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REGULAR ARTICLES

Prevalence and antimicrobial resistance of porcine O157 and non-O157 Shiga toxin-producing *Escherichia coli* from India

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Abstract The aims of this study were to determine the prevalence of Shiga toxin-producing *Escherichia coli* (STEC) strains in pigs as a possible STEC reservoir in India as well as to characterize the STEC strains and to determine the antimicrobial resistance pattern of the strains. A total of 782 *E. coli* isolates from clinically healthy (n=473) and diarrhoeic piglets (309) belonging to major pig-producing states of India were screened by the polymerase chain reaction (PCR) assay for the presence of virulence genes characteristic for STEC, that is, Shiga toxin-producing gene(s) (*stx1*, *stx2*), intimin (*eae*), enterohemolysin (*hlyA*) and STEC autoagglutinating adhesin (Saa). Overall STEC were detected in 113 (14.4 %) piglets, and the prevalence of *E. coli* O157 and non-O157 STEC were 4 (0.5 %) and 109 (13.9 %), respectively. None of the O157 STEC isolates carried gene encoding for H7 antigen (*fliCh7*). The various combinations of virulence genes present in the strains studied were *stx1* in 4.6 %, *stx1* in combination with *stx2* gene in 5.1 %, *stx1* in combination with *stx2* and *ehxA* in 0.6 %, *stx1* in combination with *stx2* and *eae* in 0.2 % and *stx2* alone in 3.7 %. All STEC isolates were found negative for STEC autoagglutinating adhesin (Saa). The number of STEC isolates which showed resistance to antimicrobials such as ampicillin, tetracycline, streptomycin, lincomycin, nalidixic acid, sulfadiazine, penicillin, gentamicin, kanamycin and ceftriaxone were 100, 99, 98, 97, 95, 94, 92, 88, 85 and 85, respectively. Ninety-seven isolates showed resistance to more than 2 antimicrobials, and 8 resistance groups (R1 to R8) were observed. This study demonstrates that pigs in India harbour both O157 and non-O157 STEC, and this may pose serious public health problems in future.

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Introduction

Pig farming in India constitutes the livelihood of rural poor belonging to the lowest socio-economic strata. Pig is considered as one of the best meat-producing animals in the world. In India, meat production is now the fastest-growing segment of livestock sector, and the consumption of pork is increasing significantly in the country, particularly in the North Eastern States where country's 40 % pig population exists (Livestock Census 2007).

Escherichia coli O157:H7 and other Shiga toxin (Stx)-producing *E. coli* (STEC) strains are associated with food and waterborne illness around the world (Karmali et al. 2010). *E. coli* O157 has been implicated as the causative agent in several human outbreaks and is the most common cause of hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS). Non-O157 STEC strains have more recently been recognized as important pathogens with an increasing impact on human health and are now also considered to be a major cause of disease (Chase-Topping et al. 2012). The ability of STEC to cause disease is related to the production of one or more Shiga-like toxins (Stx1, Stx2 or their variants), which inhibits protein synthesis of host cells, thus leading to cell death (Growther et al. 2013). In addition to toxin production, another virulence-associated factor expressed by STEC is a protein called intimin (encoded by the chromosomal gene *eae*), which is responsible for the intimate attachment of STEC to intestinal epithelial cells, causing effacing lesions in the intestinal mucosa (Helmy et al. 2013). The large plasmid of STEC O157 carries determinant characteristic for STEC that presumably harbour additional virulence factors: *hlyA* (the enterohemorrhagic *E. coli* hemolysin gene), which

acts as a pore-forming cytolysin on eukaryotic cells (Schmidt et al. 1995). The pathogenic capacity of STEC also resides in virulence factor such as STEC autoagglutinating adhesin (Saa) (Gyles 2007).

