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Salmonella enterica serovar Miami Possessing Both Virulence and Extended-Spectrum β-Lactamase Resistant Genes Isolated from Diarrhoeic Piglets of North East India (Mizoram)

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A B S T R A C T

The present study report findings of an acute gastroenteritis outbreak in an unorganized pig farm in the tribal area of North East India (Mizoram). Faecal samples were collected from 12 pigs including 2severely diarrhoeic piglets. A total of 25 *E. coli* were isolated, all of which were found to be negative for any putative virulence genes of STEC, ETEC, EHEC and EPEC pathotypes. However, no samples were found to be positive for *Clostridium* spp, viral (*Rotavirus*, *Picobirna virus*) and parasitic pathogens (eggs or larvae) under the study. *Salmonella* isolates (n=2) were isolated and confirmed as *Salmonella enterica* serovar Miami (9,12:a:1,5).PCR amplification of the genes in study showed present of enterotoxin (*stn*), invasive (*invA*) as well as ESBL bla_{TEM-1} and bla_{CMY-2} genes. Analysis of the strains by RAPD-PCR indicates that the strains were genetically different from other *Salmonellae*. The resistance trait from the isolates could not be transferred to the recipient host by the conjugation method. The isolated *Salmonella* strains were found to be multi drug resistant (MDR) strains. The present study report the isolation of *Salmonella* Miami strains possessing both virulence and ESBL genes, from severely diarrhoeic piglets in the tribal area of North East India (Mizoram).

Introduction

Salmonellosis is one of the major causes of acute gastroenteritis around the world and there are more than 2579 serovars of *Salmonella* that has been recorded across the globe (World Health Organization, 2007). Among the serovars, most commonly isolated from human are *S. typhimurium* and *S.* Enteritidis, whereas, most common serovars of animals origin are *S. typhimurium* and *S.* Newport (CDC, 2006). *Salmonella* spp. are usually susceptible to many antimicrobial agents especially to the third generation cephalosporins such as cefotaxime, ceftriaxone and ceftazidime but a recent increase in the rates of resistance to these drugs is of serious concern globally particularly in developing countries. The present investigation reports the isolation of rare *Salmonella* strains possessing both virulence and ESBL genes, from severe

diarrhoeic piglets of an unorganized farm in the tribal area of North East India (Mizoram).

Materials and Methods

Collection of fecal samples

The fecal samples were collected from pigs from an outbreak of acute gastroenteritis in two unorganized pig farms of Mizoram, India during 2014. Samples (n=12) were collected from all the animals including 2 severely diarrhoeic piglets, and transported to laboratory under cold chain.

Bacteriological screening of clinical specimens

All the samples were processed for isolation and identification of possible enteric bacteria including *E. coli, Salmonella* and *Clostridium* spp.as per the method described by Ewing (1986) and also for detection of enteric viruses, including *Rotavirus* and *Picobirna virus* by RNA-PAGE and RT-PCR. Samples were also examined for the presence of parasitic eggs and larvae by standard floatation technique.

Screening of samples for parasites

All the faecal samples were tested for presence of common parasitic eggs by standard floatation technique.

Detection of selected viral pathogens

The faecal samples were screened for the presence of *Rotavirus* and *Picobirna virus* by RNA-PAGE analysis following standard protocol. In brief, samples were diluted in phosphate buffered saline (pH 7.4) to prepare a 10% (w/v) faecal 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). *Rotavirus* and *Picobirna virus* was also detected by reverse transcription-PCR (RT-PCR). Detection of *Rotavirus* group A and C was performed by targeting VP7 gene (designed primers) and VP6 gene (Gabbay *et al.,* 2008), respectively. For detection of *Picobirna virus* genogroup I (Rosen *et al.,* 2000) and genogroup II specific primers (Smits *et al.,* 2011) were used.

Molecular characterization of the isolates

DNA lysate for PCR analysis was prepared by standard boiling and snap chilling method. Detection of putative virulence genes of EPEC (*eaeA*), STEC (stx_1, stx_2), EHEC (*hly*A) was evaluated by multiplex PCR (Paton and Paton 1998) and ETEC (*LT* and *ST*) by Phipps *et al.,* (1995). *Salmonella* isolates was subjected to analysis by specific PCR for presence of putative virulence genes *stn* (Prager *et al.,* 1995), *invA* (Galan *et al.,* 1992), *pef* (Rahman *et al.,*2000) and ESBL resistant genes bla_{TEM} (Weill *et al.,* 2004), bla_{SHV} (Schjorring *et al.,* 2008), bla_{CMY-2} and bla_{CTX} (Perez and Hanson, 2002)as depicted in Table 1. PCR amplification was performed in a Master cycler gradient (Eppendorf, Germany) with PCR mixture in a thin walled 0.2ml PCR tube included a final volume of 25 µl containing 10X Dream taq buffer with $MgCl_2$ (20mM), dNTP mix (25 mM each), 5 U/µl Taq DNA polymerase, 1µl (20 pmol) each of forward and reverse primer, 4µl of template DNA and nuclease free water to make up the volume 25µl.Amplified products were separated by 1% agarose gel in 1X Tris-borate-EDTA buffer by electrophoresis and stained with ethidium bromide (0.5µg/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).

