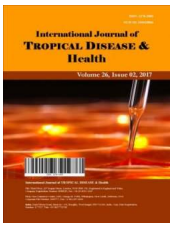

*International Journal of TROPICAL DISEASE & Health*

*27(2): 1-8, 2017; Article no.IJTDH.37109*
*ISSN: 2278–1005, NLM ID: 101632866*

# Prevalence and Molecular Characterization of Shiga-toxigenic *Escherichia coli* in Piglets of North East Region of India

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***Authors' contributions***

*This work was carried out in collaboration between all authors. Author HK designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors TKD and LK managed the analyses of the study. Author HK managed the literature searches. All authors read and approved the final manuscript.*

***Article Information***

DOI: 10.9734/IJTDH/2017/37109
Editor(s):
(1) Ravindra Nath Sharma, Professor, Department of Pathobiology, School of Veterinary Medicine, St. George's University, Grenada, West Indies.
Reviewers:
(1) Ilham Zahir, Sultan Moulay Slimane University, Morocco.
(2) Mona Zaki Zaghloul, Ain Shams University, Egypt.
Complete Peer review History: http://www.sciencedomain.org/review-history/21778

***Original Research Article***

***Received 30th September 2017***
***Accepted 27th October 2017***
***Published 7th November 2017***

## ABSTRACT

**Background:** Shiga-toxigenic *E. coli* also known as verotoxin–producing *E. coli* is one of the diarrhoeagenic *E. coli* strains which also include its well-known subgroup enterohaemorrhagic *E. coli*. It is increasingly recognized as the cause of severe gastrointestinal and systemic diseases, such as haemorrhagic colitis and haemolytic uraemic syndrome.
**Materials and Methods:** The present research study was conducted during 2013-15 to investigate the prevalence and molecular characterization of Shiga-toxigenic *Escherichia coli* associated with gastroenteritis of piglets in organized and unorganized farms of North East Region of India. A total of 457 faecal samples were collected from unweaned piglets in organized and unorganized farms of North East States of India. All the isolates were screened by multiplex PCR assay for presence of putative virulence genes (*stx*$_1$, *stx*$_2$, and *hlyA*), serotyped and further characterized for resistance against 15 selected antimicrobial drugs.
**Results:** Of the 1286 *E. coli* isolates screened by multiplex PCR, a total of 30 isolates (2.33%)

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were recorded as STEC and were isolated from diarrhoeic piglets only. Of the 30 STEC, 28 isolates were positive for *stx*$_2$ gene and 2 isolates possessed *hlyA* gene. Nineteen STEC (2.9%) were recovered from organized farms and 11 (1.74%) from unorganized farms, further, 5 STEC isolates (1.51%) were isolated from local breed and 25 (2.61%) from cross breed piglets. The 30 STEC isolates belonged to 9 different serogroups, 4 isolates (13.33%) were untypable and 5 isolates (16.66%) were rough strains. Serogroups, O1, O8, O156 were found to be the most prevalent serotype (10.0%). All the isolates showed resistance to at least three antimicrobial agents. None of the isolates were found resistance against imipenem, whereas the highest resistance was observed against cefalexin (80.0%) and amoxycillin (76.66%).

*Keywords: Piglets; Escherichia coli; shigatoxin; serotypes; antimicrobial resistance.*

## 1. INTRODUCTION

*Escherichia coli* is a gram-negative, rod-shaped, flagellated, nonsporulating, and facultative anaerobic bacterium which belongs to Enterobacteriaceae family. The bacterium is classified into several categories based on its virulence factors causing debilitating and sometimes fatal diseases [1, 2, 3]. Shiga-toxigenic *E. coli* (STEC) also known as verotoxin–producing *E. coli* (VTEC) is one of the diarrhoeagenic *E. coli* strains which also includes its well-known subgroup enterohaemorrhagic *E. coli* [4,5]. STEC is increasingly recognized as the cause of severe gastrointestinal and systemic diseases, such as haemorrhagic colitis and haemolytic uraemic syndrome [6, 7]. STEC strains includes various serologically group of O:H serotypes that cause disease in humans and animals [8,9]. One of the common feature of the strains is the production of shiga toxins (stx) that are considered to be the major virulence factors. The two main groups of toxins consist of Stx1, which is almost identical to the toxin of *Shigella dysenteriae* type 1 and Stx2, which shares less than 60 per cent amino acid sequence with Stx1 [10]. Genetic information responsible for production of both the toxins is situated in the genome of lambdoid prophages integrated in the STEC chromosome [10].

