Salmonella daarle and Salmonella hiduddify associated with acute gastroenteritis in piglets in India

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ABSTRACT

The present study was conducted to investigate an acute gastroenteritis outbreak in an unorganized pig farm in the North Eastern Hilly Region of India. Fecal samples were collected from 20 pigs including 5 piglets, which were suffering from acute gastroenteritis and were processed for detection of E. coli, Salmonella, Clostridium sp., Rotavirus, Picobirnavirus as well as parasitic eggs and larvae by standard laboratory techniques. Virulence genes for pathogenic E. coli and Salmonella were detected by specific PCR assays. A total of 77 E. coli were isolated, all of which were found to be negative for any putative virulence genes of STEC/VTEC, ETEC, EHEC and EPEC pathotype by PCR. A total of 5 salmonellae were also isolated from 5 affected piglets, of which 1 and 4 were recorded as Salmonella daarle and Salmonella hiduddify, respectively. All the Salmonellae were positive for enterotoxin (stx) and invasion (invA) genes by PCR. In conclusion it may be stated that this is the first report of S. daarle and S. hiduddify associated with piglet diarrhoea and also first report from India with any type of enteric infection in man and animals

Key Words: Piglets, Salmonella daarle, Salmonella hiduddify, India.

Introduction

Pigs are most commonly affected by salmonellae infections from weaning to 4 months of age, of which piglet diarrhoea is very significant (Hut et al., 2011). Although a wide range of bacteria and viruses along with few common parasitic agents can affect young pigs producing diarrhoea or scour, salmonellae remain one of the major causes of acute gastroenteritis. Amongst various existing serovars, Salmonella choleraesuis is considered the most common and important agent of swine salmonellosis. Among the other serovars, viz., S. typhimurium, S. heidelberg, S. anatum, S. dublin, S. derbysand S. enteritidis are also reported to be associated with enteric infections of pigs (Reed et al., 1985; Bedia et al., 1986).

Salmonella enterica subspecies enterica serovar Hiduddify is reported to be associated with gastroenteritis in new born nursery in USA (Acute Communicable Disease Control Program, Special studies report 2006) and from chicken and poultry meat in Nigeria (Kaufa et al., 2009). So far, only one report is available on Salmonella enterica subspecies enterica serovar Daarle from Germany (Rohr and Aleksic, 1987). To the best of our knowledge, there is no further published report on association of Salmonella enterica subspecies enterica serovar Daarle and Salmonella enterica subspecies enterica serovar Hiduddify with piglet diarrhoea.

The present study was conducted to report an association of Salmonella enterica subspecies enterica serovar Daarle and Salmonella enterica subspecies enterica serovar Hiduddify with piglet diarrhoea in pigs from the North Eastern region of India.

Material and Methods

Animals and sampling

An outbreak of acute gastroenteritis appeared in one unorganized pig farm of Nagaland, India during the month of July 2015. The farm was having 20 animals including 8 adults and 12 piglets. Of the 12 piglets, 5 were suffering from acute gastroenteritis and rest was apparently healthy. Fecal samples were collected from all the animals using sterile cotton swabs and transported to laboratory under cold chain. All the samples were processed for isolation and identification of possible enteric bacteria including E. coli, Salmonella and Clostridium sp. as per the method described by Ewing (1986) and also for detection of enteric viruses, including Rotavirus and Picobirnavirus by RNA-PAGE and RT-PCR. Samples were also examined for the presence of parasitic eggs and larvae by standard flotation technique.

Bacteriological screening of clinical specimens

E. coli and Salmonella were cultured aerobically followed by isolation and identification by standard biochemical tests. Clostridium sp. was cultured anaerobically followed by isolation and identification by standard biochemical techniques (Ewing, 1986; Safiullah et al., 2016). All the pure isolates were stored in glycerol at -80°C for further use.