STEC strains have been isolated from a variety of animals and healthy cattle, and other ruminants appear to be the main reservoir of STEC strains (Durand et al. 2010). It has been reported that pigs routinely carry STEC and high (9.5 %) prevalence of EC O157 in swine faeces was noted in South Africa (Witold and Carolyn 2011). Pig as a potential reservoir of STEC O157:H7 has also been reported from Japan (Nakazawa et al. 1999). Faecal contamination of the farm environment favours animal reinfection and persistence of the pathogen in the farm. Hence, production units represent a major reservoir for STEC and human infection occurs primarily via faecal contamination of food products or by ingestion of untreated water contaminated with livestock wastes and slurries (Fairbrother and Nadeau 2006). Therefore, to reduce the risk of human infection, it is critical to reduce bacterial population in the live animal at the primary production level before entry to the slaughterhouse.

As *E. coli* infection is one of the common diseases of livestock (including pigs) particularly in tropical countries which have tremendous economic impact on livestock productions and the paucity of information on the prevalence of STEC in pigs in India, the present study was undertaken to determine the prevalence of STEC in healthy and diarrhoeic pigs as well as to characterize and determine the antimicrobial resistance of the STEC isolates.

Materials and methods

Sample collection, culture and STEC screening

Rectal swabs were collected in duplicate from clinically healthy (473) and diarrhoeic (309) piglets (1–8 weeks of age) during the period from February 2010 to May 2013 from organized and unorganized pigs farms located in Assam (251 nos.), Arunachal Pradesh (152 nos.), Meghalaya (192 nos.) and Nagaland (187 nos.) states of India. Rectal swabs were collected from each piglet. Samples were collected directly from the rectum by using individual sterilized cotton swabs to avoid piglet to piglet cross-contamination. All the samples were collected in sterile polypropylene containers, were transported to the laboratory under ice cover and were processed within 4 h.

For isolation of non-O157 STEC, faecal samples were plated directly onto MacConkey agar (Hi Media, Mumbai, India). Following overnight incubation, 10 suspected *E. coli* colonies were tested for the genes encoding Stx1 and Stx2 toxins (*stx1* and *stx2* genes) by polymerase chain reaction (PCR) as previously described (Rey et al. 2003). The resulting STEC isolates were also confirmed biochemically as per the method of Cowan and Steel (1993) and tested for the genes encoding intimin (*eae* gene), enterohaemolysin (*ehxA* gene) and Saa (*saa* gene) as previously described (Sanchez et al. 2009). When isolates from a given sample exhibited similar genetic characteristics in terms of the presence or absence of virulence genes, only one colony was selected and stored until further characterization. Otherwise, when isolates with different genetic characteristics were obtained, one colony of each was selected and stored for further characterization.

For isolation of O157 STEC, the protocol described by De Boer and Heuvelink (2000) was followed. Briefly, each of the rectal swabs was added to 10 ml of modified tryptic soy broth (TSB containing 20 mg/l novobiocin) for enrichment culture and incubated for 6–8 h at 37 °C. Enrichment culture in modified TSB was streaked onto sorbitol MacConkey agar (SMAC) and SMAC containing 0.05 mg/l of cefixime and 1 mg/l potassium tellurite (CTSMAC). The plates were incubated for 18–24 h at 37 °C. Both sorbitol-fermenting and non-fermenting colonies were picked (5–10 from each plate) and identified biochemically. Following overnight incubation, both sorbitol-fermenting and non-fermenting colonies (5–10 from each plate) were tested for the genes encoding O157 and H7 antigens (O157 *rfbE* and *fliCh7* genes) by PCR as previously described (Garcia-Sanchez et al. 2007). The resulting *E. coli* O157 isolates were confirmed biochemically as *E. coli* and tested for the genes *stx1*, *stx2*, *eae*, *ehxA* and *saa* Table 1.

Serotyping

E. coli isolates were serotyped based on their somatic (O) antigens at National Salmonella and Escherichia Centre, Central Research Institute, Kasauli, Himachal Pradesh, India.