*E. coli* infection result in production losses and high mortality in piglets, causing considerable loss in terms of economic viability and profitability of swine industry. Another major concern is the emergence of drug resistant *E. coli* in which one of the reason is due to indiscriminate use of antimicrobial agents especially in food producing animals particularly in pigs and poultry. As compared to human cattle and sheep, a limited number of surveys about the prevalence and characteristics of STEC from porcine species have been published across the globe. Keeping in view the importance of the *E. coli* infection in piglets, the present study was conducted for detection and molecular characterization of STEC from piglets of organized and unorganized farms of North East India.

## 2. MATERIALS AND METHODS

### 2.1 Collection of Fecal Samples

A total of 457 fresh fecal samples were collected from piglets under 9 weeks old from organized (n=225) and unorganized (n=232) farms of four North Eastern Hilly states of India, viz., Manipur (n=108), Meghalaya (n=124), Mizoram (n=120), and Nagaland (n=105). Samples were collected from diarrhoeic (n=339) and non-diarrhoeic (n=118) piglets including indigenous local (n=130) and cross breed (n=327) piglets. Samples were collected in four different seasons of the year during June, 2013 to May, 2015.

### 2.2 Isolation and Identification of *E. coli*

The samples so collected were directly inoculated on MacConkey's Agar (Hi-Media, India) plates for *E. coli* isolation and incubated at 37ºC overnight. After incubation, four to five pink coloured colonies were randomly selected and picked up from each plate and streaked on Eosin methylene blue agar (EMB) plates followed by overnight incubation at 37ºC. Colonies with characteristic metallic sheen on EMB agar were selected and studied for their morphological characteristics, viz., shape, size, arrangement and picked up for staining reaction and various biochemical tests such as indole test, methyl red test, Voges Proskauer test, citrate utilization test, $H_2S$ production on triple sugar iron etc [11]. Isolates were stored as pure culture on semi-solid LB agar at 4ºC and also in glycerol stock at -20℃.

### 2.3 Detection of STEC by Multiplex PCR

Template DNA used in PCR was prepared by boiling and snap chilling method as per standard procedure. Molecular characterization of STEC isolates was performed by multiplex PCR [8] targeting *stx*$_1$, *stx*$_2$ and *hly*A genes (Table 1). PCR was performed in a thin-walled PCR tube in total volume of 25 µl reaction mixture containing 1X PCR buffer, 10 mM (each) dNTP, 1.5 mM $MgCl_2$, 1U Taq polymerase (Fermentas), 20pM of each primers and 4.0 µl of previously prepared DNA template. The PCR was performed in a Mastercycler Gradient (Eppendorf, Germany) in a cyclic condition: initial denaturation at 94℃ for 5 min followed by 30 cycles of denaturation at 94℃ for 45 sec, annealing at 61℃ for 45 sec and elongation at 72℃ for 45 sec. This was followed by a final extension at 72℃ for 10 min. The amplicons were analyzed by electrophoresis on 1.5% agarose gel containing ethidium bromide (0.5 µg/ml) in Tris-borate buffer and a 100 bp DNA ladder (Fermentas) was used as a molecular size marker in all the gels. The amplicons were visualized with a UV transilluminator and photographed by gel documentation system (AlphaImager, USA).

### 2.4 Serotyping

All the *E. coli* isolates possessing virulence gene were serotyped on the basis of their somatic antigen [12] at National *Salmonella* and *Escherichia* Centre, Central Research Institute, Kasauli, Himachal Pradesh (India).