Randomly amplified polymorphic DNA-PCR for molecular typing of the isolates was performed using a single primer (5'-AAGAGCCCGT-3') according to Akopyanz *et al.,* 1992.Conjugation was carried out using the method of Hasman *et al.,* (2005). A plasmid-free, amoxycillin-susceptible, but nalidixic acid and rifampin-resistant *Salmonella enterica* serovar *Typhimurium* isolate was the recipient for mating experiments. Trans conjugants were selected on LB agar plates containing ampicillin (32µg/ml) and nalidixic acid (50µg/ml).

Serotyping of *Salmonella* isolates

The *Salmonella* isolates were serotyped on the basis of their somatic antigen at National Salmonella and Escherichia Centre, Central Research Institute, Kasauli, Himachal Pradesh (India).

Antimicrobial sensitivity assay

The *Salmonella* isolates were subjected to *in vitro* drug sensitivity test by disc diffusion method against 15 commonly used antibiotics (Hi-Media): ampicillin (10mcg), amoxycillin (30mcg), aztreonam (30mcg), cefalexin (30mcg), ceftazidime (30mcg), cefixime (5mcg), ceftriaxone (30mcg), cefotaxime (30mcg), ciprofloxacin (5mcg), enrofloxacin (10mcg), gentamicin (10mcg), imipenam (10mcg), nalidixic acid (30mcg), piperacillin (10mcg) and streptomycin (10mcg). Antimicrobial susceptibility test was done on Mueller-Hinton agar (Hi-Media) plate as per criteria of Clinical Laboratory Standard Institute (CLSI, 2013). Zones of inhibition were measured using zone size interpretative chart furnished by the manufacturer.

Results and Discussion

No clostridial organisms were detected by anaerobic culture. *Salmonella* isolates (n=2) were recovered from 2 severely diarrhoeic piglets based on standard bacteriological and biochemical tests. All the *E. coli* (n=25) isolated from the samples were found to be negative for any putative virulence genes *eaeA, stx*$_1$*, stx*$_2$*, hlyA, la* and *st* genes by PCR. On laboratory examination as stated earlier, no samples were found to contain parasitic eggs or larvae. *Rotavirus* and/or *Picobirna virus* also could not be detected by either RNA-PAGE or by RT-PCR.

Both the two *Salmonella* isolates were serotyped/assigned as *Salmonella enterica* serovar Miami (9, 12: a: 1, 5).

PCR amplification of the genes in study revealed the strains yielded a 617 bp product in the enterotoxin (*stn*) gene segment and presence of invasive (*invA*) gene which yielded amplicon size 941 bp. Both the isolates were found negative for *pef* gene.

Furthermore, of the four ESBL genes tested by PCR, the isolates were found to harboured drug resistance ESBL -*bla*$_{\text{TEM-1}}$(1080 bp) and *bla*$_{\text{CMY-2}}$(462 bp) genes (Fig. 1).

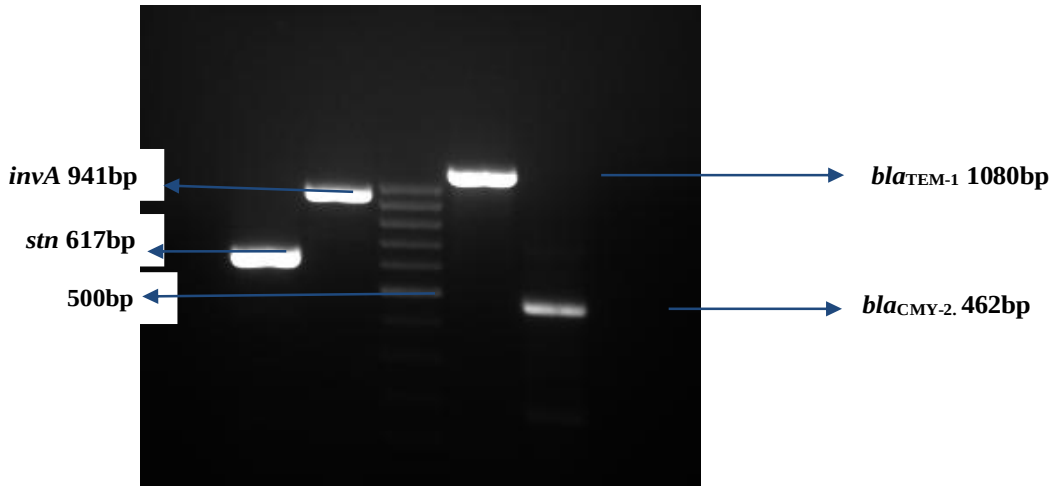
Analysis of *S.* Miami strains of the present study by RAPD-PCR revealed similar bands between them, but when comparing with other *Salmonella* spp. revealed unidentical banding patterns which indicate that the strains were genetically different from other *Salmonellae.* The resistance trait from the isolates could not be transferred to the recipient host by conjugation method.

