



## Characterization of Pig and Poultry Origin *Escherichia coli* for Beta-lactamase Type Antimicrobial Resistance

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### ABSTRACT

A study was conducted to know the prevalence of antimicrobial resistant *Escherichia coli* in fecal samples of pigs and broiler chicken. Fecal swabs were collected from apparently healthy animals and birds using sterile swabs. From a flock of 6000 birds, and a herd of 550 pigs, 50 samples each, were collected randomly. *E. coli* was isolated from all the broiler chicken samples, and 82% pig samples. AMR studies revealed 42% and 36.58% *E. coli*, respectively from chicken and pigs as ESBL producers. Beta lactam genes, *bla*<sub>TEM</sub> and *bla*<sub>CTX-M</sub> were detected, however, the isolates were negative for *bla*<sub>SHV</sub> and *bla*<sub>OXA</sub> genes. About 47.61% and 46.66% *E. coli* of chicken and pigs, respectively showed presence of beta lactam genes. Chicken isolates were resistant to cefixime (96%), tetracycline (88%), and ciprofloxacin (94%). While, pig isolates were resistant to cefazolin (100%), ciprofloxacin (65.85%) and gentamicin (51.21%). High degree of sensitivity was recorded for imipenem and trimethoprim. Multidrug resistant *E. coli* showing resistance to three or more group of antimicrobials were also recorded. This study is significant in the context of AMR highlighting the importance of pigs and chicken in carrying ESBL type *E. coli*.

### HIGHLIGHTS

- ESBL type of antimicrobial resistance was detected in *E. coli* isolated from pigs and broilers.
- *bla*<sub>CTX-M</sub> and *bla*<sub>TEM</sub> genes encoding ESBL resistance were detected.
- Multi-drug resistant *E. coli* strains are present in the feces of pigs and broilers.

**Keywords:** *E. coli*, beta lactam, ESBL, resistance, pigs, poultry

*Escherichia coli* (*E. coli*) is a very diverse and predominant group of bacterial community ubiquitous to animal, human and plant environments. It is often referred as a indicator for fecal contamination in food and water. Simultaneously, it is also considered an indicator of antimicrobial resistance (AMR). Therefore, monitoring of *E. coli* from diverse sources to explore the epidemiology of AMR is a valuable approach. Antibiotic resistance is a global challenge of

recent times and all the major national and international organizations are working together to mitigate this challenge (WHO, 2014). If AMR will continue to spread

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at a current pace, it is estimated that AMR will cause approximately 10 million human deaths per annum and 10% loss in livestock production by 2050 (O'Neill, 2014). Therefore awareness of all the stakeholders on AMR issue and its management at all the portals of spread is mandatory. Ample of studies were conducted at international level on detection AMR in commensal *E. coli* of human, animals and foods (Pormohammad *et al.*, 2019), however, limited studies are undertaken on its characterization in terms of prevalence of extended spectrum beta lactamases, beta lactam genes and *bla*<sub>CTX-M</sub> groups from India (Samanta *et al.*, 2014; Kar *et al.*, 2015; Samanta *et al.*, 2015). Poultry and pigs are considered as the most immediate sources of AMR transmission from animals to humans. Broiler farming is very common in most of the states of India, however pig farming is not very popular except some north eastern and southern states. In this preliminary study, attempts were made to characterize commensal *E. coli* isolated from fecal samples for detection of ESBL production and presence of beta lactam genes.

## MATERIALS AND METHODS

### Sampling and bacterial culture

During this study, a total of 100 fecal samples were collected, 50 samples each from broiler chicken and large white Yorkshire pigs. Chicken samples were collected from a single farm with 6000 broiler birds under intensive farming and pig samples were collected from two different farms (A) and (B). Farm A was having 300 pigs and farm B was holding 250 pigs. History of antibiotics used at these farms could not be obtained. All the birds and pigs sampled were apparently healthy. Without any specific criteria, random sampling was performed. In case of pigs, 25 samples were collected from each farm. All the samples were labelled and transported under ice to the laboratory and cultured for bacterial growth on the same day of collection. Rectal/ cloacae swabs were enriched in the *Enterobacteriaceae* enrichment broth (EEB, HiMedia Mumbai) for 24 hr at 37 °C. A loop full of enriched culture was inoculated on the EMB agar and after incubation at 37 °C for 24 hr, plates were observed for presumptive colonies. Isolates were further purified on EMB agar and representative 4-5 colonies were further confirmed by staining, morphology and biochemical characteristics

namely catalase, oxidase, indole and methyl red, Voges-Proskauer and citrate utilization as per the Bergey's Manual of Systematic Bacteriology.