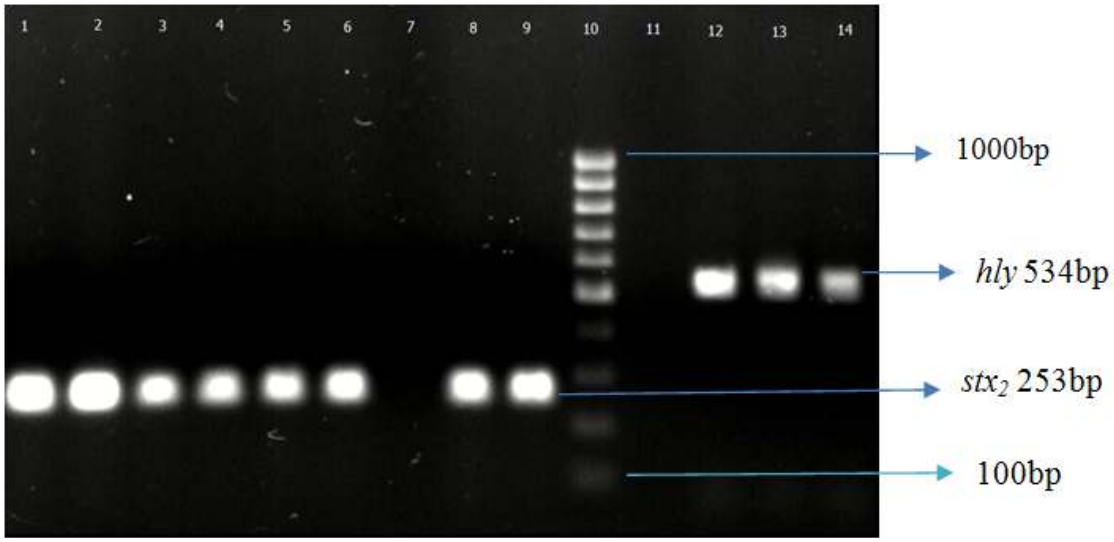


**Fig. 1. Agarose gel electrophoresis showing the PCR amplicons of *E. coli stx2* gene (253bp) and *hly* gene (534bp)**

*Lane 1: Positive control (stx2)*
*Lane 2 to 6, 8, 9: Positive field sample for E. coli stx2 gene (253bp)*
*Lane 7 and 11: No template controls for stx2 and hly*
*Lane 12: Positive control (hly); Lane 13, 14: Positive field sample for E. coli hly gene (534bp)*
*Lane 10: 100 bp DNA ladder*

**Table 1. Oligonucleotide primers used for detection of STEC genes**

| Primer | Sequences (5' – 3') | Product size | References |
|---|---|---|---|
| $stx_1$ F | ATAAATCGCCATTCGTTGACTAC | 180 bp | Paton and Paton (1998) |
| $stx_1$ R | AGAACGCCCACTGAGATCATC | | |
| $stx_2$ F | GGCACTGTCTGAAACTGCTCC | 253 bp | Paton and Paton (1998) |
| $stx_2$ R | TCGCCAGTTAATCTGACATTCTG | | |
| hlyA F | GCATCATCAAGCGTACGTTCC | 534 bp | Paton and Paton (1998) |
| hlyA R | AATGAGCCAAGCTGGTTAGCT | | |

## 2.5 Antimicrobial Sensitivity Assay

All the isolates possessing virulence genes were subjected to *in vitro* antibiotic sensitivity test by disc diffusion method against 15 commonly used antibiotics. Antimicrobial susceptibility test was done on Mueller-Hinton agar (Hi-Media, India) plate as per criteria of [13] using commercially available antibiotic discs, viz., ampicillin (10 mcg), amoxycillin (30 mcg), aztreonam (30 mcg), cefalexin (30 mcg), ceftazidime (30 mcg), cefixime (5 mcg), ceftriaxone (30 mcg), cefotaxime (30 mcg), ciprofloxacin (5 mcg), enrofloxacin (10 mcg), gentamicin (10 mcg), imipenem (10 mcg), nalidixic acid (30 mcg), piperacillin (10 mcg) and streptomycin (10 mcg). A pure colony of each isolate was inoculated into 5 ml of sterile Luria bertani broth under constant shaking at 37℃ overnight and then spread uniformly over previously prepared agar plates, allowed to dry for 5 min before the antibiotic discs were applied using sterile forceps. The plates were incubated at 37℃ for 18-24 hrs and diameters of the zones of inhibition were measured and compared with zone size interpretative chart.