Antimicrobial susceptibility test

Antibiotic susceptibility testing was performed by the disk diffusion method using 16 commercially available antimicrobials disks (Table 2) as per the method of the Clinical and Laboratory Standards Institute guidelines (CLSI 2008).

Results

A total of 782 *E. coli* (309 from diarrhoeic and 473 from healthy piglets) were isolated from 782 faecal samples. Overall STEC strains were detected in 113 (14.4 %) piglets (19.4 % from diarrhoeic piglets and 11.2 % from healthy piglets). *E. coli* O157 was isolated from four (0.5 %) piglets, and non-O157 STEC was isolated from 109 (13.9 %) piglets. The STEC isolates were placed into 18 serogroups, and the most predominant serogroup was O5 (Table 1). Gene encoding O157 (O157 *rfbE*) could be detected in all O157

Table 1 Serogroups and distribution of virulence genes among porcine STEC isolates

Serogroup	Number of STEC having virulence gene					
	stx1	*stx2*	*stx1* + *stx2*	*stx1* + *stx2* + *ehxA*	*stx1* + *stx2* + *eae*	*stx1* + *stx2* + *ehxA* + *eae*
O2	3	0	2	0	0	0
O4	0	1	1	0	0	0
O5	5	4	0	0	0	0
O7	0	1	5	0	0	1
O8	3	3	1	1	0	0
O9	0	4	0	1	1	0
O20	1	1	6	0	0	0
O25	1	0	2	0	0	0
O21	1	0	0	0	0	0
O22	0	0	2	0	0	0
O63	2	0	0	0	0	0
O75	1	0	0	0	0	0
O89	1	2	5	0	0	0
O103	1	0	1	0	0	0
O123	2	5	0	0	0	0
O138	3	0	1	0	0	0
O147	0	0	2	1	0	0
O157	1	0	2	1	0	0
UT	8	6	9	1	1	0
R	3	2	1	0	0	0

UT untypeable, *R* rough

Table 2 Antimicrobial susceptibility of porcine STEC isolates (*N*=113)

Antimicrobials used	Number of STEC		
	Sensitive	Intermediately sensitive	Resistant
Amikacin (AK)	78 (69.0)	5 (4.4)	30 (26.5)
Ampicillin (A)	9 (7.9)	4 (3.5)	100 (88.4)
Ceftriaxone (CI)	21 (18.5)	7 (6.1)	85 (75.2)
Chloramphenicol (C)	59 (52.2)	13 (11.5)	41 (36.2)
Chlortetracycline (CT)	82 (72.5)	9 (7.9)	22 (19.4)
Ciprofloxacin (CF)	76 (67.2)	7 (6.1)	30 (26.5)
Enrofloxacin (EX)	79 (69.9)	11 (9.7)	23 (20.3)
Gentamicin (G)	21 (18.5)	4 (3.5)	88 (77.8)
Kanamycin (K)	18 (15.9)	10 (8.8)	85 (75.2)
Lincomycin (L)	10 (8.8)	6 (5.3)	97 (85.8)
Nalidixic acid (NA)	14 (12.3)	4 (3.5)	95 (84.0)
Norfloxacin (NX)	77 (68.1)	8 (7.0)	28 (24.7)
Penicillin (P)	12 (10.6)	9 (7.9)	92 (81.4)
Streptomycin (S)	11 (9.7)	4 (3.5)	98 (86.7)
Sulfadiazine (SZ)	13 (11.5)	6 (5.3)	94 (83.1)
Tetracycline (T)	9 (7.9)	5 (4.4)	99 (87.6)

