Molecular characterization of STEC isolated from Ducks and its relation to ESBL production

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Abstract

The ESBL producing genes are responsible for bacterial resistance to number of antibiotics whereas Shiga toxin producing genes are responsible for bacterial virulence. The association between ESBL producing genes and Shiga toxin producing E. coli (STEC) may pose bigger threat in the battle of antibiotic resistance. This study was conducted to determine the prevalence of Shiga-toxin-producing Escherichia coli (STEC) in ducks reared in organized and unorganized sectors from different agro climatic zones of West Bengal, India and to study their relationship with extended spectrum beta-lactamase (ESBL) production. Total 202 cloacal swab samples were collected from both indigenous ducks reared in backyards sector (110 samples) and Khaki Campbell Ducks reared in organized farm (92 samples). Initially the samples were screened for detection of E. coli on the basis of their cultural, morphological and biochemical properties followed by PCR confirmation for E. coli 16S rRNA. E. coli isolates were subjected to multiplex PCR to detect the presence of shiga toxin producing genes such as stx1, stx2, eaeA and ehxA. STEC isolates were screened phenotypically for production of ESBL and ACBL by double disk diffusion method and subsequently PCR detection for blaCTX-M, blaTEM, blaSHV and blaAmpC genes were performed. Serotyping of all the STEC was also done. Out of 202 samples total 109 were confirmed to be E. coli positive. Out of them total 27 (24.77 %) E. coli isolates were detected to be positive for STEC. Higher prevalence of STEC was observed in unorganized sector compared to the organized sector. Positive association (P < 0.05) was observed between STEC and ESBL production. This study indicates that the duck may play an important role in transmission of Siga-toxin-producing E. coli (STEC) as well as antibiotic resistance genes to human beings, other birds, animals and the environment.

Key words: E.coli, STEC, ESBL, Duck.

1. Introduction

Shiga toxin producing E. coli (STEC) also known as Vero toxin producing E. coli are food borne pathogens associated with different types of human infection ranging from bloody diarrhea to life threatening infection such as hemolytic uremic syndrome (HUS), thrombotic thrombocytopenic purpurae (TTP), haemorrhagic colitis (HC) etc. (Paton & Paton, 1998a; Jamshidi et al., 2016). The ability of STEC to cause human infection is due to production of Shiga toxins which inhibit protein synthesis of the host cell leading to cell death (Karmali et al., 2010). These toxins are subdivided into two major groups: Shiga toxin 1 (stx1) and Shiga toxin 2 (stx2). Lysogenic bacteriophages are responsible for production of E. coli Shiga toxin encoded by stx1 and stx2 gene (O’Brien et al., 1984). The eaeA gene is responsible for the production of “intimin” protein, helps in intimate adhesion of bacteria to the enterocytes and production of attaching and effacing (AE) lesion (Paton & Paton, 1998a). Some of the STEC strain also exhibit haemolysis in washed sheep blood agar due to production of enterohaemolysin encoded by ehxA gene (Beutin et al., 1993). There are several reports of prevalence and characterization of STEC in domestic and wild ruminants (Wani et al., 2004; Mahanty et al., 2013; Mahanty et al., 2014) and from poultry avian species including pigeon (Farooq et al., 2009). A few reports of antibiotic resistance in STEC are available (Shroeder et al., 2002). Extended spectrum beta-lactamase (ESBL) producing E. coli shows resistance against higher generation of cephalosporin and monobactum. Now a day ESBL producing organisms are becoming a major threat for human beings and animals as well. There are three major classes of ESBL viz. TEM, SHV and CTX-M encoded by blaTEM, blaSHV and blaCTX-M genes respectively (Geser et al., 2012). Production of ACBL mediated by blaIMP gene is also another major threat. It becomes difficult when there is association between STEC and ESBL production. Treatment for STEC infection with antibiotics also increases the chances of in vivo production of Shiga toxin (Paton & Paton, 1998a). E. coli O:157, H:7 serotype is well known for its numerous
outbreaks in human (Karch et al., 2005) and animals (Oseki et al., 2002; Keen et al., 2006). Several other serogroups of STEC (O26, O91, O103, O104, O111, O113, O121, O123 and O145) have also been isolated from several outbreaks of human disease in different countries (Hussein, 2007; Espié et al., 2008; King et al., 2009). However, to the author’s best knowledge, there is no such data available from India regarding isolation of STEC from duck (organized and unorganized sector), their comparison and relation to ESBL production. India has 2nd largest duck population next to China. According to the 19th Livestock Census India has 32.76 million duck population out of which about 1/3 rd of total duck population belongs to West Bengal. So a need of systemic study on sector wise, zone wise prevalence of STEC, their serotype and relation to ESBL production from duck of West Bengal, India was felt.