## 3. RESULTS

A total of 1286 *E. coli* were isolated from 457 faecal samples, of which higher rate of isolation were from piglets of organized farms (n=654) than from unorganized farms (n=632) of the 4 NE states. Higher number of isolates (n=1042) were recovered from diarrhoeic samples compared to non-diarrhoeic samples (n=244).

### 3.1 PCR Based Screening for Virulence Genes

Of the 1286 *E. coli* isolates screened by multiplex PCR assay for presence of STEC virulence genes (*$stx_1$*, *$stx_2$*, and *hlyA*), thirty isolates (30/1286; 2.33%) were found belonging to STEC. Again, of the 30 STEC, 28 isolates were positive for *$stx_2$* gene and two isolates possessed *hlyA* gene. None of the STEC isolates harboured *$stx_1$* gene. Nineteen isolates (19/654; 2.9%) were isolated from organized farms and 11 isolates (11/632; 1.74%) were recovered from unorganized farms, further, 5 STEC isolates (5/330; 1.51%) were isolated from local breed and 25 (25/956; 2.61%) from cross breed piglets. Amongst the 30 STEC, 2, 3, 17 and 8 were isolated from piglets samples of Manipur, Meghalaya, Mizoram and Nagaland state, respectively including 2 isolates harbouring *hlyA* gene which were recovered from unorganized farm of Nagaland.

### 3.2 Serotyping of *E. coli*

All the 30 STEC isolates were serotyped based upon their somatic antigens at National Salmonella and Escherichia Centre, Central Research Institute, Kasauli, HP (India). Out of 30 STEC isolates, 21 belonged to 9 different serogroups, 4 isolates (13.33%) were untypable (UT) and 5 isolates (16.66%) were recorded as rough strains (Table 2). Out of the 9 serogroups, O1, O8, O156 were found to be the most prevalent serotype (3/30; 10.0%) followed by O26, O33, O80, O114, O125, O169 (2/30; 6.66%).

**Table 2. Serotypes and virulence genes profile of STEC isolates**

| Serial Number | Sero-groups | Number of positive *$stx_2$* | Number of positive *hlyA* |
|---|---|---|---|
| 1 | O1 | 1 | 2 |
| 2 | O8 | 3 | - |
| 3 | O26 | 2 | - |
| 4 | O33 | 2 | - |
| 5 | O80 | 2 | - |
| 6 | O114 | 2 | - |
| 7 | O125 | 2 | - |
| 8 | O156 | 3 | - |
| 9 | O169 | 2 | - |
| 10 | Untypable | 4 | - |
| 11 | Rough | 5 | - |
| Total | | 28 | 2 |

### 3.3 Antimicrobial Drugs Susceptibility of STEC Isolates

All the 30 STEC isolates were subjected to antimicrobial sensitivity test against 15 selected antimicrobial agents. All of them showed resistance to at least 3 antimicrobial agents, of which resistance to cefalexin (80.0%) was highest followed by amoxycillin (76.66%) ampicillin (60.0%), enrofloxacin (53.33%), piperacillin (46.66%), nalidixic acid (26.66%), cefixime (16.66%), gentamicin (13.33%), aztreonam (10.0%), cefotaxime (10.0%), streptomycin (10.0%), ciprofloxacin (6.66%), ceftazidime (6.66%), ceftriaxone (3.33%) and imipenem (0.0%). Conversely, all the isolates were sensitive to imipenem followed by ceftriaxone (96.66%). Result is depicted in Table 3.