Detection of selected viral pathogens

The fecal samples were screened for the presence of Rotavirus and Picobirnavirus by RNA-PAGE analysis with certain modifications. In brief, samples were diluted in phosphate buffered saline (pH 7.4) to prepare a10% (w/v) fecal suspension. Clarified supernatant was collected and processed for RNA extraction using Trizol method (WHO, 2009). The extracted RNA was subjected to RNA-PAGE followed by silver staining as per the standard procedure (Laemmli, 1970; Herrin et al., 1982).

Table 1: Expected amplicons size of the target genes under the study.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Amplicon size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.6S</td>
<td>617</td>
<td>Prager et al. (1995)</td>
</tr>
<tr>
<td>invA</td>
<td>941</td>
<td>Galan et al. (1992)</td>
</tr>
<tr>
<td>pef</td>
<td>700</td>
<td>Rahman et al. (2000)</td>
</tr>
<tr>
<td>eae</td>
<td>384</td>
<td>Paton and Paton (1998)</td>
</tr>
<tr>
<td>sst1</td>
<td>180</td>
<td>Paton and Paton (1998)</td>
</tr>
<tr>
<td>sst2</td>
<td>255</td>
<td>Paton and Paton (1998)</td>
</tr>
<tr>
<td>hlyA</td>
<td>534</td>
<td>Paton and Paton (1998)</td>
</tr>
<tr>
<td>LF</td>
<td>450</td>
<td>Philipp et al. (1995)</td>
</tr>
<tr>
<td>ST</td>
<td>190</td>
<td>Philipp et al. (1995)</td>
</tr>
<tr>
<td>VP7, Rota A</td>
<td>304</td>
<td>Husain et al. (1995)</td>
</tr>
<tr>
<td>VP6, Rota C</td>
<td>356</td>
<td>Gabbay et al. (2008)</td>
</tr>
<tr>
<td>GG1, (PBV)</td>
<td>201</td>
<td>Rosen et al. (2000)</td>
</tr>
<tr>
<td>GG2L (PBV)</td>
<td>369</td>
<td>Smits et al. (2011)</td>
</tr>
</tbody>
</table>

Rotavirus and Picobirnavirus was also detected by reverse transcription-PCR (RT-PCR). Detection of Rotavirus group A and C was performed by targeting VP7 gene (Husain et al., 1995) and VP6 gene (Gabbay et al., 2008), respectively. For detection of
Picobirnavirus genogroup I (Rosen et al., 2000) and genogroup II specific primers (Smits et al., 2011) were used. Details of primer sequence and PCR conditions are given in Table 1.

Detection of bacterial virulence genes by PCR
DNA lysate for PCR analysis was prepared by boiling and snap chilling method. Detection of putative virulence genes of EPEC (eaeA), STEC/VTEC (stx1, stx2), EHEC (hlyA) and ETEC (lta, sta and stb) was evaluated by multiplex PCR (Paton and Paton, 1998). Detection of 
\( \text{stn} \) (Pragger et al., 1995), invA (Galán et al., 1992) and pef (Rahman et al., 2000) genes was carried out for Salmonella isolates by specific PCR assay in a thermal cycler (Eppendorf, Germany) (Table 1).

Amplified products were separated by agarose gel (1% agarose in 1× Tris-borate-EDTA buffer) electrophoresis at 5v/cm for 2 h and stained with ethidium bromide (0.5 μg/ml). Standard molecular size marker (100 bp DNA ladder) was included in each gel. DNA fragments were observed by ultraviolet trans-illuminator and photographed in a gel documentation system (Alpha Imager, Germany). All the PCR were performed three times to ensure the repeatability of the technique and to make sure that isolates were correctly assigned to respective patterns.

Screening of samples for parasites
All the fecal samples were tested for presence of common parasitic eggs by standard floatation technique (FAO).

Serotyping of Salmonella isolates
All the 5 Salmonella isolates were serotyped at National Salmonella and Escherichia Centre, Central Research Institute, Kasauli, India.

