

Extended Spectrum and AmpC Beta-lactamase Producing *Salmonella* rough Strains Associated with Gastroenteritis in Piglets of Meghalaya (India)

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Short Research Article

ABSTRACT

Aim: An investigative study for gastroenteritis in unorganised private pig farms of Meghalaya, a North East State of India.

Materials and Methods: Faecal samples were collected from 32 pigs including severely diarrhoeic piglets and were processed for detection of *Escherichia coli*, *Salmonella*, *Clostridium* spp., *Rotavirus*, as well as parasitic eggs and larvae. The isolates were screen by polymerase chain reaction (PCR) for rotavirus and the presence of virulence associated genes of *E. coli* and *Salmonella*. An antibiotic sensitivity test was performed on isolated *Salmonella* and screened for the presence of drug resistance beta-lactamase genes.

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Results: A total of 40 *E. coli* were isolated, all of which were found to be negative for any putative virulence genes of shigatoxigenic *E. coli*, enterotoxigenic *E. coli*, enterohaemorrhagic *E. coli* and enteropathogenic *E. coli* pathotypes. From severely diarrhoeic piglets, four salmonellae were isolated, which were recorded as *Salmonella* Rough strains. All the 4 isolates detected positivity for ESBL (*bla*_{TEM}) and ampC β -lactamase (*bla*_{CMY-2}) genes. However, no samples were found to be positive for viral and parasitic pathogens. Antibiotic susceptibility test revealed the *Salmonella* strains were resistant to commonly used drugs, such as enrofloxacin, cefalexin, tetracycline, amoxycillin, ampicillin and aztreonam. RAPD-PCR revealed the homogeneity of the strains associated with *Salmonella* infection in the area.

Conclusion: The study indicated the involvement of *Salmonella* Rough strains with piglet diarrhoea in unorganised farms of Meghalaya. Detection of *Salmonella* possessing antibiotic resistance genes in food animal warrants immediate attention to hygienic practices and regulated use of antibiotics.

Keywords: *Salmonella*; drug resistance; PCR; *bla*_{TEM}; *bla*_{CMY-2}.

1. INTRODUCTION

Salmonellosis is one of the major bacterial diseases affecting both human and livestock species, and porcine due to their scavenging habit are one of the most affected animal species. *Salmonella* species are important bacterial agents causing many manifestations of enteric and septicaemic disease affecting particularly young ones [1]. So far, there are more than 2,579 serovars of *Salmonella* that have been recorded with *S. enterica* subspecies *enterica* contains almost all important pathogenic *Salmonella* serovars [2,3,4]. Among all serovars, the most common serovar of animal origin is *S. typhimurium* followed by *Salmonella newport* whereas serovars most commonly isolated from human are *S. typhimurium* and *S. enteritidis* [5]. There are many factors contributing to the degree of severity of salmonellosis, most importantly the host condition and the *Salmonella* serovar involved. Infections in several host species have been attributed to the acquisition and expression of virulence genes linked to a combination of many chromosomal and plasmid factors [6,7,8].

ESBL-producing strains represent an enormous therapeutic challenge as they are resistant to currently available beta-lactam antibiotics except for cephamycins and carbapenems groups [9]. AmpC β -lactamases or class C beta-lactamases, the intrinsic cephalosporinases found on the chromosome of many gram-negative bacteria are enzymes which confer resistance to penicillins and to first, second, and third generation cephalosporins, including β -lactam. A growing number of AmpC enzymes have “escaped” onto plasmids; termed as acquired or plasmidic AmpCs, with CMY-2 being the most common cephalosporinase [10]. *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 study reports the isolation of *Salmonella* rough strains possessing ESBL and ampC β -lactamase genes; from severe diarrhoeic piglets of unorganised farms in the tribal area of North East India of Meghalaya.