Figures in the parentheses indicate the percentage

STEC isolates whereas none of the O157 STEC isolates carried gene encoding for H7 antigen (*fliCh7*). *stx1* gene alone was present in 36 (4.6 %) isolates, *stx1* in combination with *stx2* gene in 40 (5.1 %) isolates, *stx1* in combination with *stx2* and *ehxA* in 5 (0.6 %) isolates and *stx1* in combination with *stx2* and *eae* in 2 (0.2 %) isolates. *stx2* gene alone was present in 29 (3.7 %) isolates (Table 1). All the STEC isolates were negative for STEC autoagglutinating adhesin (Saa). Antimicrobial susceptibility of the STEC isolates is presented in Table 2. The number of STEC isolates which showed resistance to antimicrobials such as ampicillin, tetracycline, streptomycin, lincomycin, nalidixic acid, sulfadiazine, penicillin, gentamicin, kanamycin and ceftriaxone were 100, 99, 98, 97, 95, 94, 92, 88, 85 and 85, respectively. The most effective antimicrobials in terms of sensitivity were chlortetracycline (72.5 %), enrofloxacin (69.9 %), amikacin (69 %), norfloxacin (68.1 %) and ciprofloxacin (67.2 %). Antimicrobial resistance patterns of the STEC isolates are shown in Table 3.

Discussion

In the present study, we recorded the prevalence of STEC in 14.4 % pigs, and the prevalence of non-O157 STEC and O157 STEC were 13.9 and 0.5 %, respectively. Several studies have examined the prevalence of STEC in swine. STEC were detected in 22 % pigs from Switzerland (Kaufmann et al. 2006), 30.2 % pigs from Germany (von Muffling et al. 2007), 4.5 % pigs from Argentina (Moredo et al. 2012) and 0.4 % pigs from South Africa (Mohlatlole et al. 2013). The prevalence of *E. coli* O157 in the present study was lower than that reported from the USA (Keen et al. 2006) and Nigeria (Ojo et al. 2010) where it ranged from 1.2 to 3 %, respectively but higher than that reported (present in two of 2,446 samples) in Swedish pigs (Eriksson et al. 2003). There is paucity of information on prevalence of porcine STEC in India. Barman et al. (2008) could isolate three STEC strains from an outbreak of oedema disease in a pig farm in India and all the isolates were untypeable. Isolation of O157 STEC from pigs in India was also reported by Sehgal et al. (2008), but the isolates were not analyzed for their virulence characteristics. The observed differences in the present study could be due to differences in husbandry practices and prevailing climatic conditions which may account for the varied prevalence of STEC from one geographical region to another. Several studies also showed that the prevalence of STEC in animals may vary with the husbandry practices (such as stocking densities, types of feeds offered etc.) as well as the climatic conditions of the area under the study (Gautam et al. 2011; Diaz-Sanchez et al. 2013). Diaz-Sanchez et al. (2013) found that the STEC

Table 3 Antimicrobial resistance pattern of STEC isolates from pigs

Resistance group	Antimicrobial resistance patterns	No. of isolates showing the multidrug resistance
R1	A,CI, CT, G, K, L, NA, P, S, SZ and T	22
R2	A,CI, C, EX, G, K, L, NA, NX, P, S, SZ and T	11
R3	A,CI, EX, G, K, L, NA, NX, P, S, SZ and T	12
R4	AK, A,CI, C, CF, G, K, L, NA, NX, P, S, SZ and T	5
R5	AK, A,CI, C, CF, G, K, L, NA, P, S, SZ and T	25
R6	A,CI, G, K, L, NA, P, S, SZ and T	10
R7	A, L, NA, P, S, SZ and T	7
R8	A, L, S, and T	5

prevalence in red deer was significantly higher where high densities of red deer ($p<0.001$) were present. Gautam et al. (2011) explored the potential role of ambient temperature on infection transmission dynamics for *E. coli* O157:H7 in a dairy herd, and their studies indicated that seasonal variation in ambient temperature could have a considerable impact on pathogen populations in the environment, specifically on barn surfaces and in water troughs, and consequently on the prevalence of infection in the host population. It was also observed that feeding of calves with lower levels of dietary wet distillers grains (15 % or less) 56 days prior to harvest significantly reduces *E. coli* O157:H7 in faeces and on hides (Wells et al. 2011).

