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ORIGINAL RESEARCH

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 (*stn*) 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* hidduddify, India.

Introduction

Pigs are most commonly affected by salmonellae infections from weaning to 4 months of age, of which piglet diarrhoea is very significant (Hur *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. derbyand S. enteritidis* are also reported to be associated with enteric infections of pigs (Reed *et al*., 1985; Ikeda *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 (Raufa *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 diarrhea 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 floatation 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; Saifullah *et al*., 2016). All the pure isolates were stored in glycerol at -80ºC 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; Herring *et al*., 1982).

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

Target gene	Amplicon size (bp)	Reference
stn	617	Prager*et al.* (1995)
invA	941	Galan *et al.* (1992)
pef	700	Rahman*et al.* (2000)
eae	384	Paton and Paton(1998)
stx1	180	Paton and Paton (1998)
stx2	255	Paton and Paton (1998)
hlyA	534	Paton and Paton (1998)
LT	450	Phipps *et al.* (1995)
ST	190	Phipps *et al.* (1995)
VP7, Rota A	304	Husain *et al.* (1995)
VP6, Rota C	356	Gabbay*et al.* (2008)
GG1, (PBV)	201	Rosen *et al.* (2000)
GGII, (PBV)	369	Smits *et al.* (2011)

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 (*stx*$_1$, *stx*$_2$), EHEC (*hly*A) and ETEC (*lta*, *sta* and *stb*) was evaluated by multiplex PCR (Paton and Paton, 1998). Detection of *stn* (Prager *et al*., 1995), *invA* (Galan *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 1X 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^{0}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^{0}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*, *stx*$_1$, *stx*$_2$, *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 (6,8:y:enx) and four were identified as *Salmonella* Hidduddify (6,8:1,z13,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 Hilly 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.

Sl. No.	Isolate No.	Serovars	Genes detected
1	NG.S5	*S.* Daarle	*stn* and *invA*
2	NG.S6	*S.* Hiduddify	*stn* and *invA*
3	NG.S1	*S.* Hiduddify	*stn* and *invA*
4	NG.S2	*S.* Hiduddify	*stn* and *invA*
5	NG.S3	*S.* Hiduddify	*stn* and *invA*

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.

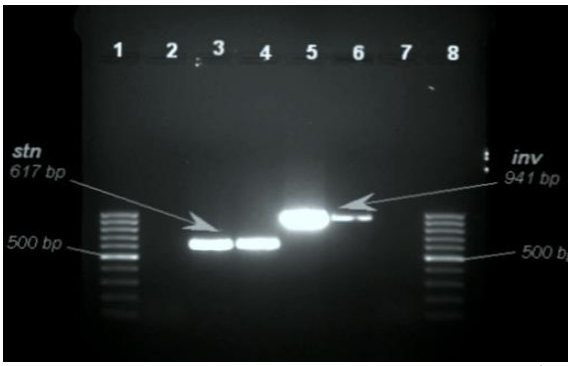

Fig. 1. Agarose gel electrophoresis showing the PCR amplicons of *stn* (617 bp) and *invA* (941 bp) genes of *Salmonella.* Lane 1 and 8:100 bp DNA ladder; Lane 2: Negative control for *stn*; Lane 3: Positive control (*stn* gene) (617 bp); Lane 4: Positive sample for *stn* (617 bp); Lane 5: Positive control 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 feces of a 29 years old man (Bader *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 (*stn)* 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.

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