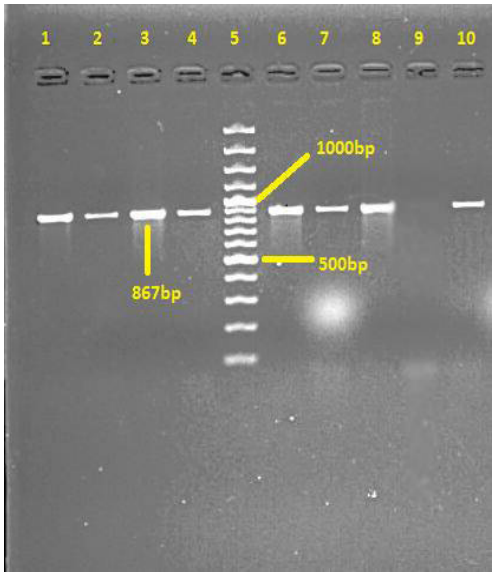

Fig. 3: Detection of *blaTEM* gene (867bp) by PCR.
Lane 1 to 4 and 6 to 8 = Samples, Lane 9= Negative Control, Lane 10= Positive Control, Lane 5= 100bp plus DNA Ladder

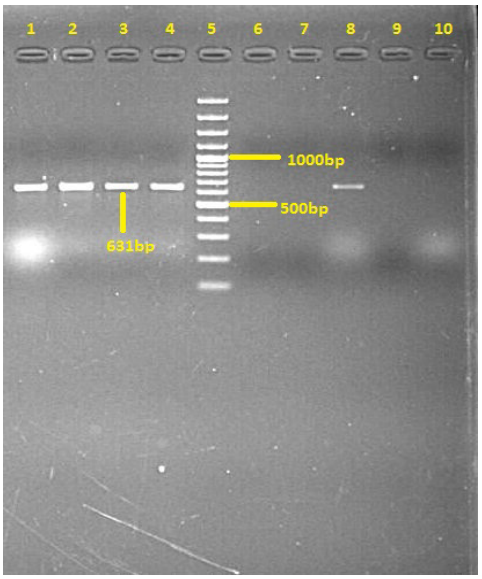

Fig. 4: Detection of the *blaAmpC* gene (631bp) by PCR.
Lane 1 to 3 and 7 to 10= Samples, Lane 4= Positive Control, Lane 6= Negative Control, Lane 5= 100bp plus DNA Ladder

and dissemination of multi-drug resistant *E. coli* pathogens among birds, animals and humans.

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