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Detection of Extended-Spectrum Beta-Lactamase-Producing Enterobacteriaceae in *Desi* Chickens in Andhra Pradesh

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ABSTRACT

A total of 150 cloacal swabs were collected from *desi* chickens, 217 *Enterobacteriaceae* isolates were identified. The phenotypic antimicrobial resistance among *Enterobacteriaceae* was studied for 14 selected antibiotics by disc diffusion method. The selection of antibiotics was based on usage of antibiotics in commercial poultry farms and also based on priority of critically important antibiotics in humans. All *Enterobacteriaceae* isolates were subjected to multiplex PCR - I and II for detection of bla_{TEM}, bla_{SHV} and bla_{OXA} genes and bla_{CTX-M}, group 1 and 2 genes. Predominant β- Lactamase genes in gut microbiota of *desi* chicken include bla_{TEM} (90.55%) followed by bla_{CTX-M} group I (25.86%) and bla_{SHV} (9.44%) genes. All the samples were found to be negative for bla_{OXA} and bla_{CTX-M} group 2 genes.

Keywords: Desi chickens, *Enterobacteriaceae*, bla_{CTX-M}, bla_{SHV}, and bla_{TEM} genes

Extended-spectrum beta-lactamase (ESBL) producers are Gram-negative bacteria that produce enzymes that bestow resistance to most beta-lactam antibiotics like penicillin's, cephalosporins and the monobactam. These ESBL producers have been noticed mainly in the *Enterobacteriaceae* family of bacteria which may harbour several antibiotic resistance determinants making treatment of infections caused by these pathogens more difficult. Extended-spectrum beta-lactamase producers have a complex epidemiology; the most prominent bacteria involved include *E. coli* and *K. pneumoniae* whose reservoirs comprise the environment (soil and water), wild animals, farm animals, food and pets (Sharif *et al*., 2017a). Extended-spectrum beta-lactamase-producing bacteria are frequently resistant to many antimicrobial agents usually recommended for the treatment of infections such as gentamicin, fluoroquinolones and trimethoprim-sulfamethoxazole. This leads to serious challenges in the treatment of ESBL-*Enterobacteriaceae* infections because the bacterial plasmids may harbour several antibiotic resistance determinants. Heavy usage of antibiotics in commercial poultry farms has been reported to be a risk factor in the acquisition of ESBL-producing organisms (Sailu *et al*., 2017). These organisms may enter into the environment and human food chain. Hence, the present study was planned with the objective to isolate the gut microbiota and to study their antimicrobial properties.

MATERIALS AND METHODS

Sample collection

A total of 150 cloacal swabs were collected from different villages in and around Tirupati, A.P. which include Chittoor, Venkatagiri, Tirupati, Nagalapuram, Pallam, Vampalli, B. Kandriga and Kalahasti.

Isolation and identification of bacteria

The samples were subjected to bacterial isolation,

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biochemical characterization with reference to *Enterobacteriaceae* as per the standard protocols.

Antibiotic Sensitivity Test (ABST)

A total of 14 antibiotic discs were selected in the present study. The discs used include ampicillin, bacitracin, cefotaxime, chloramphenicol, ciprofloxacin, colistin, doxycycline HCL, gentamicin, enrofloxacin, vancomycin, furazolidone, nitrofurazone, virginomycin and tylosine. ABST was carried out using standard protocols. The selection of antibiotics was based on usage of antibiotics in commercial poultry farms and also based on priority of critically important antibiotics in humans. Inhibition zone diameters were interpreted according to CLSI (2014) guidelines (M100-S24).

DNA Extraction

DNA extraction was carried out by boiling and snap chilling method as described by Rao (2009) with minor modifications.

Polymerase Chain Reaction (PCR)

Multiplex PCR I and II were standardised and cycling conditions include initial denaturation at 94 ˚C for 10 minutes. 30 cycles of denaturation at 94 ºC for 40 seconds, annealing at 60 ºC for 40 seconds, elongation at 72 ºC for 1 minute and final elongation at 72 ºC for 7 minutes and hold at 4ºC. Oligonucleotide primers used and their respective amplicon sizes were given in Table 1.

RESULTS AND DISCUSSION

Phenotypic antibiotic resistance in *Enterobacteriaceae*

In this study, a total of 217 isolates with reference to the family *Enterobacteriaceae* were obtained from the faeces of *desi* chicken. 130 (59.9%) were characterized as *E.coli*, 42 (19.35%) were characterized as *Salmonella* spp. and 45 (20.73%) isolates were characterized as *Klebsiella* spp. Hundred percent resistance was recorded against bacitracin, colistin, furazolidone, nitrofurazone, vancomycin, virginomycin and tylosine. 70.96, 58.52, 30.8, 26.72, 22.5 17.5 and 16.1% resistance was observed against Doxycycline Hcl, ampicillin, ciprofloxacin, cefotaxime, chloramphenicol, enrofloxacin and gentamicin respectively.

