Molecular characterization of shiga like toxin-producing Escherichia coli (STEC) isolates from pigs oedema

N.N. Barman, Reema Deb, T. Ramamurthy*, R.K. Sharma, P. Borah, S.A. Wani** & D. Kalita*

Department of Microbiology, College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati, *National Institute of Cholera & Enteric Diseases, Kolkata, **Division of Microbiology & Immunology, Faculty of Veterinary Sciences & AH, S.K. University of Agriculture & Technology of Kashmir, Srinagar, +AICRP on Pigs, College of Veterinary Science, Khanapara, Guwahati, India

Received March 7, 2007

Background & objectives: An oedema outbreak occurred in a Guwahati pig farm. Escherichia coli isolates from different necropsy samples collected from the dead piglets with oedema were characterized to confirm the virulence.

Methods: Haemolytic E. coli isolates recovered from liver, lung and intestine of pigs with oedema were examined for presence of genes encoding pathogroups such as enteropathogenic Escherichia coli (EPEC), (eae/bfpA), enteroaggregative Escherichia coli (EAggEC), (eagg), enterotoxigive Escherichia coil (ETEC), (elt/est) and shiga like toxin producing Escherichia coli (STEC), (stx1/stx2) by PCR and molecular typing by randomly amplified polymorphic DNA-PCR (RAPD-PCR).

Results: The three haemolytic E. coli recovered from diseased pigs were STEC because of presence of the stx2 and eae genes. Analysis by RAPD-PCR indicated that two of the three isolates were genetically related.

Interpretation & conclusions: The isolation of STEC isolates from pigs with oedema was shown. Although the three isolates were untypable, presence of eae and stx2 genes clearly indicated these as prime cause of pig oedema disease. Further, demonstration of STEC in pigs becomes a public health concern, as pigs are potential reservoir of such agents, which may cause human illness.

Key words Oedema - pigs - PCR - STEC

Shiga toxin-producing Escherichia coli (STEC), also known as verotoxin–producing E. coli (VTEC) comprises a serologically diverse group of pathogens that cause disease in humans and animals. STEC infections have been described in a wide range of both domestic and wild animals, but the natural pathogenic role of bacteria has been demonstrated only in young calves (diarrhoea or dysentery), weaning pigs (oedema disease), and dogs (cutaneous and renal vasculopathy in grey hounds). Studies have demonstrated that these animals including pigs harbouring STEC are also associated with human illness.

The common feature of STEC is the production of shiga toxins (Stx) that are considered to be the major
virulence factors. The two main groups consist of Stx1, which is nearly identical to the toxin of *Shigella dysenteriae* type 1 and Stx2, which shares less than 60 per cent amino acid sequence with Stx1. The genetic information for the production of Stx1 and Stx2 is located in the genome of lambdoid prophages integrated in the STEC chromosome. Stx1 shows only little sequence variations, but several variants of Stx2 with altered antigenic or biological characteristics have been reported. This study was undertaken to examine haemolytic *E. coli* isolated from pigs with oedema for the presence of genes encoding various virulence factors.

**Material & Methods**

In an organized pig farm of Guwahati, Assam, where a total of 163 adult and 208 newborn Hampshire crossbred pigs were maintained, 24 litters of piglets were born between May and June 2005. After 56 days of age, a total of 125 piglets were weaned and their average weight at weaning was 10.5 kg. Immediately after weaning, the piglets in a litter showed rough hair coat, off-fed condition, rise of temperature (103°F) followed by respiratory distress, swelling of head and eyelids and inco-ordination of movements due to ataxia. The affected piglets soon became recumbent. Diarrhoea was not recorded and the body temperature at this stage was normal. The piglets died within 3-4 days after the onset of clinical symptoms. The disease then spread to the other litters and seven more litters were affected. Number of affected piglets was 27, of which 9 piglets died. Morbidity and mortality rate was 21.6 and 33.3 per cent, respectively. On postmortem examination, the abdominal cavity was found to be filled with fluid; there was oedema in the subcutaneous tissue, submucosa of the stomach and intestine, particularly in colon.

**Necropsy and isolation of Escherichia coli:** Heart blood, liver, lungs and intestinal contents from 10 piglets died of infection in May and June 2005, were collected and processed for isolation and identification of bacteria as per the method described earlier. Three isolates were haemolytic and were sent to National Institute of Cholera and Enteric Diseases, Kolkata for molecular study, where they were identified as *E. coli*. The O-serogroups (somatic antigen) of *E. coli* isolates were determined by slide agglutination test using a commercially available antisera kit (Denka Seiken Co., Tokyo, Japan).

**Antibiotic susceptibility tests:** Drug susceptibility was determined by the disc diffusion method using the following commercial discs (Hi-Media, Mumbai, India), ampicillin (10 µg), ciprofloxacin (10 µg), gentamicin (10 µg), tetracycline (10 µg), cephalosporin (30 µg), enrofloxacin (10 µg), chloramphenicol (10 µg) and nalidixic acid (30 µg).

