Discrimination of Beta-lactam Resistant *Escherichia coli* isolated from Poultry and Poultry Farm Workers using (GTG)$_5$-Rep-PCR

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Received: 29 July, 2017 Revised: 27 Sept., 2017 Accepted: 15 Nov., 2017

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): cefazidime (CAZ, 30 µg), cefazidime 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<sub>CTX-M</sub> group-1 (688 bp) and group-2 (404 bp) beta-lactamase genes in the phenotypically resistant E. coli isolates, using the oligonucleotide primers (bla<sub>CTX-M</sub> group-1, F: 5’-TTA ATC AAC CGA GAT TCC CCC AGT-3’ and R, 5’- TCA CTA TCG GTC AGT CAG GAG-3’)(Sharif et al., 2017a). 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; MgCl<sub>2</sub> (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 94ºC for 10 min, 30 cycles of denaturation at 94ºC for 40 sec, annealing at 60ºC for 40 sec, elongation at 72ºC for 1 min, final elongation at 72 ºC for 7 min and hold at 4ºC.

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)<sub>5</sub>-Rep-PCR genotyping

Rep-PCR genomic fingerprinting beta-lactam resistant E. coli isolates was done using single oligonucleotide primer (GTG)<sub>5</sub>-GTG 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 MgCl<sub>2</sub> [25mM]; (GTG)<sub>5</sub> 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 95ºC for 5 min, 30 cycles of denaturation at 94ºC for 30 sec, annealing at 40ºC for 1 min, elongation at 65ºC for 8 min, final elongation at 65ºC 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 dendograms 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) Σ n_i (n_i-1) where D corresponds to the Discriminatory power, N corresponds to the total number of strains, n_i corresponds to the number of strains belonging to the jth 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 EM 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, ceftriaxone 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 ceftriaxone versus ceftriaxone/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...
et al., 2016). Among the isolates (five) that exhibited 'non-ESBL' resistant phenotype (positive screening and negative confirmatory test), \( \text{bla}_{\text{CTX-M}} \) 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 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 (\( C_1 \) to \( C_4 \)) for 70% similarity cut-off, viz., \( C_1 \) with isolates from fancy birds (F1, F2), \( C_2 \) with isolates from humans (H1, H2), \( C_3 \) with isolates from chicken (Ch2, Ch3) and \( C_4 \) with isolates

<table>
<thead>
<tr>
<th>No. of samples tested</th>
<th>E. coli positive for beta-lactam resistance</th>
<th>Frequency of beta-lactam resistance detected</th>
<th>ESBL phenotype</th>
<th>Beta-lactamase genes detected</th>
<th>Serotypes detected</th>
<th>Rep-PCR genotypes detected</th>
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<td>Ch2</td>
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Table 1: Serotypes and Rep-PCR genotypes detected in beta-lactam resistant \( E. \ coli \)

(134/165)
Genotyping of beta-lactam resistant *E. coli* by Rep-PCR

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.

ACKNOWLEDGEMENTS

This work was supported by Sri Venkateswara, Veterinary University, Tirupati (Andhra Pradesh, India). Thanks to the Director, National *Salmonella* and *Escherichia* Centre, Central Research Institute, Kasauli, Himachal Pradesh, India, for serotyping of the isolates.

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