2. MATERIALS AND METHODS

2.1 Collection of Fecal Samples

The fecal samples were collected from piglets and growers (3-10 weeks old) suffering from gastroenteritis in unorganised pig farms of Meghalaya, India during the winter season of January-February 2018. Faecal samples (n=32) were collected in sterile swaps and containers from all the animals including five emaciated diarrhoeic piglets. The samples were transported to the laboratory under cold chain and immediately processed for a screening of bacterial pathogens. However, samples for viral detection were preserved at -20°C for further processing.

2.2 Bacteriological, Virological and Parasitic Screening of Clinical Samples

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 [11]. All the faecal samples were examined for the presence of parasitic eggs and larvae by standard

floatation technique. The faecal samples were also processed for RNA extraction for the screening of *Rotavirus*. In brief, samples were diluted in phosphate buffered saline (pH 7.4) to prepare a 10% (w/v) faecal suspension. The clarified supernatant was collected and processed for RNA extraction using Trizol method [12]. Reverse transcription PCR (RT-PCR) targeting VP7 gene (designed primers) and VP6 gene [13] were used for detection of *Rotavirus* in the samples.

2.3 Molecular Characterisation of the Isolates

Detection of putative virulence genes of EPEC (*eaeA*), STEC (*stx1*, *stx2*), EHEC (*hlyA*) was evaluated by multiplex PCR according to [14] and ETEC (*LT* and *ST*) by [15]. *Salmonella* isolates were subjected to analysis by specific PCR for the presence of putative virulence genes *stn* [16], *invA* [17], and ESBL resistant genes *bla_{TEM}* [18], *bla_{SHV}* [19], *bla_{CMY-2}* and *bla_{CTX-M}* [20] as depicted in Table 1. PCR amplification was

performed in a Master cycler (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 MgCl₂ (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 and photographed in a gel documentation system (Vilber Lourmat, Gel Imaging system E Box CX5, France).

Molecular typing of the isolates was also performed by randomly amplified polymorphic DNA-PCR (RAPD-PCR) using a single RAPD primer (5'-AAGAGCCCGT-3') according to the method of [21].

Table-1. PCR amplification of target genes
Please insert ref. no. for below highlighted refs.

Target gene	Sequence (5' – 3')	Annealing	Amplicon size (bp)	Reference
stn	(F) TTGTGTCGCTATCACTGGCAAC (R) ATTCGTAACCCGCTCTCGTCC	60°C, 1m	617	Prageret al. (1995)
invA	(F) ACCACGCTCTTTCGTCTGG (R) GAACTGACTACGTAGACGCTC	61°C, 1m	941	Galan et al. (1992)
eae	(F) GACCCGGCACAAGCATAAGC (R) CCACCTGCAGCAACAAGAGG	61°C, 45s	384	Paton and Paton(1998)
stx1	(F) ATAAATCGCCATTCTGTTGACTAC (R) AGAACGCCCACTGAGATCATC	61°C, 45s	180	Paton and Paton(1998)
stx2	(F) GGCAGTGTCTGAACTGCTCC (R) TCGCCAGTTATCTGACATTCTG	61°C, 45s	255	Paton and Paton(1998)
hlyA	(F) GCATCATCAAGCGTACGTTCC (R) AATGAGCCAAGCTGGTTAGCT	61°C, 45s	534	Paton and Paton(1998)
LT	(F) GGCGACAGATTATACCGTGC (R) CGGTCTCTATATTCCCTGTT	50°C, 45s	450	Phipps et al. (1995)
ST	(F) ATTTTMTTCTGTATTTRTCTT (R) CACCCGGTACARGCAGGATT	50°C, 45s	190	Phipps et al. (1995)
VP7	TTGACTAARGGRTGGCCAACWGG	56°C, 45s	540	Designed primers
RotaA	TCGCATCATHCKYTCNGTTTGTGG			
VP6	1.CTCGATGCTACTACAGAATCAG	46°C, 30s	356	Gabbay et al. (2008)
RotaC	2.AGCCACATAGTTCACATTTTCATCC			
bla _{TEM}	(F) ATAAAATTCTTGAAGACGAAA (R) GACAGTTACCAATGCTTAATC	53°C, 30s	1080	Weill et al., 2004
bla _{SHV}	(F) CGCCTGTGTATTAATCAGTGAGGCAC (R) TTGCCAGTGCTCGATCAGCG	60°C, 30s	842	Schjorring et al., 2008
bla _{CMY-2}	(F) TGGCCAAGAACTGACAGGCAAA (R) TTTCTCCTGAACGTGGCTGGC	60°C, 45s	462	Perez and Hanson, 2002
bla _{CTX-M}	(F) CAATGTGCAGCACCAAGTAA (R) CGCGATATCGTTGGTGGTG	58°C, 45s	540	Perez and Hanson, 2002

