



Discrimination of Beta-lactam Resistant *Escherichia coli* isolated from Poultry and Poultry Farm Workers using (GTG)₅-Rep-PCR

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ABSTRACT

The study was aimed at examining the discrimination of beta-lactam resistant *E. coli* isolated from poultry and poultry farm workers in Andhra Pradesh (India). Faecal swabs from different poultry species (n=150) and poultry farm workers (n=15) were analyzed for *E. coli* and screened for beta-lactam resistance phenotypically, by disc diffusion method and genotypically, by PCR targeting *bla*_{CTX-M} group-1 and 2 beta-lactamase genes. Beta-lactam resistant *E. coli* were serotyped based on their 'O' antigen and then genotyped using (GTG)₅ repetitive sequence based PCR (Rep-PCR). Out of 134 *E. coli* isolates (122/150 from poultry and 12/15 from farm workers) recovered, 12 isolates were found to be beta-lactam resistant, of which seven carried extended-spectrum beta-lactamase (ESBL) phenotype. All the phenotypically resistant isolates carried multiple beta-lactamase genes. Serological typing of the 12 resistant *E. coli* revealed nine different serotypes (O2, O49, O60, O63, O83, O101, O120, UT and Rough). Rep-PCR fingerprinting allowed the discrimination of 12 resistant *E. coli* isolates into 11 Rep-PCR genotypes. The numerical index of discrimination was 0.999. Present findings enabled the observation of genetic diversity between beta-lactam resistant *E. coli* isolated from healthy poultry and poultry farm workers in Andhra Pradesh (India) based on their Rep-PCR genotype.

Keywords: Beta-lactam resistant, *E. coli*, (GTG)₅-Rep-PCR, Poultry

Beta-lactamases are the bacterial enzymes that hydrolyze and inactivate beta-lactam antibiotics (Bush and Jacoby, 2010). Extended Spectrum Beta-Lactamases (ESBLs) are variants of beta-lactamases that hydrolyze penicillins, first, second, third generation cephalosporins and monobactams and are inhibited by beta-lactamase inhibitors (Bush and Jacoby, 2010). In the last few decades, dominance of CTX-M (cefotaximase-Munich) type beta-lactamases was reported globally among several Gram negative bacteria (Carattoli, 2008). The (GTG)₅ repetitive sequence based PCR (Rep-PCR) has been widely employed as a high-throughput genotyping tool for the typing of *Escherichia coli* strains (De Moura *et al.*, 2001; Jonas *et al.*, 2003

and Brocchi *et al.*, 2006 and Mohapatra *et al.*, 2007). Amplification of DNA sequences flanked between the polytrinucleotide (GTG)₅ repetitive sequences was known to generate typical DNA fingerprints for discriminating individual *E. coli* strains (De Vuyst *et al.*, 2008). Although studies regarding beta-lactam resistance in *E. coli* of poultry origin were reported from India (Kar *et al.*, 2015), paucity of information regarding their molecular typing was noticed. Hence, the present study is aimed at the (GTG)₅-Rep-PCR based genotyping of beta-lactam resistant *E. coli* isolates, recovered from different poultry species and poultry farm workers in Andhra Pradesh, India.

MATERIALS AND METHODS

Sample collection and isolation of *E. coli*

Faecal swabs (n=150) from different poultry species viz., chicken, ducks, quails, turkey, fancy birds (each 30) and poultry farm workers (n=15) were collected from three different poultry farms in Andhra Pradesh, India. Isolation and identification of *E. coli* were done using Trypticase soya broth (TSB, Hi-Media), eosin methylene blue (EMB, HiMedia) agar, MacConkey agar medium and standard biochemical tests (Sneath and Holt, 2001) as well as by the PCR amplification of the *E16S* gene of *E. coli* using the primer pair (F, 5'- ATC AAC CGA GAT TCC CCC AGT-3' and R, 5'- TCA CTA TCG GTC AGT CAG GAG-3') (Sharif *et al.*, 2017a). Whole cell DNA extraction was carried out by boiling and snap chilling method (Sekhar *et al.*, 2017a).