Even though, antibiotics are not used in *desi* chicken, they found to harbour resistant gut microflora. Antibiotic resistance was also reported in *desi* chicken by other workers. Kakkar *et al*. (2017) tested samples from backyard poultry in New Delhi. High level of resistance was reported to chloramphenicol, ciprofloxacin, gentamicin, levofloxacin, norfloxacin and oxytetracycline. Ibrahim, (2017) tested poultry cloacal swabs from Sudan and reported 60% resistance to enrofloxacin, 40, 20, 10 and

Table 1: Primers used for multiplex PCR I and II for the detection of beta lactamasegenes

Target gene	Primer sequence (5'-3')	Amplicon size	Reference
	Multiplex PCR I		
bla_{TEM} gene	F: CATTTCCGTGTCGCCCTTATTC	800bp	
	R: CGTTCATCCATAGTTGCCTGAC		Sharif *et al*. (2017b)
bla_{SHV} gene	F: AGCCGCTTGAGCAAATTAAAC	713bp	
	R: ATCCCGCAGATAAATCACCAC		
bla_{OXA} gene	F: GGCACCAGATTCAACTTTCAAG	564bp	
	R: GACCCCAAGTTTCCTGTAAGTG		
	Multiplex PCR II		
bla_{CTX-M} group1 gene	F: TTAGGAAATGTGCCGCTGTA	688 bp	
	R: CGATATCGTTGGTGGTACCAT		Sharif *et al*. (2017b)
bla_{CTX-M} group 2 gene	F: CGTTAACGGCACGATGAC	404 bp	
	R: CGATATCGTTGGTGGTACCAT		

5% resistance to tetracycline, ciprofloxacin, gentamicin and colistin respectively. The samples which were found to be resistant to ampicillin and cefotaxime in ABST were selected and further screened for the presence of beta lactamase genes.

Multiplex PCR I and II for the detection of beta lactamase genes

A total of 127 isolates showing phenotypic resistance to ampicillin were selected. The DNA extraction was carried out and the isolates were tested for the presence of bla_{TEM}, bla_{SHV} and bla_{OXA} genes. Out of 127 (85 *E.coli*, 18 *Klebsiella* and 16 *Salmonella*), 103 (81.10%) isolates were found positive for the presence of bla_{TEM} gene and 2 (1.57%) samples were found to be positive for the presence of bla_{SHV} gene alone and 10 (7.87%) samples were found to possess both bla_{TEM} and bla_{SHV} genes (Fig. 2). None of the samples harboured bla_{OXA} gene. Out of 115 bla_{TEM} PCR positive samples, 85 (73.91%) belonged to *E.coli*, 14 (12.17%) were *Klebsiella* spp. and 16 (13.91%) were *Salmonella* spp. Out of 12 bla_{SHV} PCR positive samples, 8 (66.66%) belonged to *E.coli* and 4 samples (33.33%) belonged to *Klebsiella* spp (Fig. 1).

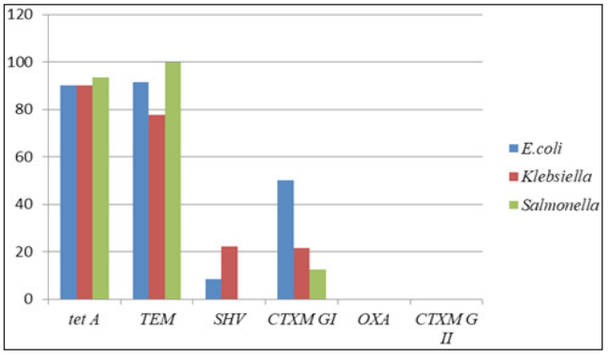

Fig. 1 Occurrence of *tet* A, bla_{TEM}, bla_{SHV}, bla_{OXA} and bla_{CTXM} Group I and II genes in enteric bacteria of *desi* chicken

A total of 58 isolates showing phenotypic resistance to cefotaxime were selected. The DNA extraction was carried out and the isolates were tested for the presence of bla_{CTXM} Group 1 and Group 2 genes. Out of 58 (20 *E.coli*, 14 *Klebsiella* and 16 *Salmonella*), 15 (25.86%) samples were found to be positive for the presence of bla_{CTXM} group I gene (Fig 3). None of the samples harboured Group 2 gene. Out of 15bla_{CTXM} Group II PCR positive samples, 10 (66.66%) belonged to *E.coli*, 3 (20%) were *Klebsiella* spp. and 2 isolates (13.33%) were *Salmonella* spp (Table 2).