2. Materials and methods

Samples
In this study, total 202 (n = 202, single sample/duck) cloacal samples were collected from both Indigenous ducks reared in backyard (110 samples) and Khaki Campbell ducks reared in organized farms (92 samples). These samples were collected on random basis from different districts of West Bengal, India.

The collection area included farms of North 24 Parganas district, Belgharia of Kolkata district, Nabadwip and Kalyani block of Nadia district (New Alluvial zone); Jangipara block of Hooghly district, Raina – 1 block of Purba Burdwan district and Jagatballavpur block of Howrah district (Old Alluvial zone) and Bankura – 1 block of Bankura district (Red Laterite zone). Among them samples of Khaki Campbell ducks were collected from organized duck farms of North 24 Parganas district and Kalyani block, Nadia district; whereas samples of Indigenous (Desi) ducks were collected from rest of the places.

The cloacal samples were collected aseptically with sterile cotton swabs (HiMedia, India). After collection, the swabs were kept in a sterile sample collecting vial containing peptone water as a media for transport. All the collected samples with proper label were placed on ice in a thermo flask and were brought to the laboratory for further processing.

Reference Strains
Extended spectrum β-lactamase producing Escherichia coli and STEC strains used in this research work as positive control were supplied by the Department of Veterinary Microbiology, West Bengal University of Animal and Fishery Sciences, Kolkata.

Isolation & Identification of Escherichia coli from collected cloacal samples
The collected samples were incubated in peptone water at 37 °C for overnight. After that it was streaked on MacConkey’s agar plates (HiMedia, India) and incubated at 37 °C for overnight for isolation of E. coli. After incubation, rose pink colonies were selected and sub cultured on EMB agar (HiMedia, India) and incubated overnight at 37°C. Next day colonies with metallic sheen were identified and single colony was preserved on nutrient agar (HiMedia, India) slant for further morphological and biochemical confirmation.

All the isolates preserved on nutrient agar slants were stained by Gram’s staining procedure and examined microscopically. The characters considered for morphological identification were Gram positive/ Gram negative, shape, size and arrangement of the organisms. Biochemical identification of the isolates was performed as per methods described by (Quinn et al., 1994) with modifications as per media/ reagent manufacturer’s recommendations.

PCR based confirmation of Escherichia coli.
For PCR based confirmation of E. coli, DNA was extracted from all the morphological and biochemically confirmed isolates. The isolates were subjected to PCR for molecular confirmation as described by Wang et al. (1996) and Jamshidi et al. (2016) with some modifications and the amplified product was visualized by gel documentation system (UVP, UK) after electrophoresis in 2 % (w/v) agarose (SRL, India) gel containing ethidium bromide (0.5μg/ml) (SRL, India).

PCR based detection for STEC
All the E. coli isolates were subjected to multiplex PCR for detection of stxl, stx2, eaeA, ehxX genes as described by Paton & Paton, (1998b) with some modification. 5μl DNA templates, 0.5 μM each primers, 200 mM deoxynuceoside triphosphate, 1U of Taq DNA polymerase (Promega, USA), 2 mM MgCl2 was added in a 25 μl reaction mixture and subjected to two regime of PCR amplification. First regime consisted of 15 cycles. Each cycle consisted of 95 °C for 1 min, 65 °C for 2 min and 72 °C for 1 min 30 sec. Second regime consisted of 20 cycles of 95 °C for 2 min, 60 °C for 2min, 72 °C for 2 mins. This was followed by final extension of 5 min at 72 °C. The amplified product was visualized by gel documentation system (UVP, UK) after electrophoresis in 1.5 % (w/v) agarose (SRL, India) gel containing ethidium bromide (0.5 μg/ml) (SRL, India). The primers and the annealing temperature and predicted length of PCR amplification product are listed in Table 1.