Phenotypic screening test for ESBL production

E. coli isolates were screened for resistance against four indicator beta-lactam antibiotics: cefotaxime (CTX, 30 µg), ceftazidime (CAZ, 30 µg), ceftriaxone (CTR, 30 µg) and aztreonam (AT, 30 µg) by disc diffusion method (Bauer *et al.*, 1966) on Mueller Hinton (MH) agar. Sensitivity and resistance patterns were interpreted as per Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2014). Resistance to at least one of the four indicator antibiotics used was considered as 'positive' screening test for possible ESBL production (Drieux *et al.*, 2008; Sharif *et al.*, 2017b).

Phenotypic confirmatory test for ESBL production

E. coli isolates that were found to be positive in the screening test were subjected to phenotypic 'confirmatory test' by the combination disc method using three pairs of antibiotic discs (i.e., with and without beta-lactamase inhibitor): ceftazidime (CAZ, 30 µg), ceftazidime plus clavulanic acid (CAC, 30/10 µg), cefotaxime (CTX, 30 µg), cefotaxime plus clavulanic acid (CEC, 30/10 µg) and ceftriaxone (CTR, 30 µg), ceftriaxone plus sulbactam (CIS, 30/10 µg). ESBL production was confirmed if the inhibition zone size was expanded by a minimum of 5 mm in the presence of beta-lactamase inhibitor (Drieux *et al.*, 2008; Sharif *et al.*, 2017b).

PCR for the detection of beta-lactamase genes

A multiplex-PCR assay (Dallenne *et al.*, 2010; Sharif *et al.*, 2007c) was carried out for the detection of *bla*_{CTX-M} group-1 (688 bp) and group-2 (404 bp) beta-lactamase genes in the phenotypically resistant *E. coli* isolates, using the oligonucleotide primers (*bla*_{CTX-M} group-1, F: 5'-TTA GGA AAT GTG CCG CTG TA-3', *bla*_{CTX-M} group-2, F: 5'-CGT TAA CGG CAC GAT GAC-3' and *bla*_{CTX-M} group 1 and 2, R: 5'- CGA TAT CGT TGG TAC CAT-3'). Reaction mixture was optimized in 25 µl volume containing 1.0 µl of DNA template prepared from each isolate; *Taq* buffer (10x) – 2.75 µl; dNTP mix (10mM) – 0.5 µl; MgCl₂ (25mM) - 1 µl; two forward primers (10 pmol/µl) - each 0.75 µl; two reverse primers (10 pmol/µl) - each 0.75 µl; *Taq* DNA polymerase (1 U/µl) - 1 µl and nuclease free water – 15.75 µl; in an Eppendorf thermal cycler (USA) under the following standardized cycling conditions - initial denaturation at 94°C for 10 min, 30 cycles of denaturation at 94°C for 40 sec, annealing at 60°C for 40 sec, elongation at 72°C for 1 min, final elongation at 72 °C for 7 min and hold at 4°C.

Serotyping of beta-lactam resistant *E. coli* isolates

Serotyping of beta-lactam resistant *E. coli* isolates was done at National *Salmonella* and *Escherichia coli* Centre (NSEC), Central Research Institute (CRI), Kasauli (Himachal Pradesh, India) on the basis of their 'O' antigen.

(GTG)₅-Rep-PCR genotyping

Rep-PCR genomic fingerprinting beta-lactam resistant *E. coli* isolates was done using single oligonucleotide primer (GTG)₅ (5'-GTG GTG GTG GTG GTG-3') (Mohapatra *et al.*, 2007). Reaction mixture was optimized in 25 µl volume containing 2.0 µl of DNA template (50 ng/µl); 2.5 µl of *Taq* buffer [10x]; 0.5 µl of dNTP mix [10mM]; 1.5 µl of MgCl₂ [25mM]; (GTG)₅ primer (10 pmol/µl) – 2.0 µl; 1 µl of *Taq* DNA polymerase (1 U/µl) and 15.5 µl of nuclease free water, under the following standardized cycling conditions - initial denaturation at 95°C for 5 min, 30 cycles of denaturation at 94°C for 30 sec, annealing at 40°C for 1 min, elongation at 65°C for 8 min, final elongation at 65°C for 16 min. Standardization of the Rep-PCR reactions was done using the DNA from reference strain *E. coli* (MTCC 1610).