**Detection of E. coli virulence genes by PCR:** The lactose fermenting colonies grown on MacConkey agar plate were enriched in 2 ml Luria Bertani Broth (Becton Dickinson Co., Sparks, USA), by incubation at 37°C for 16-18 h under shaking condition. Two hundred µl of each enriched culture was subjected to heating in a boiling water bath for 10 min, snap cooled in ice and directly used as a template DNA for PCR for screening of virulence genes. PCR for the detection of virulence genes was performed using a thermal cycler (GeneAmp 9700, Applied Biosystems, Foster city, USA) in a total reaction volume of 25µl. The reaction mixture contained 2.5µl of culture lysate (template DNA), 2.5µl of 10×PCR buffer with 15mM MgCl₂ (Genei, Bangalore), 2.0µl of dNTP containing 2.5 mM each of the four deoxynucleotide triphosphates, 10 pmol of each primer (Isogen Life Sciences, Ijsseltstein, The Netherlands) and 1U of Taq DNA polymerase (Genei, Bangalore). The primer sequences of the targeted virulence genes and their PCR conditions are presented in the Table. STEC 1, STEC 2, STEC 3 harbouring stx₁, stx₂, and stx₁ and stx₂, respectively were used as positive control strains (*E. coli* Repository centre, NICED, Kolkata).

The PCR amplified products were separated by electrophoresis with 2 per cent (w/v) agarose (Sisco Research Laboratories, Mumbai) gels in 1xTAE [0.04M Tris acetate (Sigma, St Louis, USA), 0.001M EDTA, pH 8.0] buffer along with 100 bp ladder (Takara, Shuzo, Otsu, Japan) as a molecular weight marker. After staining with ethidium bromide (Sigma, USA), the gels were viewed with the help of a gel documentation system (Gel Doc 2000, BioRad, Hercules, USA).

**Randomly amplified polymorphic DNA-PCR (RAPD-PCR):** The molecular typing of the *E. coli* was performed by RAPD-PCR using a single primer 1247 (Isogenen, The Netherlands) (5'-AAGAGCCCGT-3') in the Gene Amp PCR system 9700 (Applied Biosystems, USA). The PCR mixture was prepared to a volume of 50 µl containing 20 ng of genomic DNA, 200 µM of each dNTP, 30 pmol of primer, 5 µl of 10xPCR buffer, 3 mM MgCl₂ and 2.0U of Taq DNA Polymerase. PCR was initiated with a hot start at 80°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 40°C for 1 min, and extension at 72°C for 2 min. A final extension step was done at 72°C for 10 min.
The RAPD-PCR amplified products were electrophoresed in 1 per cent agarose gel along with a 1 kb molecular weight marker (New England Biolabs, Beverly, MA, USA). After staining with ethidium bromide, the gel was documented (Gel Doc 2000, BioRad) for comparison and to determine the clonal relationship between the strains.

**Results & Discussion**

Oedema disease usually occurs 1-2 wk after weaning and is most common in rapidly growing piglets. Passive immunity probably protects the neonates from STEC infection and when the maternal antibody diminishes, the piglets become susceptible. Pregnant sows immunized with K88ac antigens excreted high antibody titre in colostrums and milk. Piglets born to such immunized mother were protected against challenge infection. Besides, nutritional factors influence propagation and colonization of bacteria. The source of infection for this outbreak may be in the pig farm premises itself. In intensively reared pig farms, the incidence of oedema disease is high compared to the free grazing pigs. Asymptomatic adult pigs might harbour and shed the pathogen in their faeces time to time.

The bacteriological examination of heart blood, liver, lungs and intestinal contents revealed the presence of Gram-negative bacilli. In the biochemical tests, the isolates were identified as *E. coli*. The haemolytic pattern of the *E. coli* isolates in 10 per cent sheep blood agar showed narrow zone of haemolysis, typical for the STEC. The antimicrobial susceptibility assay in the present study showed that all the three isolates were susceptible to gentamicin, chloramphenicol, nalidixic acid, cephalosporin, enrofloxacin and resistant to ampicillin, ciprofloxacin, and tetracycline. Multidrug resistance was previously reported from India STEC isolated from beef samples.

The three representative alpha haemolytic *E. coli* isolates recovered from liver, lung and intestine of affected pigs were examined for presence of genes encoding various virulence factors associated with pathogroups such as EPEC (*eae/bfpA*), EAggEC (*eagg*), ETEC (*elt/est*) and STEC (*stx1/stx2*) by PCR. All the three isolates harboured *eae* and *stx2* genes, as they gave 454 and 110 bp amplicons, respectively (Fig. 1). Fratamico et al reported isolation of *stx2* and *stx2e* variants from healthy as well as pigs with oedema disease.