Most of the serogroups associated with porcine STEC in this study have been reported in different animal species including pigs by various workers from different countries (Fratamico et al. 2004; Hussein 2007). Vu Khac et al. (2006) found that porcine pathogenic *E. coli* involved in post weaning diarrhoea typically belonged to serogroups O8, O138, O139, O141, O147, O149 and O157. We have also recorded the presence of serogroups such as O8, O138, O147 and O157 in the present study thereby indicating that pigs in India are potential reservoirs' of pathogenic *E. coli*. Interestingly, the serogroups such as O103 and O157 recorded in the present study have been generally reported to be associated with HUS in human being (OIE 2008). Therefore, isolation of these serogroups from pigs in India warrants possibility of transmission of this pathogen from pigs to human being and may pose serious public health problem in future. Several STEC strains are responsible for human gastrointestinal illnesses, from mild diarrhoea to bloody diarrhoea to haemorrhagic colitis and HUS. Most outbreaks and sporadic cases of bloody diarrhoea and HUS have been attributed to strains of STEC serotype O157:H7. However, recently in many countries, the role of non-O157 STEC strains as the cause of HUS, bloody diarrhoea and other gastrointestinal illnesses are increasingly being recognized (European Food Safety Authority 2010).

It was observed that although four of the *E. coli* O157 isolates carried genes for O157 *rfbE*, they were negative for gene encoding for H7 antigen (*fliCh7*). The present finding is consistent with the findings of Farzan et al. (2010) who detected *E. coli* O157 in 3 % samples from pigs in Canada, but no *E. coli* O157:H7 could be recovered from those samples.

In this study, all O157 *E. coli* and non-O157 *E. coli* isolates had one or both Shiga toxin genes. A greater percentage (5.1 %) of STEC isolates in this study possessed both *stx* genes as against *stx1* (4.6 %) and *stx2* (3.7 %). Studies on the prevalence of STEC in pigs from France revealed that all (48) STEC isolates were non-O157:H7 and positive for *stx2* gene (Bouvet et al. 2002). Similarly, while conducting studies for determining the prevalence of STEC in swine in the USA, Fratamico et al. (2004) found that out of 687 faecal samples, 70 % (484 of 687) were positive for Shiga toxin genes and 54 % (370 of 687), 64 % (436 of 687) and 38 % (261 of 687) were positive for *stx*1, *stx*2 and both toxin genes, respectively. It has also been reported that only 8.8 % (283 of 3,218) of faecal *E. coli* isolates from piglets with edema disease in Germany were *stx* positive (Barth et al. 2007). Ateba and Bezuidenhout (2008) observed that out of 20 STEC isolates from pigs, none of the isolates were positive for *stx* genes, but the number of isolates positive for genes *eae*, *hlyA* and both *eae* and *hlyA* were 7, 18 and 5, respectively. The variations in the prevalence of these genes as observed in the present study could be due to geographical variations or differences in age of the animals as observed by Shaw et al. (2004).

The main virulence factor of STEC contributing to pathogenicity is the production of Stx1, Stx2 or both, each including several variants (Growther et al. 2013). Strains producing Stx2 alone or both Stx1 and Stx2 cause more serious illness than those producing only Stx1 (Jeong et al. 2010). The mode of toxic action starts with the toxin molecule binding to host cell receptors, facilitating transfer of the toxin into the cells. Once inside the host cell, the A-subunit of the toxin, which possesses enzyme activity, interferes with host cell protein synthesis and induces an inflammatory response (Tesh 2012).

Numerous investigators have underlined the strong association between the carriage of *eae* gene and the capacity of

STEC to cause severe human disease, especially HUS (Suardana et al. 2011). In the current study, the combination of *eae* and *stx2* in addition to *stx1* was detected in two (0.2 %) isolates. Therefore, the prevalence of STEC isolates with such gene combination in pigs in India may pose threat to human health as porcine STEC isolates frequently share virulence-associated traits, i.e. genes with human enterohaemorrhagic *E. coli* (EHEC) strains (Meng et al. 2014).