Analysis of Rep-PCR fingerprinting patterns

The Rep-PCR products were subjected to 2% agarose gel electrophoresis under 110V for 2 h (Sambrook and Russell, 2001). DNA fingerprints were photographed and analyzed using BIO-RAD Gel Documentation system (USA) and the supplied image lab software. The position of bands was compared using 100 bp and 1 kb DNA ladder (Genei™, Bengaluru) as an external reference. Binary matrix was generated using binary coding based on presence (1) or absence (0) of a particular band in the given isolate. The binary data was analyzed using dollop programme of phylip version 3.6 software and dendrograms were constructed. Clusters were considered at a 70% similarity cut-off and the similarity of band patterns was calculated using the Pearson's correlation coefficient. The numerical index of discrimination was calculated using the Simpson's index of diversity, $D = 1 - 1/N (N-1) \sum n_j (n_j - 1)$, where D corresponds to the Discriminatory power, N corresponds to the total number of strains, n_j corresponds to the number of strains belonging to the j^{th} type (Sekhar *et al.*, 2017b).

RESULTS AND DISCUSSION

Isolation and identification of *E. coli*

Out of 150 poultry faecal swab samples analyzed, 122 (81.3%) *E. coli* isolates were recovered (26/30 from chicken, 24/30 ducks, 23/30 quails, 24/30 turkey and 25/30 fancy birds). Out of 15 human faecal swab samples analyzed, 12 (80%) *E. coli* were recovered. All the *E. coli* isolates were able to produce green metallic-sheen colonies on EMB agar, lactose fermenting pink colonies on MacConkey agar and amplified *E16S* gene giving 231 bp amplicon. The present results were in accordance with the findings of earlier studies from Srinagar, India (Wani *et al.*, 2004) and Sao Paulo, Brazil (Saidenberg *et al.*, 2013), where *E. coli* was isolated from 80.2% and 78.3% of the poultry faecal samples tested.

Phenotypic beta-lactam resistance

A total of 12 out of 134 *E. coli* isolates screened were found to be resistant to one or more of the cephalosporin antibiotics tested with an overall frequency of 8.9% beta-lactam resistance and were designated as 'suspect ESBL producers' (Table 1). Resistance to cefotaxime was

observed in 11 isolates, ceftazidime in eight, ceftroxone in six and aztreonam in five isolates (Table 1). Mamza *et al.* (2010) observed beta-lactam resistance in 11.1% of the *E. coli* strains isolated from poultry. Isolates that were found positive in the screening test were confirmed for ESBL production by beta-lactamase inhibitor based test. ESBL production was confirmed in seven isolates (out of 12 suspected) (Table 1). All these seven isolates were resistant to at least one of the indicator cephalosporin used in the screening test, but were found susceptible to the combination of indicator cephalosporin with clavulanic acid or sulbactam in the confirmatory test. As clavulanic acid or sulbactam are beta-lactamase inhibitors, we can conclude that in these seven isolates the cephalosporin resistance mechanism could be mediated by beta-lactamase production (Drieux *et al.*, 2008; Sharif *et al.*, 2017b). In the remaining five isolates, beta-lactamase inhibitor synergy was not detected, likely due to the co-production of other non-ESBL beta-lactamases that confer resistance to beta-lactamase inhibitors or could be due to the existence of other resistance mechanisms conferring resistance to beta-lactam antibiotics, like porin proteins or efflux pumps, which are unaffected by the beta-lactamase inhibitors used in the confirmatory test (Sidjabat *et al.*, 2006; O'Keefe *et al.*, 2010). In the present study, four of the ESBL producing *E. coli* strains were only detected with cefotaxime versus cefotaxime/clavulanate combination while three of the ESBL producers were detected with both ceftazidime versus ceftazidime/clavulanate combination and ceftroxone versus ceftroxone/sulbactam combination. The present results were in accordance with the findings of Chah *et al.* (2007), Kar *et al.* (2015) and Chishimba *et al.* (2016) who observed ESBL phenotype in 9.3 (16/172), 9.4 (16/170) and 20.1% (77/384) of the *E. coli* strains isolated from poultry.