Results

Epidemiological details of the animals
All the piglets under the study were in the age group of 2-8 weeks. All the 5 piglets were suffering from acute gastroenteritis with non-haemorrhagic diarrhoea and fever in some occasions. The adult, gilt and rest of the piglets were apparently healthy without any clinical signs. No animals received any treatment against bacterial or parasitic infection till the date of collection of fecal samples. There was no report of death of any piglets. Samples were collected on the second or third day after onset of symptoms.

Detection of parasitic and viral pathogens
On laboratory examination as stated earlier, no samples were found to contain parasitic eggs or larvae. Rotavirus and/or Picobirnavirus also could not be detected by either RNA/PAGE or by RT-PCR.

Isolation of bacterial organisms
No clostridial organisms were detected by anaerobic culture. A total of 77 E. coli were isolated and identified from all the 20 samples under the study. In brief, fecal samples were streaked on MacConkey’s Agar medium and incubated for 24 hours at 37°C. Five randomly selected pink colored colonies were studied by Gram’s staining followed by inoculation on EMB agar medium and incubated for 24 hours at 37°C. Colonies with characteristics metallic sheen were further characterized by battery of sugar fermentation and biochemical assays (Ewing, 1986). On the other hand, 5 Salmonella were isolated and identified from the 5 diarrhoeic piglets based on standard bacteriological and biochemical tests.

Detection of bacterial virulence genes
All the E. coli (n=77) isolates were found to be negative for eaeA, stx1, stx2, hlyA, lta, sta and stb genes by PCR. On the other hand, all the 5 Salmonella isolates were positive for enterotoxin (stn, 617 bp) and invasin (invA, 941 bp) genes but none were positive for pef gene (Table 2, Fig. 1).

Serotyping of Salmonella isolates
Of the 5 isolates, one was identified as Salmonella Daarle (6B:3y:y:exy) and four were identified as Salmonella Hiduddify (6B:8:1.13:z28:1.5).

Discussion
Pathogenic E. coli is a common agent responsible for a variety of intestinal disorders, such as diarrhea and edema disease syndrome in pigs (Kim et al., 2010). Majority of the diarrheal diseases of piglets caused by E. coli are categorized under STEC/VTEC or EPEC or ETEC or EHEC (Kim et al., 2010; Vu-Khac et al., 2006). During the present study, all the E. coli isolated from diarrhoeic and healthy pigs of the farm were found to be avirulent type based upon the result of PCR on detection of STEC/VTEC, ETEC and EPEC. Based upon the observation, it was presumed that E. coli was not responsible for diarrhea in the piglets under the study.

Neonatal piglet diarrhoea is commonly of viral origin, of which Rotavirus and Picobirnavirus are mainly incriminated. Dubal et al. (2013) reported the presence of Rotavirus in piglet diarrhoea from the North Eastern Hill Region of India from 10.18% by SDS-PAGE to 30.00% by RT-PCR, which is indicative of the role of Rotavirus in piglet diarrhoea in this region. In another study from our laboratory, Picobirnavirus was detected in 4.59% diarrheic and 5.60% healthy pigs from NER India (unpublished data). In the present study, both the organisms were found to be negative, hence ruled out as a possible cause of diarrhoea.

Table 2: Salmonella Daarle and Salmonella Hiduddify isolated from piglets suffering from acute gastroenteritis in an unorganized farm of Nagaland, India.

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Isolate No.</th>
<th>Serovars</th>
<th>Genes detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NG.S5</td>
<td>S. Daarle</td>
<td>stn and invA</td>
</tr>
<tr>
<td>2</td>
<td>NG.S6</td>
<td>S. Hiduddify</td>
<td>invA and stn</td>
</tr>
<tr>
<td>3</td>
<td>NG.S1</td>
<td>S. Hiduddify</td>
<td>invA and stn</td>
</tr>
<tr>
<td>4</td>
<td>NG.S2</td>
<td>S. Hiduddify</td>
<td>invA and stn</td>
</tr>
<tr>
<td>5</td>
<td>NG.S3</td>
<td>S. Hiduddify</td>
<td>invA and stn</td>
</tr>
</tbody>
</table>