Serogrouping of STEC isolates
STEC producing E. coli were sent for Serogrouping to National Salmonella and Escherichia Centre, HP, India.

Double disc diffusion test
All the STEC producing E. coli isolates were subjected to screening for extended spectrum beta lactamase production by antibiotic sensitivity test containing cefotaxime (30 μg, HiMedia, India) and ceftazidime (30 μg, HiMedia, India) antibiotic discs with or without clavulinate (10 μg, HiMedia, India) (CLSI, 2014). A difference of ≥5 mm between the zone diameters of either of the cephalosporin discs and their respective Cephalosporin/clavulanate discs was considered to be phenotypically positive for ESBL production. Further, cefoxitin-cloxacillin double disc synergy (CC-DDS) was performed for phenotypic confirmation of ACBL production (Tan et al., 2009).

Molecular detection of ESBL and ACBL genes
All the Physically detected STEC producing isolates were subjected to PCR for detection of blaxTEM genes (Weill et al., 2004), blaxSHV genes (Cao et al., 2002), blaxCTX-M genes (Weill et al., 2004).
The PCR for detection of ACBL producing gene (bla\text{AmpC} gene) was performed as per the procedure described by Féria et al. (2002).

The PCR products were electrophoresed in 1.5 % (w/v) agarose (SRL, India) gel containing ethidium bromide (0.5 μg/ml) (SRL, India) and the gel was visualized in gel documentation system (UVP, UK) and photographed. The primers, corresponding annealing temperature and predicted length of PCR amplification products are listed in Table 1.

| Sl. No. | Target gene amplified | Primer sequence (5’→3’ | Annealing temp. | Product size (bp) | Reference |
|--------|-----------------------|-------------------------|-----------------|------------------|-----------|
| 01     | E. coli 16S rRNA      | F: GACCTCGGTTTAGTCACAGA | 58 °C           | 585              | Wang et al. (1996) |
|        |                       | R: CACACCGGTCAGCGTCCA   |                 |                  |           |
|        |                       | R: TCACCCGACGTTAAAGACCT |                 |                  |           |
|        |                       | R: TTCACTCTGAAGTCTGTTC  |                 |                  |           |
| 02     | bla\text{TEM}         | F: ATAAAATTCTTTGAGAAGCAA | 53 °C           | 1080             | Weill et al. (2004) |
|        |                       | R: GACAGTTACCAATGCTAACT |                 |                  |           |
| 03     | bla\text{SHV}         | F: TTA TCT CCC TGT TAG CCA CC | 52 °C           | 792              | Cao et al. (2002) |
|        |                       | R: GAT TGT CTG ATT TCG TCG GG |                |                  |           |
| 04     | bla\text{CTX-M}       | F: CCCCCTTTAATAGACACCAA | 53 °C           | 540              | Weill et al. (2004) |
|        |                       | R: TCAATGGTGCACCTTCACC |                 |                  |           |
|        |                       | F: ATAAATGCGCATTGGTACAC |                 |                  |           |
| 05     | bla\text{AmpC}        | F: GCCGACCTGGTAAACGTCGC | 60 °C           | 634              | Féria et al. (2002) |
|        |                       | R: AGAACGCCCATGAGATCATC |                 |                  |           |
| 06     | stx1                  | F: GGCACTGCTGAAACTGCGTC | 65 °C in first regime and 60 °C in second regime | 255 | Paton & Paton (1998) |
| 07     | stx2                  | R: TTCGCCAGTTATGCAATTTCTG |                  | 384              |           |
| 08     | eaeA                  | F: GACCCGGCAACAAGCATAAGC |                 |                  |           |
|        |                       | R: CCACCTGCAACAAAGAGG |                 |                  |           |
| 09     | ehxA                  | F: GCATCATCAAGCGTGACGTCC |                 |                  |           |
|        |                       | R: AATGAGGCCAAGCTGGTAAAGCT |              | 534              |           |