The ability to produce one or more Shiga toxins is an important virulence characteristic of STEC (Growther et al. 2013). However, production of Shiga toxins alone may not be sufficient for STEC to be pathogenic. Other virulence factors such as the intimin protein, the presence of a plasmid-encoded hemolysin or both, are important in the pathophysiology of hemorrhagic disease (Lorenz et al. 2013). As the isolates carrying *stx*, *eae* and *ehxA*, *stx* plus *eae* and *stx* plus *ehxA* have also been recovered in this study; these isolates can potentially cause disease and should be considered pathogenic to humans. In the present study, none of the STEC isolates was found to carry *saa* gene which corroborated with the findings of Sonntag et al. (2005).

The number of STEC isolates in the present study which showed resistance to antimicrobials such as ampicillin, tetracycline, streptomycin, lincomycin, nalidixic acid, sulfadiazine, penicillin, gentamicin, kanamycin and ceftriaxone were 100, 99, 98, 97, 95, 94, 92, 88, 85 and 85, respectively. The present finding is in agreement with the findings of Choi et al. (2002) who also observed resistance of *E. coli* isolates from diarrhoeic pigs to several antimicrobials and they found that out of 285 *E. coli* isolates, the number of isolates resistant to ampicillin, tetracycline, lincomycin, sulfadiazine, penicillin and gentamicin were 249, 259, 254, 257, 258 and 191, respectively. Similarly, while evaluating the antimicrobial resistance of STEC isolates from pigs, Ojo et al. (2010) observed that out of 154 STEC isolates, the number of isolates resistant to ampicillin, tetracycline and streptomycin were 127, 116 and 78, respectively. In the present study, 97 isolates showed multidrug resistance where eight resistance groups (R1 to R8) were observed. Similarly, Ojo et al. (2010) detected multidrug resistance STEC isolates from pigs in Nigeria where they observed 38 resistance groups (R1-R38, showing resistance against more than two antimicrobials), and the resistance pattern which the maximum number of isolates (16) exhibited was ampicillin, chloramphenicol, streptomycin and tetracycline followed by ampicillin, chloramphenicol, ciprofloxacin, enrofloxacin, nalidixic acid, neomycin, norfloxacin, streptomycin, and tetracycline (13 isolates). The findings of the present study also corroborated with the findings of Schroeder et al. (2002) who observed that highest number of STEC isolates (71 %, $n=70$) from pigs in comparison to humans (12 %, $n=131$) and cattle (20 %, $n=133$) were resistant to tetracycline, and the corresponding values for ampicillin were 24, 21 and 3 %, respectively. The emergence of resistant bacterial strains could be due to common use of these antimicrobials for the treatment of animal diseases in India. Antimicrobial resistant strains of STEC may have selective advantage over other bacterial flora of the gastrointestinal tract of animals thereby contributing to their predominance and persistence in animals to establish a carrier status (Zhao et al. 2001). An increase in antimicrobial resistance in zoonotic bacterial isolates from animals, food and humans is a concern in veterinary and human medicine. The monitoring of antimicrobial resistance in enteric bacteria especially of animal origin is important in tracking and controlling the emergence and dissemination of antimicrobial resistant bacterial strains in human and animal populations.

As the pathogenicity of pathogenic *E. coli* can be determined by virulence factor combinations (Döpfer et al. 2012) and we also observed different combinations of virulence factors in STEC isolates from pigs in this study, these isolates may be important potential pathogens of public health significance. However, further studies should be undertaken to establish the role of pigs in transmitting this pathogen to human being.

Conflict of interest The authors declare no potential conflicts of interest with respect to research, authorship and publication of this article.

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