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Short communication

Prevalence and molecular characterization of *Salmonella* species associated with piglet diarrhea in North East India

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Abstract

Salmonellosis is a public health concern worldwide and also causes huge losses to the piggery industry. A total of 457 fecal samples were collected from organized and unorganized farms including indigenous and crossbreed piglets of North East India. *Salmonella* isolates were serotyped, screened for their virulence genes, characterized for drug resistance pattern and representative isolates were cloned and sequenced for their partial length enterotoxin (*stn*) gene. A total of 8.31% *Salmonella* were identified with higher prevalence observed in unorganized compared to organized farms and higher detection level in cross breed compared to indigenous piglets. *Salmonella* typhimurium (65.78%) was found to be the predominant serovar and irrespective of serovars high number of isolates (68.4%) harboured enterotoxin gene. The isolates were multidrug resistant showing highest resistance against cefalexin (77.31%). Sequence analysis of *stn* gene showed two isolates having diverse sequence compared to other isolates. Our study revealed the significance of *Salmonella* as important pathogen with zoonotic potential between porcine and human populations. This is probably the first systematic study of *Salmonella* species associated with piglet diarrhea in India.

Key words: *Salmonella*, enterotoxin, sequencing, drug resistance, piglets

Introduction

India has a total of 10.29 million pig population in which the North East Region alone accounted for 3.95 million pigs in the country (19th Livestock census 2012). Salmonellosis is one of the economically important diseases associated with various disease symptoms, morbidity and in some cases heavy mortality in farm animals (Malkawi and Gharaibeh 2004). The World Health Organization have recorded more than 2,579 serovars of *Salmonella* species across various countries of the globe (WHO 2007) and among these, the most common serovar of non-human origin is *S.* typhimurium followed by *S.* newport.

The attributing factor of enteric infection in many host species is mainly due to acquisition and expression of associated virulence genes (Pfeiffer et al. 2012). Widespread drug-resistance mediated by plasmid

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Table 1. Antimicrobial resistance pattern of *Salmonella* isolated from piglets.

Antibiotic disc and content	Sensitive (%)	Resistant (%)
Ampicillin (AMP) 10mcg	22 (57.89)	16 (42.1)
Amoxycillin (AMX) 30mcg	19 (50.0)	19 (50.0)
Aztreonam (AT) 30mcg	37 (97.36)	1 (2.63)
Cefalexin (CN) 30mcg	9 (23.68)	29 (77.31)
Ceftazidime (CAZ) 30mcg	32 (84.21)	6 (15.78)
Cefixime (CFM) 5mcg	32 (84.21)	6 (15.78)
Ceftriaxone (CTR) 30mcg	38 (100)	0 (0.0)
Cefotaxime (CTX) 30mcg	38 (100)	0 (0.0)
Ciprofloxacin (CIP) 5mcg	38 (100)	0 (0.0)
Enrofloxacin (EX) 10mcg	23 (60.52)	15 (39.47)
Gentamicin (GEN) 10mcg	35 (92.1)	3 (7.89)
Imipenem (IPM) 10mcg	38 (100)	0 (0.0)
Nalidixic acid (NA) 30mcg	35 (92.1)	3 (7.89)
Piperacillin (PI) 10mcg	20 (52.63)	18 (47.36)
Streptomycin (S) 10mcg	38 (100)	0 (0.0)

encoded genes in *Salmonella* has been reported worldwide and in past few decades, various countries have witnessed a significant increase in drug resistant salmonellae of human as well as animal origin (Izumiya et al. 2001). Compared to other livestock and human, limited studies of *Salmonella* from porcine species have been published across the globe. Keeping in view the importance of the *Salmonella* infection, our research was conducted to study the prevalence, drug resistance profile and molecular characterization of *Salmonella* species from piglets of North East India.

Materials and Methods

A total of 457 fresh fecal samples were collected from piglets under 9 weeks of age from organized (n=225) and unorganized (n=232) farms of North East states of India, viz., Manipur (n=108), Meghalaya (n=124), Mizoram (n=120), and Nagaland (n=105) during 2013-15. Samples were collected from diarrhoeic (n=339) and non-diarrhoeic (n=118) piglets including indigenous (n=130) and cross breed (n=327) piglets. *Salmonella* organisms were isolated and identified on the basis of cultural, morphological and biochemical reactions (Ewing 1986) and were serotyped on the basis of their somatic antigen at National Salmonella and Escherichia Centre, Kasauli, HP (India) and at National Salmonella Centre, IVRI, Bareilly, India.