The isolated *Salmonella* strains were found to be multi drug resistant (MDR) strains revealing resistant to ampicillin, amoxycillin, cephalexin, enrofloxacin, piperacillin, cefixime, cefotaxime and showing sensitivity to imipenem, ceftriaxone, aztreonam, streptomycin, nalidixic acid, gentamicin, ciprofloxacin and ceftazidime.

Table.1 Details of the oligonucleotide primers used in the present study

Primer	Sequences (5'- 3')	Annealing temp.	Amplicon size (bp)	References
stn	(F) TTGTGTCGCTATCACTGGCAACC (R) ATTCGTAACCCGCTCTCGTCC	61^{0}C	617	Prager *et al.*, 1995
invA	(F) ACCACGCTCTTTCGTCTGG (R) GAACTGACTACGTAGACGCTC	60^{0}C	941	Galan *et al.*, 1992
pef	(F) TGTTTCCGGGCTTGTGCT (R) CAGGGCATTTGCTGATTCTTCC	57^{0}C	700	Rahman *et al.*, 2000
***bla*$_{TEM}$**	(F) ATAAAATTCTTGAAGACGAAA (R) GACAGTTACCAATGCTTAATC	53^{0}C	1080	Weill *et al.*, 2004
***bla*$_{SHV}$**	(F) CGCCTGTGTATTAATCAGTGAGGCAC (R) TTGCCAGTGCTCGATCAGCG	60^{0}C	842	Schjorring *et al.*, 2008
***bla*$_{CMY-2}$**	(F) TGGCCAAGAACTGACAGGCAAA (R) TTTCTCCTGAACGTGGCTGGC	60^{0}C	462	Perez and Hanson, 2002
***bla*$_{CTX-M}$**	(F) CAATGTGCAGCACCAGTAA (R) CGCGATATCGTTGGTGGTG	58^{0}C	540	Perez and Hanson, 2002

Fig.1 *Salmonella* Miami positive for *stn* (617bp), *invA* (941bp), *bla*$_{TEM-1}$ (1080bp), *bla*$_{CMY-2}$ (462bp) genes



Resistance to cephalosporin drugs and detection of such resistance genes from resistant *Salmonella* isolates in food animals are to be associated with human disease (Blanc *et al.*, 2006).

The two putative virulence genes of *Salmonella* spp. viz., Enterotoxin (*stn*) and invasion (*invA*) are extensively present in *Salmonella* spp. irrespective of various serovars; and many workers considered molecular characterization of *invA* gene as a standard for detection of *Salmonella* genus (Prager *et al.*, 1995; Rahman, 1999; Malorny *et al.*, 2003). *Salmonella Typhimurium* is most commonly associated with enteric infections in man and animals (Rahman, 2002; Murugkar *et al.*, 2005). In another report, *S. enteritidis*, *S. typhimurium* and *S. typhi* are the main serovars consisted of 76.1% of all isolates reported from 104

countries (Herikstad *et al.*, 2002). Isolation of *Salmonella* Miami is of significant as this serovar has been rarely reported in India and this is the first published report of the involvement of *Salmonella* Miami in piglet diarrhoea in India.

Detection of *Salmonella* strains with simultaneous combination of virulence and ESBL resistance genes mentioned in present research article is a cause of public health concern, and it indicate the nature of continuous selective pressure which promote combinations of virulent and resistant strains. Hence, regular monitoring for *Salmonella* spp. in food animals is very much necessary which must include adequate antimicrobial susceptibility testing and molecular characterization.

There are reports around the globe of *Salmonella* spp. possessing either virulence or ESBLs genes, but to the best of our knowledge, this is probably the first information in India or elsewhere of ESBL-producing *Salmonella* isolates possessing virulence genes belonging to *Salmonella enterica* serovar Miami (9, 12: a: 1, 5).

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Competing Interests

Authors have declared that no competing interests exist.

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