### Antibiogram

Phenotypic resistance and sensitivity patterns of *E. coli* isolated from pigs and broiler birds were recorded against 10 different antimicrobials (HiMedia Laboratories, Mumbai) by Kirby-Bauer method and results were interpreted in accordance to the recommendations of Clinical and Laboratory Standards Institute (CLSI, 2017). All the *E. coli* isolates were further studied for extended spectrum beta lactamase (ESBL) production by disc diffusion synergy test (DDST) in accordance to the CLSI. All the isolates were first subjected for initial screening using five antimicrobial discs namely cefpodoxime (10 µg<sup>-1</sup>), ceftazidime (30 µg<sup>-1</sup>), aztreonam (30 µg<sup>-1</sup>), cefotaxime (30 µg<sup>-1</sup>), ceftriaxone (30 µg<sup>-1</sup>). Initial screening was followed by ESBL confirmation using cephalosporin discs alone and cephalosporin discs in combination with ESBL inhibitor clavulanic acid. Antimicrobials used for ESBL confirmation were cefotaxime (30 µg<sup>-1</sup>), cefotaxime/clavulanic acid (10 µg<sup>-1</sup>), and ceftazidime (30 µg<sup>-1</sup>), ceftazidime/clavulanic acid (10 µg<sup>-1</sup>). *E. coli* isolates showing a zone difference of ≥5 mm between cephalosporin alone and cephalosporin in combination with clavulanic acid were confirmed as ESBL producers. For AMR studies, *E. coli* were enriched overnight at 37°C in the Brain Heart Infusion (BHI) broth and streaking was done on the Mueller Hinton Agar (MHA) plates.

### Detection of beta-lactamases genes

All the *E. coli* strains resistant to ESBL type of AMR were further studied for the presence of four beta lactam genes namely *bla*<sub>TEM</sub>, *bla*<sub>CTX-M</sub>, *bla*<sub>SHV</sub> and *bla*<sub>OXA</sub> by multiplex PCR assay described previously (Fang *et al.* 2008). Bacterial DNA was extracted by boiling and snap chilling method. Pure bacterial colony was suspended in 100 µl nuclease free water, boiled for five minutes and immediately snap chilled in crushed ice for one minute. Tubes were centrifuged at 10000 rpm for two minutes and supernatant was used as DNA template. Multiplex PCR was performed in a total volume of 25 µl volume containing 12.5 µl 2x PCR master mix supplied with Taq polymerase, buffer, MgCl<sub>2</sub> and dNTPs (HiMedia Laboratories Mumbai); 1 µl

each forward and reverse primers, 2 µL DNA template and 2.5 µL nuclease free water. Reactions were carried out in the thermal cycler (Veriti™, Applied Biosystems). Cycling conditions involved, one cycle of initial denaturation (95°C/15 min) followed by 30 cycles of denaturation (94°C/30 sec), annealing (62°C/90 sec), and extension (72°C/60 sec). Final extension of DNA was achieved at 72°C for 10 min. Amplified PCR products were separated in 1.5% agarose gel electrophoresis stained with ethidium bromide. Primers used for detection of beta lactam genes are presented in Table 1.

## RESULTS AND DISCUSSION

This study was conducted to estimate the prevalence of ESBL producing *E. coli* in the fecal samples of broilers and pigs. Out of 100 fecal samples obtained from broiler birds and pigs, *E. coli* strains were isolated from 91 samples. Occurrence of *E. coli* was recorded in 100% broiler fecal samples and 82% of the pig fecal samples. *E. coli* is predominant prototype Gram negative organism of *Enterobacteriaceae* family and it is generally present in the gastrointestinal tract of the mammal, birds and other invertebrates. Therefore, its presence in the fecal matter is not surprising. All the *E. coli* strains isolated from broiler and pigs were studied for antimicrobial resistance with special reference to ESBL production and beta lactam genes. It was revealed that pigs and poultry origin *E. coli* are multi-drug resistant strains and ESBL producers. *E. coli* isolates from broiler birds exhibited maximum resistance to cefazolin (96%), tetracycline (88%), and ciprofloxacin (94%), while pig origin isolates showed higher resistance to cefazolin (100%), ampicillin (73.17%), ciprofloxacin (65.85%), cefixime (51.21%) and gentamicin (51.21%).