Detection of *Salmonella* enterotoxin, *stn* (Prager et al. 1995), invasive, *invA* (Galan et al. 1992) and plasmid efficacy fimbrial, *pef* (Rahman et al. 2000) was carried out by specific PCR assay performed in a Mastercycler Gradient cycler (Eppendorf, Germany) and PCR products were separated on 1.5% agarose gels, and analyzed using gel documentation system (AlphaImager, USA). Antimicrobial susceptibility test against 15 antibiotics was performed on Mueller-Hinton agar plate according to criteria of Clinical Laboratory Standards Institute (CLSI 2015) and *Salmonella* Enteritidis ATCC 13076 was used as control organism. Extracted PCR products were cloned using InsTAclone (Thermo Scientific) and sequencing was performed at the DNA sequencing facility, University of Delhi (India) using sequencer Excel Applied Biosystem 3730 (USA). Sequencing data were analyzed using MegAlign program. Phylogenetic and bootstrap analyses were performed using neighbor joining program.

Results and Discussion

A total of 38 *Salmonella* isolates (8.31%) were recovered from diarrhoeic samples of organized (11; 28.94%) and unorganized farms (27; 71.05%) of the region. The isolates belonged to 5 different serovars in which the most prevalent serovars detected were *S.* typhimurium (25; 65.78%), followed by *S.* hiduddify (4; 10.52%), *S.* miami (3; 7.89%), *S.* infantis (2; 5.26%) and *S.* daarle (1; 2.63%). One isolate was designated as rough strain and 2 isolates could not be serotyped and were designated as *Salmonella* unidentified serotype. In total, 26 isolates were found to be positive for at least one virulence gene with 18 *Salmonella* found to harbour both *stn* and *invA* genes, whereas all the isolates were negative for *pef* gene. Irrespective of serovars, *stn* gene (26/38; 68.42%) was more frequently detected than *invA* gene (14/38; 36.84%).

Table 2. NCBI GenBank Accession number for nucleotide sequences of *stn* gene.

Sl. No.	*Salmonella* Serovar	Isolate No.	Accession numbers
1	*S.* Typhimurium	ML. S1	KR054360
2	*Salmonella* Rough	ML. S2	KR054361
3	*S.* Typhimurium	MZ. S3	KR054362
4	*S.* Typhimurium	MZ. S4	KR054363
5	*S.* Daarle	NG. S5	KR054364
6	*S.* Hiduddify	NG. S6	KR054365
7	*S.* Typhimurium	MN. S9	KR054366