3. Results and discussion

Results

From 202 duck cloacal swab samples total 109 E. coli isolates were identified, 61 were isolated from unorganized sector farm samples and 48 were obtained from organized sector farm samples (Table 2). Out of 109 E. coli isolates, 27 (24.77 %) isolates were found to be positive for STEC bearing gene. Among them 02 (7.4 %) samples were found to be positive for all stx1, stx2 and eaeA; 02 (7.4 %) samples were found to be positive for both stx1 & stx2; 17 (62.96 %) samples were detected positive for stx1 only; 04 (14.81 %) samples were found to be positive for stx2 only and 02 (7.4 %) samples were found to be positive for eaeA only. However no sample was found to be positive for ehxA (Figure 1).

In zone wise as well as sector wise analysis 13 (27.08%) isolates were detected from New Alluvial zone of West Bengal, India and organized sector. In case of unorganized sector and Old Alluvial zone of West Bengal, India 14 (22.95%) isolates were found to be positive for STEC. So, slightly higher prevalence of STEC was observed in organized sector compared to the unorganized sector (Table 2).

Out of 27 STEC isolates 25 (92.59%) were found to be positive for ESBL/ACBL production (Table 3). So, positive association between ESBL/ACBL producing genes and Shiga toxin producing genes was observed. Total 15 (60%) STEC isolates were found to be positive for either one or more than one of the bla\text{CTX-M}, bla\text{TEM}, bla\text{SHV} and bla\text{AmpC} genes. Total 25 STEC isolates were detected to be positive for ACBL production.

![Fig. 1. Distribution of Shiga toxin genes in STEC positive isolates](image)

The serotyping report of these STEC isolates revealed 11 different serogroup (O119, O83, O84, O5, O2, O8, O88, O157, O35, O128, O119) and 11 isolates were untypeable (Table 3).
Table 2
Virulence genes of *Escherichia coli* (Zone and Sector wise)

| Source          | Zone wise      | Sector wise         | Sample no. | PCR ASSAY FOR STEC Genotypes | Genotypes for ESBL/ACBL Genes |
|-----------------|----------------|---------------------|------------|------------------------------|--------------------------------|
| North 24 parganas | New Alluvial zone | Organized (Khaki Campbell) | GD 1E | + | - | - | - |  
|                 |                 |                     | GD 2E | + | - | - | - |  
|                 |                 |                     | GD 4E | + | - | - | - |  
|                 |                 |                     | GD 6E | + | - | - | - |  
|                 |                 |                     | GD 8E | + | - | - | - |  
|                 |                 |                     | GD 9E | + | + | - | - |  
|                 |                 |                     | GD 12E | + | - | - | - |  
|                 |                 |                     | GD 16E | + | - | - | - |  
|                 |                 |                     | GD 25E | + | - | - | - |  
|                 |                 |                     | GD 31E | + | - | - | - |  
| Nadia (Kalyani) |                 |                     | KE35/4 | + | - | - | - |  
|                 |                 |                     | KE35/9 | + | - | - | - |  
|                 |                 |                     | KE35/10 | + | - | - | - |  
| Total           | 13             | 13                  | 12        | 02                           | 01                             | 00                             |
| Hooghly         | Old Alluvial zone | Unorganized (Desi)  | HE2       | + | - | - | - |  
|                 |                 |                     | HE3       | + | - | - | - |  
|                 |                 |                     | HE6       | + | - | - | - |  
|                 |                 |                     | HE7       | + | - | - | - |  
|                 |                 |                     | HE8       | + | - | - | - |  
|                 |                 |                     | HE9       | + | + | - | - |  
|                 |                 |                     | HE10      | + | + | - | - |  
|                 |                 |                     | HE12      | - | + | - | - |  
| Total           | 14             | 14                  | 09        | 06                           | 03                             | 00                             |
| Burdwan         |                 |                     | BE1       | - | + | - | - |  
|                 |                 |                     | BE2       | - | + | - | - |  
|                 |                 |                     | BE3       | + | - | - | - |  
|                 |                 |                     | BE18      | + | + | - | - |  
| Howrah          |                 |                     | HWE 22    | - | - | + | - |  
|                 |                 |                     | HWE 29    | - | - | + | - |  
| Total           | 14             | 14                  | 09        | 06                           | 03                             | 00                             |
| Grand Total     | 27             | 21                  | 08        | 04                           | 00                             | 00                             |