Isolates under study were highly sensitive to imipenem and trimethoprim. Chloramphenicol sensitive (56.09%) pig isolates were also recorded during the study (Table 2). ESBL producing *E. coli* were comparatively isolated in high numbers which is indicative of the fact that food animals and poultry are the reservoirs of emerging antimicrobial resistance. Over 30% of the *E. coli* from poultry and pigs were ESBL producers. Moreover similar pattern of antimicrobial resistance has also been recorded in the *E. coli* isolated from poultry and pigs by previous investigators (Miles *et al.*, 2006; Pholwat *et al.*, 2020).

All the ESBL producing strains of *E. coli* were studied by multiplex PCR for the presence of beta lactam genes. Prevalence of *bla*<sub>CTX-M</sub> and *bla*<sub>TEM</sub> was recorded, however *bla*<sub>SHV</sub> and *bla*<sub>OXA</sub> could not be detected. Prevalence of *bla*<sub>CTX-M</sub> was more in broilers (33.33%), while *bla*<sub>TEM</sub> was detected in higher percentage in pigs. Variations are recorded in the resistance and sensitivity pattern of the *E. coli* from pigs and broilers (Table 3 & 4). Multi drug resistant *E. coli* exhibiting resistance to three or more group of antimicrobials has also been recorded (16.48%) in this study. Present findings are consistent to previous reports from India. Very high prevalence of ESBL producing *bla*<sub>CTX-M</sub> and *bla*<sub>TEM</sub> positive *E. coli* in piglets with diarrhea was recorded, wherein samples were collected from eight states of India (Vinodh Kumar *et al.*, 2019). In another study from Mizoram, *E. coli* from pig feces resistant to ampicillin, tetracycline and trimethoprim were recorded. Similarly *E. coli* were ESBL type and *bla*<sub>TEM</sub> and *bla*<sub>CTX-M</sub> positivity was recorded in pigs from organized and unorganized farms (Mandakini *et al.*, 2020). Almost similar prevalence of ESBL type *E. coli* was also reported in the fecal samples of pigs slaughtered in United

**Table 1:** Oligonucleotide sequences used for detection of beta lactam genes

Sl. No.	Name of the primers	Oligonucleotide sequence (3'-5')	Genes encoded	Product size in bp	Reference
1	blaSHV-F	CTT TATCGG CCC TCA CTC AA	<i>bla</i> <sub>SHV</sub>	237	Fang <i>et al.</i> (2008)
2	blaSHV-R	AGG TGC TCA TCA TGG GAA AG			
3	blaTEM-F	CGC CGC ATA CAC TAT TCT CAG AAT GA	<i>bla</i> <sub>TEM</sub>	445	
4	blaTEM-R	ACG CTC ACC GGC TCC AGA TTT AT			
5	blaCTX-M-F	ATG TGC AGY ACC AGT AAR GTK ATG GC	<i>bla</i> <sub>CTX-M</sub>	593	
6	blaCTX-M-R	TGG GTR AAR TAR GTS ACC AGA AYC AGC GG			
7	blaOXA-F	ACA CAA TAC ATA TCA ACT TCG C	<i>bla</i> <sub>OXA</sub>	813	
8	blaOXA-R	AGT GTG TTT AGA ATG GTG ATC			

**Table 2:** Antibiogram and prevalence of *E. coli* isolated from broiler birds and pigs

Antimicrobials used	Concentration per disc ( $\mu\text{g}$ )	Resistant pattern				Sensitive pattern			
		Broiler birds (n=50)		Pigs (n=41)		Broiler birds (n=50)		Pigs (n=41)	
		No	%	No	%	No	%	No	%
Ampicillin (AMP)	10	8	16	30	73.17	14	28	4	9.75
Cefazolin (CZ)	30	48	96	41	100	0.00	0.00	0	0.00
Cefixime (CFM)	5	12	24	21	51.21	26	52	4	9.75
Gentamicin (GNE)	10	14	28	21	51.21	14	28	11	26.82
Kanamycin (K)	30	12	24	8	19.51	8	16	4	9.75
Tetracycline (TE)	30	44	88	18	43.90	1	2	7	17.07
Ciprofloxacin (CIP)	5	47	94	27	65.85	1	2	4	9.75
Imipenem (IPM)	10	0.00	0.00	0.00	0.00	49	98	38	92.68
Chloramphenicol (C)	30	17	34	11	26.82	7	14	23	56.09
Trimethoprim (TR)	5	0.00	0.00	0.00	0.00	43	86	41	100