Table-2: Antimicrobials resistance pattern of *Salmonella* isolated from diarrhoeic faecal samples of piglets (Meghalaya, India)

Antibiotic disc and content	Resistant	Sensitive
Ampicillin (AMP) 10mcg	5 (100)	0 (0)
Amoxycillin (AMX) 30mcg	5 (100)	0 (0)
Aztreonam (AT) 30mcg	5 (100)	0 (0)
Cefalexin (CN) 30mcg	5 (100)	0 (0)
Ceftazidime (CAZ) 30mcg	0 (0)	5 (100)
Cefixime (CFM) 5mcg	0 (0)	5 (100)
Ceftriaxone (CTR) 30mcg	0 (0)	5 (100)
Cefotaxime (CTX) 30mcg	0 (0)	5 (100)
Ciprofloxacin (CIP) 5mcg	0 (0)	5 (100)
Enrofloxacin (EX) 10mcg	4 (80)	1 (20)
Gentamicin (GEN) 10mcg	4 (80)	1 (20)
Imipenem (IPM) 10mcg	0 (0)	5 (100)
Nalidixic acid (NA) 30mcg	3 (60)	2 (40)
Piperacillin (PI) 10mcg	3 (60)	2 (40)
Streptomycin (S) 10mcg	0 (0)	5 (100)
Tetracycline (T) 10mcg	5 (100)	0 (0)

Note: Percentage (%) is given in parentheses

2.4 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).

2.5 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), imipenem (10mcg), nalidixic acid (30mcg), piperacillin (10mcg), streptomycin (10mcg) and tetracycline (10mcg). Antimicrobial susceptibility test was done on Mueller-Hinton agar (Hi-Media) plate as per criteria of Clinical Laboratory Standard Institute [22]. Zones of inhibition were measured using zone size interpretative chart furnished by the manufacturer.

3. RESULTS AND DISCUSSION

All the five piglets were suffering from acute gastroenteritis with non haemorrhagic diarrhoea and fever. However, the adults were apparently healthy without any clinical signs. Many of the affected piglets have not received any treatment

against any infection till the date of sample collection. There was no report of death of any piglets. On laboratory examination, as stated earlier for detection of parasitic and viral pathogens, it was found that no samples contain parasitic eggs or larvae. *Rotavirus* also could not be detected by RT-PCR. In case of bacterial isolation, no clostridial organisms could be detected by anaerobic culture. A total of 40 *E. coli* were isolated and identified from all the 32 samples under the study. On the other hand, from five samples of diarrhoeic piglets, both *Salmonella* and *E. coli* were isolated and identified based on standard bacteriological and biochemical tests. On molecular detection of bacterial associated virulence genes by PCR; all the *E. coli* isolates were found to be negative for *eaeA*, *stx1*, *stx2*, *hlyA*, *la* and *st* genes.

Serotyping of *Salmonella* isolates revealed that four isolates belonged to Rough strains, however, one isolate could not be typed due to the report of contamination probably during long distance and other factors. PCR amplification of *Salmonella* targeted genes in study revealed that all the four *Salmonella* isolates yielded a 1080bp product in the *bla*_{TEM} and 462bp product in the *bla*_{CMY-2} gene segment (Fig.1). However, *stn* and *invA* gene could not be detected in any of *Salmonella* isolates. All the four rough strains showed identical banding patterns when subjected to RAPD-PCR indicating the homogeneity of the strains associated with *Salmonella* infection in the area.