Table 3
Serogroup of the STEC isolates along with corresponding ESBL/ACBL genes

| STEC Isolate No. | Serogroup | Genotype for STEC associated genes | Genotype for ESBL/ACBL associated genes |
|------------------|-----------|------------------------------------|----------------------------------------|
| GD1E             | O119      | stx1                               | blampC                                 |
| GD2E             | O83       | stx1                               | blampC                                 |
| GD4E             | O84       | stx1                               | blampC & blctxM, blashV & blampC         |
| GD5E             | UT        | stx1                               | blampC, blctxM, blashV, & blampC         |
| GD8E             | O5        | stx1                               | blampC, blctxM, blashV, & blampC         |
| GD9E             | O2        | stx1, stx2 & eaeA                   | blampC                                 |
| GD12E            | O83       | stx1                               | blampC                                 |
| GD16E            | O2        | stx1                               | blampC                                 |
| GD25E            | O2        | stx1                               | blampC                                 |
| GD31E            | O83       | stx1                               | blampC                                 |
| KE 35/4          | O8        | stx1                               | --                                     |
| KE 35/9          | UT        | stx2                               | blatem                                 |
| KE 35/10         | UT        | stx1                               | --                                     |
| HE2              | UT        | stx1                               | blatem & blampC                         |
| HE 3             | UT        | stx1                               | blatem, blctxM, blashV & blampC         |
| HE6              | O88       | stx1                               | blatem, blctxM, blashV & blampC         |
| HE7              | UT        | stx1                               | blatem, blctxM, blashV & blampC         |
| HE8              | O35       | stx1                               | blatem, blctxM, blashV & blampC         |
| HE9              | O 157     | stx1 & stx2                         | blatem & blampC                         |
| HE 10            | O128      | stx1 & stx2                         | blatem, blctxM, blashV & blampC         |
| HE 12            | UT        | stx2                               | blatem, blctxM, blashV & blampC         |
| BE1              | O119      | stx2                               | blampC                                 |
| BE2              | O83       | stx2                               | blampC                                 |
| BE 5             | UT        | stx1                               | blampC                                 |
| BE 18            | UT        | stx1, stx2 & eaeA                   | blampC                                 |
| HWE22            | UT        | eaeA                               | blampC                                 |
| HWE29            | O83       | eaeA                               | blampC                                 |
Discussion

Ducks have free access to land and water bodies, screening duck samples can give an idea about the distribution pattern of STEC in both land & water and the antibiotic resistance genes carried by those organisms; very little of which was known so far.

In the present study a total of 202 duck cloacal swab samples were collected from different agro-climatic zones of West Bengal to minimize sampling error, out of which 110 samples were collected from backyard ducks kept by small and marginal farmers while 92 samples were collected from organized farms and each of the samples were screened individually. Successful isolation of \( E.\) \( \text{coli} \) from about 54 % (109 out of 202) of total duck samples containing about 55 % (61 out of 110) from backyard unorganized sectors and about 52 % (48 out of 92) from organized sector farms; invariably represent the ubiquitous prevalence of this organisms in their respective habitat.