**Table 3. Antimicrobial drugs resistance pattern of STEC isolated from diarrhoeic faecal samples of piglets of the North East India**

| Antibiotic disc and content | Sensitive | Resistant |
|---|---|---|
| Ampicillin (AMP) 10mcg | 12 (40.0) | 18 (60.0) |
| Amoxycillin (AMX) 30mcg | 7 (23.33) | 23 (76.66) |
| Aztreonam (AT) 30mcg | 27 (90.0) | 3 (10.0) |
| Cefalexin (CN) 30mcg | 6 (20.0) | 24 (80.0) |
| Ceftazidime (CAZ) 30mcg | 28 (93.33) | 2 (6.66) |
| Cefixime (CFM) 5mcg | 25 (83.33) | 5 (16.66) |
| Ceftriaxone (CTR) 30mcg | 29 (96.66) | 1 (3.33) |
| Cefotaxime (CTX) 30mcg | 27 (90.0) | 3 (10.0) |
| Ciprofloxacin (CIP) 5mcg | 28 (93.33) | 2 (6.66) |
| Enrofloxacin (EX) 10mcg | 14 (46.66) | 16 (53.33) |
| Gentamicin (GEN) 10mcg | 26 (86.66) | 4 (13.33) |
| Imipenem (IPM) 10mcg | 42 (100.0) | 0 (0.0) |
| Nalidixic acid (NA) 30mcg | 22 (73.33) | 8 (26.66) |
| Piperacillin (PI) 10mcg | 16 (53.33) | 14 (46.66) |
| Streptomycin (S) 10mcg | 27 (90.0) | 3 (10.0) |

## 4. DISCUSSION

Shigatoxigenic *E. coli* is one of the leading cause of diarrhoea, especially among infants in the developing world and they are commonly recovered from faeces of food producing animals and pose threats to health of humans and livestock [14,15]. As compared to human, cattle and sheep, a limited number of surveys about the prevalence and characteristics of STEC from porcine have been published across the globe. In the present study, $stx_2$ gene consist of 93.33% of the STEC which is in agreement with many workers that the dominant gene in STEC isolates is $stx_2$. [16] recorded 100% prevalence rate of $stx_2$ gene from diarrhoeic piglets in Argentina. [17,18] also recorded higher prevalence of $stx_2$ in pigs in USA and India, respectively. Shiga-toxin 2 gene ($stx_2$) is considered to be the most important virulence factor associated with human disease [19]. Therefore, presence of high number of STEC with $stx_2$ indicate the magnitude of virulence in pig and also the possibility of transmission of such organisms between pig and human beings due to close association. Transmission of STEC is probably very high in North East India, as rural people rear 5 to 10 pigs near their houses and mostly share common source of water, and most importantly pork is the major protein source for human consumption in the region.

Prevalence of diarrhoea associated with STEC is recorded slightly higher in organized farms compared to unorganized farms. Prevalence of STEC in cross breed animals is recorded as higher than local piglets population. Although the variation is not very significant, but it may be possible that the local non-descriptive piglets possess better protective nature against natural infection than the exotic or cross breed animals. Another reason may be that the weaning age of piglets of local animals is generally 8 to 10 weeks in comparison with the cross breed animals in organized farms which is within 6 weeks. Maternal immunity might also play an important role in resisting the infection in piglets. The close proximity of the cross bred animals reared in the commercial farms may also be one of the reasons for this fact. Paucity of literatures regarding prevalence of *E. coli* infection in exotic/cross breed and non-descriptive animals and also between organized and unorganized farms did not allow us to compare our results.