During the entire study, only Salmonella Daarle and Salmonella Hiduddify could be detected from the 5 affected piglets in the farm. In addition to that, all the 5 salmonellae isolates were carrying two important virulence genes (invA and stn), which are known to be associated with acute gastroenteritis in man and animals. Based upon the facts, it may be assumed that all the animals were suffering from acute salmonellosis. In a global survey of 104 countries, 3 serovars, viz., S. Enteritidis, S. Typhimurium and S. Typhi were accounted for 76.1% of all Salmonella isolates (Herikstad et al., 2002). In India, Rahman (2002) reported that among all serovars of Salmonella enterica, Salmonella Typhimurium was most commonly associated with enteric infections in man and animals. In another study, Murugkar et al. (2005) reported the detection of 95 isolates of Salmonella enterica belonging to 5 serovars – S. Typhimurium, S. Enteritidis, S. Gallinarum, S. Paratyphi B and S. Bareilly from man and animals including pigs in North Eastern India. Recently, Borah et al. (2013) also recovered 5 Salmonella isolates from 20 diarrhoeic pigs in Assam, India as S. Typhimurium.

Fig. 1. Agarose gel electrophoresis showing the PCR amplicons of stn (617 bp) and invA (941 bp) genes of Salmonella. Lane 1 and 8: 100 bp DNA ladder; Lane 2: Negative control for stn; Lane 3: Positive control (stn gene) (617 bp); Lane 4: Positive sample for stn (617 bp); Lane 5: Positive control for invA gene (941 bp); Lane 6: Positive isolate for invA (941 bp); Lane 7: Negative control for invA gene.

Till date, S. Daarle and S. Hiduddify have very rarely been reported as pathogen causing acute diarrhoea. Salmonella Hiduddify was first isolated from Germany in 1970 from faeces of a 29 years old man (Baider et al., 1972). The serovar was again reported in 1978 from faeces of dogs in Nigeria (Britt et al., 1978), which suggested that these animals may act as a source for transmission of salmonellosis to humans and domestic animals. During October 2006, three confirmed cases of S. Hiduddify were reported in three infants in Los Angeles (USA) hospital. It is believed that animal skins imported from West Africa and used by the father of one infant for making drums were the vehicle of salmonellosis (Acute Communicable Disease Control Program, 2006). The first isolation of S. Hiduddify from chickens was
reported in 2009 from Nigeria (Raufa et al., 2009), where 39 out of 41 samples yielded S. Hiduddify. S. Daarle was first reported in 1987 from human (Rohr and Aleksic, 1987) but since then no official report of isolation of this serovar is available. This is probably the first report of S. Hiduddify and S. Daarle associated with piglet diarrhea.

Enterotoxin (sten) gene is widely distributed among the salmonellae irrespective of their serovars and source of isolation. This gene has been reported to be absent in S. bongori strains and also from other members of Enterobacteriaceae or Vibrio, having enterotoxigenic potential (Prager et al., 1995; Rahman, 1999). invA gene encodes a protein in the inner membrane of bacteria, which is responsible for invasion to the epithelial cells of the host (Darwin and Miller, 2009). In the present investigation, all the Salmonella isolates (100%) were positive for stn and invA genes, which further indicated that these two genes are highly conserved in Salmonella spp. Murugkar et al. (2003) and Borah et al. (2013) also reported S. Typhimurium possessing stn and invA genes most commonly associated with enteric infections in man and animals.

Detection of Salmonella enterica serovars Daarle and Salmonella enterica serovars Hiduddify possessing both virulence genes stn and invA is of major significance, as these serovars have never been reported from India. This is also the first report of the involvement of these two Salmonella serovars in piglet diarrhoea. Detection of new serovars in piglet’s diarrhoea is of great concern from public health point of view, as they have been reported previously from humans and chickens.

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Conflict of interest

The authors declared that there is no conflict of interest.

References