STEC were identified on the basis of presence of four set of genes viz. \( \text{stx1, stx2, eaeA} \) and \( \text{ehxA} \) considered to be specific for production of four major Shiga toxin components \( \text{stx1, stx2, eaeA} \) and \( \text{ehxA} \). Out of 109 \( E.\) \( \text{coli} \) isolates 27 isolates could be identified through PCR for carriage of one or more of the above mentioned genes. The result showed that about 25 % (27 out of 109) of the isolates were positive for one or more components of Shiga toxin producing gene. A similar study from Srinagar, India by Farooq et al. (2009) reported only 4.24 % STEC isolates. However, the present study revealed a much higher percentage i.e. about 25 % of STEC isolated which may be attributed to wide geographical distance between the places from where samples were collected. It was quite fascinating to see that organized farms contained slightly less STEC carriers compared to unorganized farms (13 and 14 STEC positive respectively) which may be due to common sharing of water body by the unorganized sector ducks with other animals and human beings.

So far the carriage of different genes responsible for production of Shiga toxin components by STEC isolates were concerned; 7.4 % (2 out of 109) isolates were found to be positive for three major genes viz. \( \text{stx1, stx2} \) and \( \text{eaeA} \); 7.4 % (2 out of 109) isolates were found to be positive for both \( \text{stx1} \) & \( \text{stx2} \); 62.96 % (17 out of 27) isolates were detected positive for \( \text{stx1} \) only; 14.81 % (4 out of 27) isolates were detected positive for both \( \text{stx1} \) & \( \text{stx2} \) and \( \text{eaeA} \); and 7.4 % (2 out of 109) isolates were found to be positive for \( \text{ehxA} \) only. No isolate was positive for presence of \( \text{ehxA} \). The result also varied with great extent to a similar study previously made by Wani et al. (2004) from Srinagar, India; carried on assorted samples from chicken and pigeon where they did not report any \( \text{stx1} \) or \( \text{stx2} \) positive isolate. Again the previously mentioned study of Farooq et al. (2009) on assorted samples collected from pigeon, duck and chicken indicated the presence of \( \text{stx1, stx2} \) and \( \text{eaeA} \) genes among STEC isolates. The difference of present results compared to their findings may be attributed to the considerable differences in size and geographical origin as well as heterogeneous nature of the samples incorporated in their experiment.

Out of 27 STEC isolates 25 (92.59 %) were found to be positive for ESBL /ACBL production. Significantly higher association (\( P < 0.05 \)) was observed by statistical analysis (chi-square test) between ESBL/ACBL producing genes and Shiga toxin producing genes. Total 15 (60 %) STEC isolates were found to be positive for either one or more than one of the \( \text{bla}_{\text{CTX-M}} \), \( \text{bla}_{\text{TEM}} \), \( \text{bla}_{\text{SHV}} \) and \( \text{bla}_{\text{IMP}} \) genes. Total 25 STEC isolates were detected to be positive for ACBL production. Present findings could not be compared as no data of similar nature was apparently available from India from available literatures during the period of this study.

The serotyping report of these STEC isolates revealed 11 different serogroup (O119, O83, O84, O5, O2, O8, O 88, O 157, O 35, O 128, O 119) and 11 isolates were untypeable as mentioned previously. The results also varied with the findings of Wani et al. (2004) from Srinagar, India possibly due to dissimilarities in origin of samples in terms of both the species of origin (pigeon and chicken) and geographical location.

4. Conclusions

These results show that, the ducks are not only the important sources of ESBL & ACBL producing \( E.\) \( \text{coli} \) but they are also potential carriers of STEC harboring ESBL & ACBL genes. Total 92.5 % STEC were found to be positive for ESBL/ACBL production. These STEC may be transmitted to human beings & other animals through environmental contamination i.e. contamination of land and water bodies with duck fecal droppings or through direct consumption of eggs and meat of ducks soiled with duck feces.

Acknowledgments

The authors sincerely acknowledge the Department of Microbiology, West Bengal University of Animal & Fishery Sciences, 37 K.B. Sarani, Kolkata 700037, West Bengal, India for the kind guidance, funding and entire laboratory facilities for this experimental work.

Conflict of interest

The authors declare that there is no conflict of interest.

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