Many studies showed that *stx2* and *eae* genes of *E. coli* were associated with oedema disease in pigs. The pathogenesis of STEC is triggered by a set of genes carried in the chromosome that includes a 35.5 kb pathogenicity island, termed locus of enterocyte

---

**Table.** Polymerase chain reaction (PCR) primers and conditions used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Nucleotide sequence of primer (5’-3’)</th>
<th>Target</th>
<th>PCR condition</th>
<th>Amplicon Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Denaturing Temp (°C)</td>
<td>Annealing Temp (°C)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Time (sec)</td>
<td>Time (sec)</td>
</tr>
<tr>
<td>EVT1</td>
<td>CAACACTGGAATGATCCTTAG</td>
<td><em>stx1</em></td>
<td>94</td>
<td>60</td>
</tr>
<tr>
<td>EVT2</td>
<td>CCCCTCAACTGCTAATA</td>
<td></td>
<td>94</td>
<td>60</td>
</tr>
<tr>
<td>EVS1</td>
<td>ATCACTGCACCTACCTGTTA</td>
<td><em>stx2</em></td>
<td>94</td>
<td>60</td>
</tr>
<tr>
<td>EVS2</td>
<td>CTCTGCAGATAAACCCCTCTGC</td>
<td></td>
<td>94</td>
<td>60</td>
</tr>
<tr>
<td>EAE1</td>
<td>AAACAGGTTAAACTGTGGCC</td>
<td>eae</td>
<td>94</td>
<td>60</td>
</tr>
<tr>
<td>EAE2</td>
<td>CTCTGCAGATAAACCCCTCTGC</td>
<td>bfpA</td>
<td>94</td>
<td>60</td>
</tr>
<tr>
<td>BFPA1</td>
<td>ATATGATCGCTTGCGCTTCGTC</td>
<td>agg</td>
<td>94</td>
<td>60</td>
</tr>
<tr>
<td>BFPA2</td>
<td>GCGCCTTTATCTCACTTGTTA</td>
<td>elt</td>
<td>94</td>
<td>60</td>
</tr>
<tr>
<td>EAGG1</td>
<td>CTGGCCGAAAGACTGTATCAT</td>
<td>est</td>
<td>94</td>
<td>60</td>
</tr>
<tr>
<td>EAGG2</td>
<td>CAATGATAGAAATCCGCTGTG</td>
<td></td>
<td>94</td>
<td>60</td>
</tr>
<tr>
<td>ELT1</td>
<td>GCCGCAAATTATACCCTGTCT</td>
<td></td>
<td>94</td>
<td>60</td>
</tr>
<tr>
<td>ELT2</td>
<td>CCAGATTCTGTATATATATAT</td>
<td></td>
<td>94</td>
<td>60</td>
</tr>
<tr>
<td>EST1</td>
<td>ATTTTTA/CTTCTCTTATATAT/CTTCTT</td>
<td></td>
<td>94</td>
<td>60</td>
</tr>
<tr>
<td>EST2</td>
<td>CACCCGTACAA/GGCCGATT</td>
<td></td>
<td>94</td>
<td>60</td>
</tr>
</tbody>
</table>

*PCR was done for 30 cycles

After 35 cycles, a final extension step of 10 min at 72°C was done.
effacement (LEE). The characteristic attaching and effacing (A/E) lesion caused by most of the STEC depends on the activity of the multiple genes in the LEE including the type III secretion system and on the initiation of the signal transduction events. The \textit{eae} gene is responsible for attaching to translocated intimin receptor (tir). Most of oedema causing STEC in pigs belonged to serogroups O138, O139, O141 and untypable. In this study, none of the STEC isolates were serologically reactive with the commercial serotyping kit, and hence were untypable. A study carried out in weaned piglets in Belgium showed that 52 per cent of the haemolytic \textit{E. coli} were verocytotoxic. The \textit{stx} harbouring serogroups O141; O85ab and OUT (untypable) strains were also found to be associated with oedema disease. A clone of oedema disease-causing serogroup O147 was reported in USA. Though STEC isolates have been reported earlier in India from human and animals, the present report might be the first recorded incidence of oedema disease in pigs in this country. Two of the three STEC isolates identified in this study were genetically related as determined in the RAPD-PCR. The \textit{E. coli} that typically cause oedema disease have been shown to have genetically diverse backgrounds.

The STEC isolates possessing \textit{stx}_{2e} variant that cause oedema disease in pigs have also been isolated from human patients with diarrhoea and haemolytic-uraemic syndrome (HUS). It has been demonstrated that STEC strains recovered from pigs harboured different virulence genes similar to the one isolated from the infected humans (\textit{stx}_{r}, \textit{stx}_{r} \textit{eae}, \textit{hly}) and thus pigs may be important reservoir for these organisms. It is thus important to make an extensive survey on STEC in pigs. Molecular characterization has proved to be a rapid and accurate diagnostic approach for establishing the epidemiology of STEC.

References


Reprint requests: Dr N.N. Barman, Associate Professor, Department of Microbiology College of Veterinary Science, Khanapara, Guwahati 781 022, India e-mail: nnbarman@gmail.com