The present study identifies different serotypes of STEC in which O1, O8, O156 (10.0%) were the predominant serotypes. This is in sharp contrast with many workers in India who reported O85, O119, O60 as predominant serotypes from piglets [18,20,21]. The predominant STEC serotype O8 causing diarrhoea in this study also agrees with [16]. However, another predominant serotype O1 recovered in this study do not come under commonest porcine pathogenic serogroups [20] which may be an indication of potential newly emerging pathogenic serogroup associated with diarrhoea in piglets in the NE region of India. Therefore, it is uncertain to say that whether this particular serogroup is specific pathogen for pigs. It may pose threat to human infections in future as pigs are one of the major reservoirs of STEC. Serogroup O26, O103, O111, O113, O145 and O157 were reported as being responsible for haemolytic uraemic syndrome in human being [22]. Thus, detection of serotypes O26 in the present study from diarrhoeic piglets were alarming, and warrant the chance of transmission of this pathogen from pig

to human, which may pose potential public health concern. High number of isolates (13.33%) remained untypable with available 'O' antisera which indicate the occurrence of a wide spectrum of *E. coli* serogroups in NE region of India. This finding also tallied with [23,24] who recovered high number of untyped *E. coli* strains from diarrheic piglets.

Antimicrobial drug resistance in Gram negative enteric bacteria has emerged as a serious problem in human and veterinary medicine. *Enterobacteriaceae* resistance to cephalosporins is mainly due to the production of extended-spectrum-betalactamases which have been increasingly detected in food animals and have gained considerable attention worldwide [25]. In the present study, none of the isolates were found resistant to imipenem which is in agreement with [26,27]. Resistance to ceftriaxone was found to be 3.33%, which partially agrees with report on ceftriaxone-resistant *E. coli* from chicken meat in 2014 of [28]. However, [29] found no resistant isolates against ceftriaxone. Imipenem is not in use for veterinary practices in any of the North East Region of India and ceftriaxone due to its high cost have limited usage. This may probably explained the high sensitivity of these drugs in the region. Even slight decrease in susceptibility to imipenem should be taken seriously, as this is the 4$^{th}$ generation cephalosporins for treating infections caused by Gram negative bacteria. Overall resistance to piperacillin was found to be 46.66%, which is in agreement with [30] who recorded 50% resistance to piperacillin in *E. coli* isolates from clinical patients. Resistance to streptomycin and ampicillin was found to be 10.0% and 60.0%, respectively, which disagrees with [31] in Poland who observed high resistance to streptomycin (88.3%) and only 49.3% resistance to ampicillin among the *E. coli* isolates from swine. Our result is in contrast with the study done by [32,33], who showed 84%, 75% and 85% resistance of *E. coli* isolates to cefotaxime, ceftriaxone and ceftazidime, respectively.

Multiple antimicrobial resistant in STEC may partly result from the spread of genetic elements including plasmids, transposons and integrons [34,35] and acquiring of drug resistant gene through horizontal gene transfer because of indiscriminate use of antibiotics. Due to the dissemination of antimicrobial resistance in STEC, it may complicate future therapeutic options that are being developed for treatment of haemorrhagic colitis (HC) and haemolytic uraemic syndrome (HUS). Therefore, continued surveillance of emerging antimicrobial resistance among zoonotic food-borne pathogens is required to ensure public health.

## 5. CONCLUSION

The present study highlighted the significance of STEC as important diarrhoeal agent in young pigs with possibility of transmission between porcine and human population. STEC (*stx2*) were very much prevalent among piglets with diarrhoea in North East India with O1, O8 and O156 being the predominant serotypes. Imipenem was highly sensitive for STEC and cefalexin and amoxycillin has highest resistance. Taking into account the huge pork consumption and also close contact between human and pigs in the region, these findings warrant a more critical appraisal of these zoonotic pathogens in pigs and humans. More emphasis may be given to detail epidemiological study and understand the mechanism of multidrug resistance pattern of diarrhoeagenic *E. coli*.

## ACKNOWLEDGEMENTS

The authors are thankful to the Institutional Biotech Hub, Department of Biotechnology, Government of India, DBT project on ADMaC and Dean, College of Veterinary Sciences & Animal Husbandry, Aizawl, Mizoram, India for providing all the facilities to conduct the present work.

## CONSENT

It is not applicable.

## ETHICAL APPROVAL

As per international standard or university standard written ethical approval has been collected and preserved by the author(s).

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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