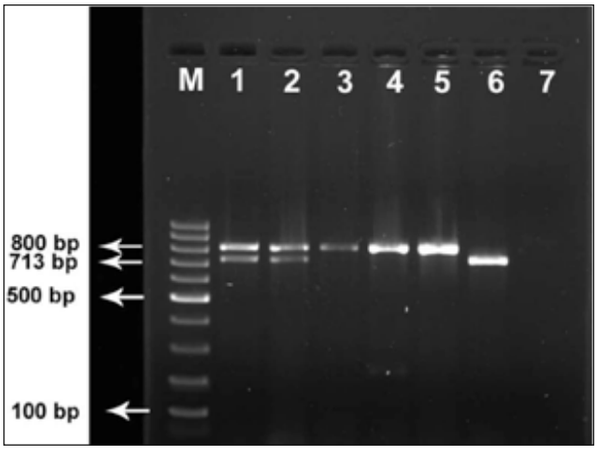

Fig. 2: Detection of Multiplex PCR I (bla_{TEM}, bla_{SHV} and bla_{OXA}) genes in *Enterobacteriaceae* of *desi* chicken

Lane M: Molecular weight marker (100bp); Lane 1 : Known DNA standard carrying bla_{TEM} and bla_{SHV} genes; Lane 2 to 6 : Desi chicken microbiota carrying bla_{TEM} and bla_{SHV} gene; Lane 7: Negative control.

Presence of ESBL genes were reported from *desi* chicken in other studies. Hasan *et al.* (2012) isolated 66 *E.coli* from *desi* chicken in Bangladesh. Out of 66, 36 *E.coli* harboured ESBL genes. 34 of them belonged to the $bla_{CTX\text{-}M\text{-}1}$ group and 2 of them belonged to $bla_{CTXM\text{-}9}$ group. Combinations of

Table 2: Detection of β- lactamase genes in *Enterobacteriaceae* of *desi* chicken

Desi chicken gut microbiota	Multiplex PCR I				Multiplex PCR II		
	No.of samples tested	bla_{TEM} +ve (%)	bla_{SHV} +ve (%)	bla_{OXA} +ve (%)	No. of samples tested	$bla_{CTX\text{-}M}$ G I +ve (%)	$bla_{CTX\text{-}M}$ +ve G II (%)
E.coli	93	85 (91.39)	8 (8.60)	0	20	10 (50)	0
Klebsiella spp.	18	14 (77.77)	4 (22.22)	0	14	3 (21.42)	0
Salmonella spp.	16	16 (100)	0	0	16	2 (12.5)	0

bla$_{CTX-M-1}$ and *bla*$_{TEM-1}$ were detected in 50% of the isolates, whereas none of the isolates harboured *SHV* genes. In a similar study conducted by Hyati *et al*. (2019) from Indonesia, *Klebsiella* spp. was isolated from *desi* chicken. Isolates that showed phenotypic resistance to ampicillin and cefotaxime were screened by PCR for *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{OXA}$ and *bla*$_{CTXM}$ genes.The isolates harboured *bla*$_{SHV}$ (9.1%), *bla*$_{TEM}$(100%), and *bla*$_{CTX-M}$ (90.9%).

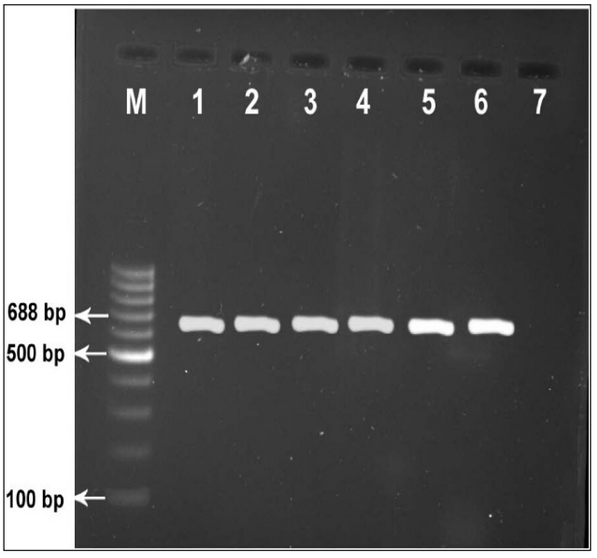

Fig. 3: Detection of Multiplex PCR II *bla*$_{CTX-M}$ Group 1 gene in *Enterobacteriaceae* of *desi* chicken

Lane M : Molecular weight marker (100bp); Lane 1 : Known DNA standard for *bla*$_{CTX-M}$ group 1 gene (688bp); Lane 2 to 6 :Desi chicken microbiota carrying *bla*$_{CTX-M}$ group 1 gene; Lane 7 : Negative control.

Samanta *et al*. (2015) tested 360 poultry samples from backyard poultry and120 samples from the farmed poultry in India. Phenotypic resistant ampicillin and cefotaxime samples were screened by PCR for ESBL genes. None of the *E.coli* isolates from the backyard poultry harboured any ESBL gene. 29.4% of isolates from the farmed poultry were found to possess the ESBL genes. These findings are contrary to our findings in the present study.

CONCLUSION AND RECOMMENDATIONS

Even though, the *desi* chicken were reared in the free range system without any routine antibiotic supplementation in the feed, they found to harbour the antibiotic resistance genes which might have acquired from the environment. These birds in turn may act as reservoirs of resistant bacteria and may play a major role in transmission of the resistant genes to other animals and humans. The results of the present study warrant the usage of antibiotics in poultry feed and suitable alternatives like probiotics, prebiotics, organic acids and synbiotics may be tried.

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