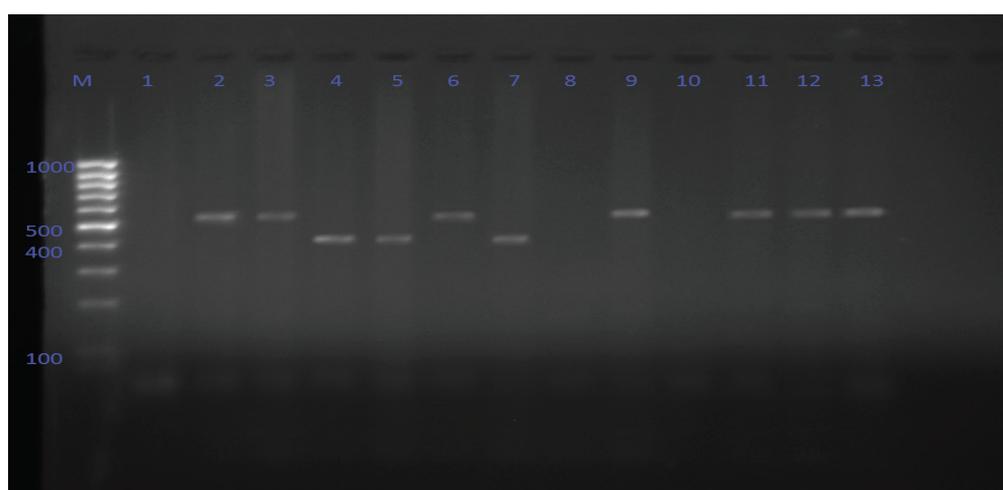
**Table 3:** Prevalence of ESBL producing *E. coli* in broiler birds and pigs

Source	No of samples	No of isolates & prevalence	ESBL strains	Beta lactam genes	<i>bla</i> <sub>CTX-M</sub>	<i>bla</i> <sub>SHV</sub>	<i>bla</i> <sub>TEM</sub>	<i>bla</i> <sub>OXA</sub>
Broiler birds	50	50 (100%)	21 (42%)	10 (47.61%)	7 (33.33%)	0.00	3 (14.28%)	0.00
Pigs	50	41 (82%)	15 (36.58%)	7 (46.66%)	2 (13.33%)	0.00	5 (33.33%)	0.00
Total	100	91 (91%)	36 (39.56%)	17 (47.22%)	9 (25%)	0.00	8 (22.22%)	0.00

**Table 4:** Most common resistant pattern of ESBL positive *E. coli*

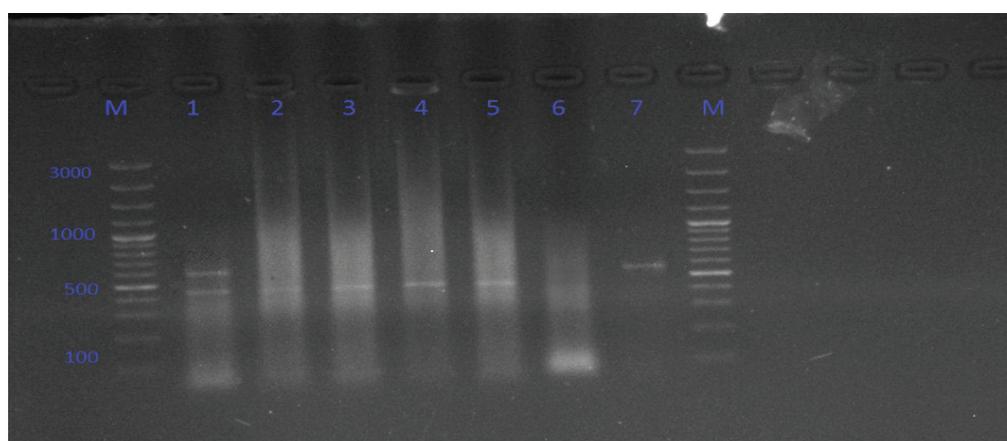
Source	<i>ESBL positive strains</i>	
Broiler birds	CZ CFM K TE CIP	
	CZ K TE CIP C	
	CZ TE	
	CZ TE CIP C	
	CZ GEN TE CIP	
	CZ CFM GEN CIP C	
	Pigs	AMP CZ CFM CIP C
		CZ CFM GEN CIP
		AMP CZ CFM GEN CIP C
		AMP CZ CFM
	AMP CZ CFM GEN TE CIP	
<hr/> <i>bla</i> <sub>CTX-M</sub> positive isolates <hr/>		
Broiler birds	CZ TE CIP C	
	AMP CZ TE CIP C	
	CZ CFM TE CIP C	
	CZ K TE CIP	
	CZ CFM GEN K TE CIP	
	CZ TE CIP C	
	CZ GEN K TE CIP	