Detection of CTX-M beta-lactamase genes

Beta-lactamase genes were detected in a total of seven isolates, with *bla*_{CTX-M} group-1 being the predominant gene detected followed by *bla*_{CTX-M} group-2 gene (Table 1). All the isolates with confirmed ESBL phenotype carried *bla*_{CTX-M} group-1 gene. The present results corroborate with the findings of other workers where predominance of *bla*_{CTX-M} genes were reported across the world both in healthy poultry and humans (Kolar *et al.*, 2010; Li *et al.*, 2010; Ewers *et al.*, 2012; Kar *et al.*, 2015; Chishimba

Table 1: Serotypes and Rep-PCR genotypes detected in beta-lactam resistant *E. coli*

No. of <i>E. coli</i> / No. of samples tested	<i>E. coli</i> positive for beta-lactam resistance	Frequency of beta-lactam resistance detected				ESBL phenotype	Beta-lactamase genes detected		Serotypes detected	Rep-PCR genotypes detected
		CTX	CTR	CAZ	AT		bla _{CTX-M}	bla _{CTX-M}		
							group 1	group 2		
Chicken (26/30)	Ch1	+	+	-	-	+	+	-	O120	R1
	Ch2	+	-	-	-	-	-	-	O120	R2
	Ch3	+	+	+	+	+	+	+	O2	R3
Ducks (24/30)	D1	+	-	-	+	-	-	-	O63	R4
	D2	-	+	+	-	-	-	-	R	R5
Quails (23/30)	Q1	+	-	+	-	+	+	-	UT	R6
	Turkey	+	+	+	+	+	+	+	UT	R7
Fancy birds (25/30)	T2	+	-	+	-	-	-	-	O83	R8
	F1	+	+	-	-	+	+	-	O60	R9
	F2	+	-	+	-	-	-	-	O60	R9
Humans (12/15)	H1	+	-	+	+	+	+	-	O49	R10
	H2	+	+	+	+	+	+	+	O101	R11
Total (134/165)	12	11	6	8	5	7	7	3	9	11

et al., 2016). Among the isolates (five) that exhibited ‘non-ESBL’ resistant phenotype (positive screening and negative confirmatory test), *bla*_{CTX-M} genes were not detected in the present study. One explanation could be the presence of broad spectrum beta-lactamase genes that were not screened for or the contribution of other resistance mechanisms, such as enhanced expression of efflux pumps and porin proteins (Drieux *et al.*, 2008; Sidjabat *et al.*, 2006; O’Keefe *et al.*, 2010). Serotyping of the 12 beta-lactam resistant *E. coli* isolates revealed nine different serotypes of which O2, O60 and UT were predominant (two isolates each) followed by O49, O63, O83, O101, O120 and Rough (one isolate each).

(GTG)₅-Rep-PCR genotypes among beta-lactam resistant *E. coli*

(GTG)₅-Rep-PCR genotyping generated 4-21 bands per isolate, ranging in size from slightly less than 100 bp to 2500 bp (Fig. 1). The binary data of Rep-PCR profiles showed highly polymorphic DNA fragments among the 12 beta-lactam resistant *E. coli* isolates; viz., 4, 7, 8, 10, 13, 14, 16, 17, 19 and 21 amplicons detected in 1, 1, 1,

1, 1, 1, 1, 2, 2 and 1 isolates, respectively. DNA standard of *E. coli* MTCC 1610 showed 11 fragments. The Rep-PCR profiles revealed 11 Rep-PCR patterns/genotypes discriminated among 12 beta-lactam resistant *E. coli* isolates. The two resistant *E. coli* isolates (F1 and F2) recovered from fancy birds were found to have identical Rep-PCR fingerprinting profile (R9) (Fig. 1 and Table 1). Interestingly, these two isolates shared same ‘O’ serotype (O60) as well. The present results were in accordance with the findings of Mohapatra *et al.* (2007), who assessed the utility of Rep-PCR technique for the discrimination of *E. coli* strains isolated from humans, poultry and wild birds. Other workers across globe also reported high sensitivity and specificity of Rep-PCR genomic fingerprinting tool for the discrimination of *E. coli* strains isolated from diverse sources (De Moura *et al.*, 2001; Dos Anjos Borges *et al.*, 2003; Jonas *et al.*, 2003; Brocchi *et al.*, 2006).