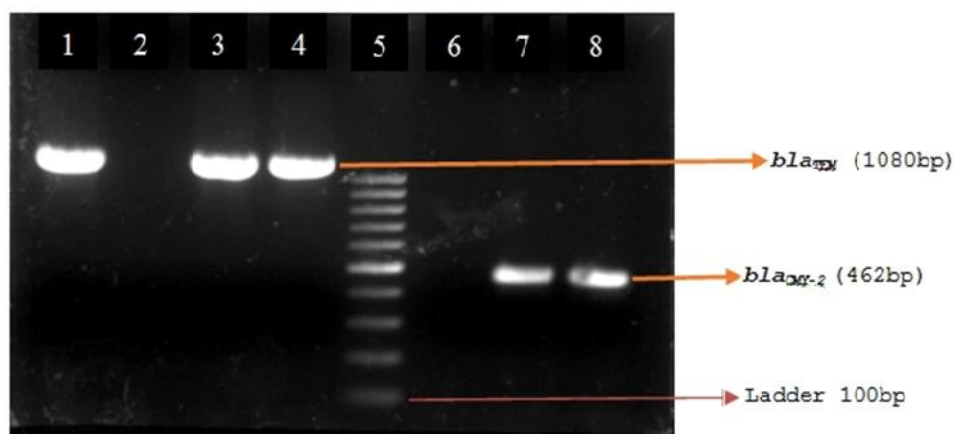


Fig. 1. Agarose gel electrophoresis showing the PCR amplicons of *Salmonella* rough strains
 Lane 1, 3, 4: Positive field isolates (1080bp, *bla*_{TEM});
 Lane 7 and 8: Positive field isolates (462bp, *bla*_{CMY-2})
 Lane 2 and 6: No template control;
 Lane 5: 100 bp DNA ladder

Majority of *E. coli* strains are classified as shigatoxigenic/verotoxigenic, enterotoxigenic, enteropathogenic, enterohaemorrhagic strains causing manifestation of many gastrointestinal disorders in livestock and human [23,24]. During the present investigation, the *E. coli* isolated from diarrhoeic and healthy pigs of the farm were found to be avirulent type based upon the result of PCR detection. Hence, it was presumed that *E. coli* was not responsible for diarrhoea in the piglets under the study. Neonatal piglet diarrhoea is also commonly of viral origin, of which *Rotavirus* is mainly incriminated. In a study of the prevalence of *Rotavirus* in North East India [25] reported upto 30% prevalence by RT-PCR, which indicate the main role of *Rotavirus* in piglet diarrhoea in this region. In the present report, all the samples were negative for *Rotavirus* and hence ruled out as a possible cause of gastroenteritis.

According to the European Food Safety Authority, salmonellosis was the second most commonly reported zoonotic infection in the European Union with *S. enteritidis* as the most frequently isolated serovar [26]. Approximately 50% of *Salmonella* infections are caused by three *Salmonella* serovars, these are Typhimurium, Enteritidis and Newport. In another research from pigs in Northeastern India, ninety five isolates of *Salmonella* enteric were isolated and characterised to five serovars viz., *S. typhimurium*, *S. enteritidis*, *S. gallinarum*, *S. paratyphi B* and *S. bareilly* [27]. In another study, five *Salmonella* isolates reported as *S.*

typhimurium were recovered from 20 diarrhoeic pigs in Assam, India [28]. In a report by Kylla et al. [29] from Mizoram state showed ESBL (*bla*_{TEM-1}) and ampC (*bla*_{CMY-2}) producing *Salmonella miami* were detected which also possessed virulence associated enterotoxin and invasive genes. However, in the present investigative study, all the four *Salmonella* isolates belong to Rough strains and were carrying drug resistance *bla*_{TEM} and *bla*_{CMY-2} gene. Hence based on cultural and molecular findings, it may be assumed that the piglets were suffering from acute salmonellosis due to *Salmonella* rough strains and non-responsive to the drugs administered such as enrofloxacin, cefalexin, tetracycline, ampicillin and amoxycillin.