<b>Pigs</b>	AMP CZ CIP
	CZ GEN TE
<hr/>	
<b><i>bla<sub>TEM</sub></i> positive isolates</b>	
<b>Broiler birds</b>	CZ CFM K TE CIP
	CZ TE
<b>Pigs</b>	CZ GEN K TE CIP
	AMP CZ CIP
	CZ GEN TE
	AMP CZ
	AMP CZ CFM GEN TE CIP
	AMP CZ GEN TE



Lanes from left to right – Lane M- 100 bp DNA ladder; Lanes 2,3,6,9,11,12,13 : positive for *bla<sub>CTX-M</sub>* (593 bp); Lanes 4,5,7: positive for *bla<sub>TEM</sub>* (445 bp).

**Fig. 1:** Multiplex PCR for detection of beta lactam genes in broiler origin *E. coli*.



Lanes from left to right – Lane M- 100 bp DNA ladder; Lanes 1,7: positive for *bla<sub>CTX-M</sub>* (593 bp); Lanes 2,3,4,5: positive for *bla<sub>TEM</sub>* (445 bp).

**Fig. 2:** Multiplex PCR for detection of beta lactam genes in pig origin *E. coli*



Kingdom, and *CTX-M* (22%) and *SHV* (2.2%) genes were detected (Randall *et al.*, 2014). ESBL and *CTX-M* positive *E. coli* were also isolated from fecal samples of piglets and farm workers at the organized farms from India (Tamta *et al.*, 2020). ESBL producing *E. coli* in poultry were also detected by other investigators across the globe and from India. A study from Punjab revealed high prevalence of *E. coli* resistant to ciprofloxacin, tetracycline and ampicillin as well as ESBL producing strains isolated from broilers as compared to layers (Brower *et al.*, 2017). *E. coli* isolated from healthy chicken gut from Nepal revealed predominance of CTXM-15 ESBL enzyme (Hosuru Subramanya *et al.*, 2020). As recorded in this study, poultry origin *E. coli* are mainly harbouring *bla*<sub>CTX-M</sub> and *bla*<sub>TEM</sub> genes as recorded by other workers (Girlich *et al.*, 2007; Gundran *et al.*, 2019).

Increasing resistance to beta lactam group of antimicrobials globally in human and animals including poultry is due to their widespread use which has increased selective pressure and diversified antimicrobial resistance mechanisms. ESBL type of bacterial resistance is due to the beta-lactamases hydrolysing the antibacterial activity of penicillins, cephalosporins, and aztreonam, but they are inhibited by clavulanic acid. After the introduction of third generation cephalosporins, resistance in the bacterial species has been recorded to these extended spectrum antimicrobials termed as ESBL resistance. *TEM*, *SHV*, *CTX-M* and *OXA* are the group of enzymes encoding ESBL type of resistance. *CTX-M* type of resistance is considered as most frequent and widespread globally in the bacterial population (Paterson and Bonomo, 2005).

## CONCLUSION

Present results reveal the prevalence of *E. coli* harbouring *bla*<sub>CTX-M</sub> and *bla*<sub>TEM</sub> ESBL genotypes in broilers and pigs. The high prevalence of multidrug resistant *E. coli* with co-existence of ESBL genotypes is alarming in the context of AMR and One health. More intensive and integrated AMR surveillance on livestock and poultry is necessary to correlate emerging type of antimicrobial resistance in the food chain.

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