The Rep-PCR profile dendrogram analysis discriminated resistant *E. coli* isolates into four major clusters (C₁ to C₄) for 70% similarity cut-off, viz., C₁ with isolates from fancy birds (F1, F2), C₂ with isolates from humans (H1, H2), C₃ with isolates from chicken (Ch2, Ch3) and C₄ with isolates

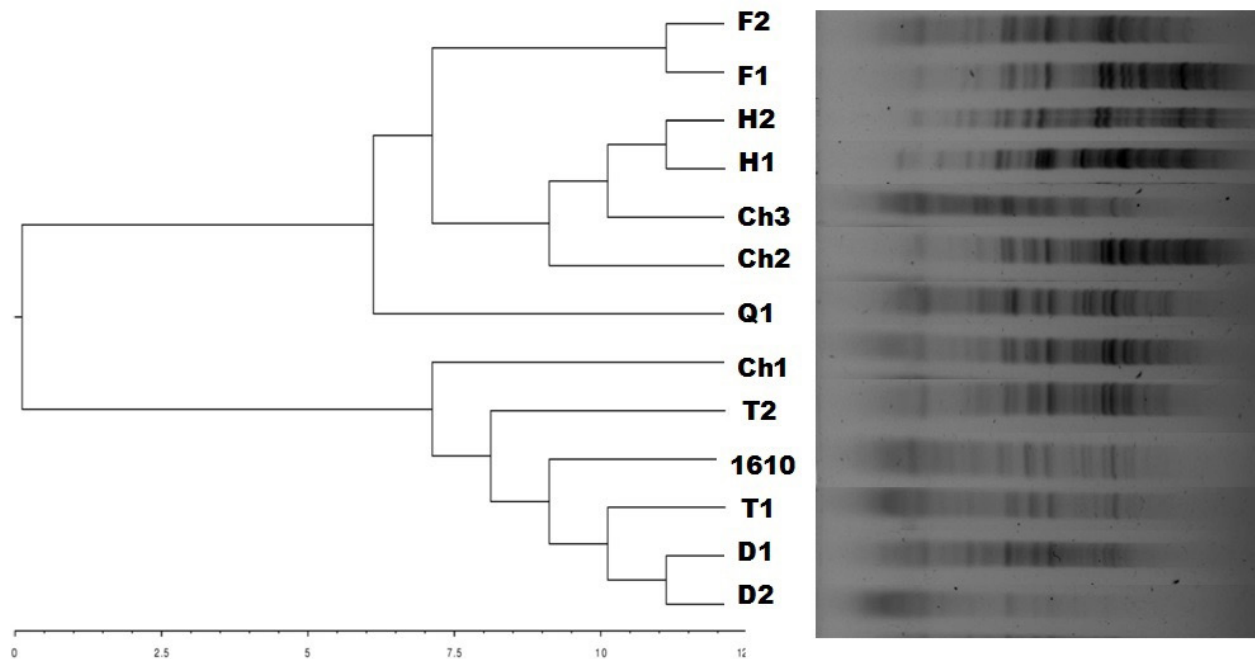


Fig. 1: (GTG)₅-Rep-PCR cluster analysis of faecal *E. coli* isolates from chicken (Ch1, Ch2, Ch3), ducks (D1, D2), quails (Q1), turkey (T1, T2), fancy birds (F1, F2) and poultry farm workers (H1, H2); standard *E. coli* MTCC 1610

from ducks (D1, D2) (Fig. 1). The remaining five isolates (turkey, T1 and T2; chicken, Ch1; quail, Q1 and reference strain *E. coli* MTCC 1610) were found to be unclustered (UC) with other isolates. Cluster analysis indicated intra-serotypic genetic diversity among the isolates affiliated to the same 'O' serotype, as evidenced by their grouping under different clusters in the dendrogram. For example, isolates of O120 serotype viz., Ch1 and Ch2 were grouped under different clusters with different Rep-PCR genotypes (R2, R3) (Table 1 and Fig. 1). The numerical index of discrimination calculated using Simpson's index of diversity was found to be 0.997, which was highly desirable, since the discriminatory powers above 0.90 are considered highly significant (Sekhar *et al.*, 2017b). The present results corroborate with earlier studies (De Moura *et al.*, 2001; Mohapatra *et al.*, 2007), where Rep-PCR was shown to have highly significant discriminatory power in discriminating the *E. coli* strains of diverse origin based on their genotype.

CONCLUSION

The present findings emphasize the utility of PCR based genotyping tools in the epidemiological investigations

to elucidate the intra-serotypic genetic diversity of beta-lactam resistant *E. coli* isolates and (GTG)₅-Rep-PCR was proved to be an effective genotyping tool for the rapid and reliable discrimination of *E. coli* strains of diverse origin based on their genotype.

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