Serotyping is one of the most commonly used methods for subtyping of *Salmonella*, which enable the source attribution and reveal the source of outbreaks. The traditional approach of serotyping is done by slide agglutination using antisera aiming at two cell surface antigens: somatic (O) lipopolysaccharides (LPS) and flagellar (H). Some *Salmonella* strains have incomplete LPS structures, referred to as rough strains, or do not express H antigens, therefore serotyping by agglutination cannot be performed on these isolates [30]. Lipopolysaccharides (LPS) are characteristic components of the cell wall of Gram-negative bacteria; they are not found in Gram-positive bacteria. They are localised in the outer layer of the membrane and are, in noncapsulated strains, exposed on the cell surface. They contribute to the integrity of the

outer membrane and protect the cell against the action of bile salts and lipophilic antibiotics [31,5]. Amplification of *invA* gene now has been recognised as an international standard for detection of *Salmonella* [32]. This gene encodes a protein in the inner membrane of bacteria which is responsible for invasion to the epithelial cells of the host [33].

All the *Salmonella* isolates in the present study showed resistance to antimicrobial drugs used commonly in the state/region including cefalexin, amoxycillin, ampicillin, enrofloxacin, piperacillin, tetracycline and aztreonam; but none of the isolates were resistance to ceftazidime, cefixime, ceftriaxone, cefotaxime, ciprofloxacin, streptomycin and imipenem (Table 2). High level of drug resistance to ampicillin, amoxycillin, tetracycline, cefalexin and enrofloxacin is alarming as these are commonly used among the livestock farmers in Meghalaya state to treat gastroenteritis infection in livestock and poultry. Regular surveillance of emerging antimicrobial resistance among zoonotic food-borne pathogens is utmost required to ensure safe public health.

Resistance to antimicrobials has emerged as a greater problem in human and veterinary medicine, which were mainly due to the production of extended spectrum betalactams (ESBLs) which have been increasingly detected in food animals and animal products and have gained widespread attention [34]. Antimicrobial resistance among diarrhoeagenic bacteria, especially the production of ESBLs, has been rising worldwide since the early 1990s [35]. In the present study, *Salmonella* rough strains harboured ESBL and ampC genes (*bla*_{TEM} and *bla*_{CMY-2}), which may be due to the wide use of extended-spectrum cephalosporins in the region, or it may be associated with the high mobilisation of the encoding genes [36,37]. Detection of *Salmonella* strain with drug resistance ESBL and ampC β -lactamase marker genes is suggestive of the nature of selective pressure that promotes resistant strains. The finding related to multidrug resistance is alarming since the drugs resistant in the present study were used frequently and effectively in the past for treatment of salmonella related gastroenteritis infection.

The factors contributing to *Salmonella* infection might be due to introduction of organisms through the introduction of infected animals from across the border and also indicated towards poor hygienic and managerial practices

prevailing in unorganised farms. Regular monitoring for *Salmonella* in food animals is very much necessary which must include adequate antimicrobial susceptibility testing and molecular characterisation.

4. CONCLUSION

Pathogenesis of salmonellosis is highly complex, and hence, its control programme is an enormous challenge to public health due to various serovars which may emerge and re-emerge in different geographical areas. Detection of *Salmonella* rough strains in the present study are of great zoonotic importance and a threat to public health, as well as animal production due to the presence of ESBL and ampC β -lactamase genes. Therefore, a comprehensive strategy involving regular surveillance at state and national level, preventive and proper treatment with judicious use of antibiotics should be initiated in controlling the disease.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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