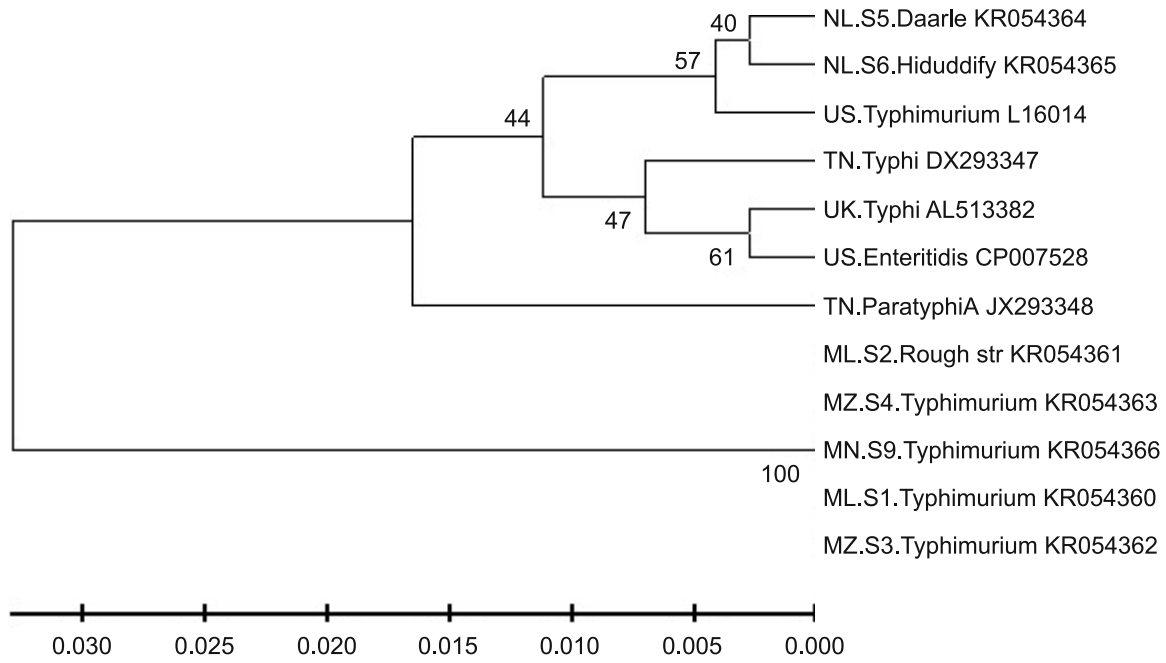

Fig. 1. Phylogenetic analysis of nucleotide sequences of *Salmonella stn* gene.

All the *Salmonella* isolates were found to be multi-drug resistant showing resistance to at least 3 antimicrobials (Table 1), of which cefalexin (77.31%) has the highest level of resistance followed by amoxycillin (50.0%), piperacillin (47.36%) and ampicillin (42.1%). Partial nucleotide sequences of representative isolates (*stn* gene) were designated with GenBank Accession number (Table 2). Phylogenetic analysis of seven enterotoxin (*stn*) gene sequences of the present study with additional 5 sequences from GenBank (Fig. 1), found that sequences of Meghalaya, Mizoram and Manipur states of India (KR054360, KR054361, KR054362, KR054363, KR054366) were separately grouped under one unique clade with 100% sequence identity between them and were not associated with any of the world/Indian isolates. Whereas, 2 sequences from Nagaland, India (KR054364, KR054365) are placed distinctly in a clade along with *S.* typhimurium isolate from USA (L16014).

Isolation of *Salmonella* from feces of human or animals at any level is an indicator of pathological conditions. Many researchers recorded different prevalence rate of *Salmonella* ranging from 2.05% (Shekhar and Singh 2015), through 7.4% (Rabins et al. 2018) to 20.5% (Murugkar et al. 2005). Variation in prevalence may reflex the suitable environment persistence for *Salmonella* and isolation rate of 8.31% in our study shows the presence of suitable ecology for the organism in the region. Prevalence of *Salmonella* was higher in cross- breed (32; 9.78%) than in indigenous pig population (6; 4.61%) which is in corroboration with the study of diarrhoegenic pathogens by Kylla et al. (2017a,b) and Kylla et al. (2018). The variation in isolation rate may be due that the non-descriptive piglets possess better protective nature against natural infection. Higher prevalence of salmonellosis in unorganized compared to organized farms might be due to direct correlation with managemental practices in the region as small private holdings are unable to maintain proper hygienic practices at their own (Kylla et al. 2017b, Kylla et al. 2018).

Salmonella serovars such as Enteritidis and Typ-

himurium has been one of the significant causes of bacterial zoonotic gastroenteritis throughout the world (Pfeiffer et al. 2012). Our finding is in corroboration with results of many scientists who also reported that *S.* typhimurium is the most predominant serovar associated with enteric infection in man and animals, including porcine (Murugkar et al. 2005, Sinwat et al. 2016, Rabins et al. 2018). *Salmonella* hiduddify and *S.* daarle have only rarely been reported around the globe. Hence, our isolation of these serovars along with *S.* miami indicate that these are the emerging serovars being introduced to India by certain sources and maybe through transboundary migration of animals from other regions or neighbouring countries. Also in our study, the recovery of unidentified *Salmonell*a serovars which was rare incidence, indicate that there is an urgent need for a more comprehensive surveillance to monitor the circulating serovars of *Salmonella* species in human as well as in animal population. Enterotoxin gene (*stn*) is an important virulence factor and is widely distributed among the *Salmonella* irrespective of their serovars and source of isolation (Prager et al. 1995, Rahman, et al. 1999). Higher resistance to ampicillin, amoxycillin, cefalexin, enrofloxacin and piperacillin in our study may be due to their indiscriminate usage for treatment of gastroenteritis and other infections in pigs. Presence of virulence and multidrug resistant *Salmonella* species may pose a threat to both food animal and human health care. Phylogenetic analysis of sequences from North East India showed variability and grouping in different clades which suggests their differences in origin, particularly with the two sequences from Nagaland state. This variation might be due to the isolation of newly introduced strains in the region.

Our findings indicate that different *Salmonella* serovars with repertoire of virulence and multidrug resistance are persistently associated with piglet's diarrhea in North East India with sequences showing variability in genetic relationship. The study indicate potential hazard of interspecies sharing of these organisms between porcine and human populations.

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