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Characterization of Shiga toxin-producing *Escherichia coli* isolated from healthy pigs in China

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Abstract

Background: Shiga toxin-producing *Escherichia coli* (STEC) is recognized as an important human diarrheal pathogen. Swine plays an important role as a carrier of this pathogen. In this study we determined the prevalence and characteristics of STEC from healthy swine collected between May 2011 and August 2012 from 3 cities/provinces in China.

Results: A total of 1003 samples, including 326 fecal, 351 small intestinal contents and 326 colon contents samples, was analyzed. Two hundred and fifty five samples were *stx*-positive by PCR and 93 STEC isolates were recovered from 62 *stx*-positive samples. Twelve O serogroups and 19 O:H serotypes including 6 serotypes (O100:H20/[H20], O143:H38/[H38], O87:H10, O172:H30/[H30], O159:H16, O9:H30/[H30]) rarely found in swine and ruminants were identified. All 93 STEC isolates harbored *stx*₂ only, all of which were *stx*_{2e} subtype including 1 isolate being a new variant of *stx*_{2e}. 53.76%, 15.05% and 2.15% STEC isolates carried *astA*, *hlyA* and *ehxA* respectively. Four STEC isolates harbored the high-pathogenicity island. Of the 15 adherence-associated genes tested, 13 (*eae*, *efa1*, *iha*, *lpfA*_{O113}, *lpfA*_{O157/O1-154}, *lpfA*_{O157/O1-141}, *toxB*, *saa*, F4, F5, F6, F17 or F41) were all absent while 2 (*paa* and F18) were present in 7 and 4 STEC isolates respectively. The majority of the isolates were resistant to tetracycline (79.57%), nalidixic acid (78.49%), trimethoprim-sulfamethoxazole (73.12%) and kanamycin (55.91%). The STEC isolates were divided into 63 pulsed-field gel electrophoresis patterns and 21 sequence types (STs). Isolates of the same STs generally showed the same or similar drug resistance patterns. A higher proportion of STEC isolates from Chongqing showed multidrug resistance with one ST (ST3628) resistant to 14 antimicrobials.

Conclusions: Our results indicate that swine is a significant reservoir of STEC strains in China. Based on comparison by serotypes and sequence types with human strains and presence of virulence genes, the swine STEC may have a low potential to cause human disease.

Keywords: Shiga toxin-producing *Escherichia coli* (STEC), Shiga toxin, Multilocus sequence typing, Adhesin genes, Putative virulence genes, Antibiotic resistance, Pulsed-field gel electrophoresis, Swine

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Background

Escherichia coli that produces one or more types of cytotoxins known as Shiga toxin (Stx) or Verocytotoxin (VT) is referred to as Shiga toxin-producing *E. coli* (STEC) or Verocytotoxin-producing *E. coli* (VTEC) [1]. STEC is a well-known pathogen as a cause of diarrhea, hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) [2]. Most cases of HC and HUS have been attributed to STEC O157:H7, but the importance of non-O157 STEC is increasingly recognized [3].

STEC possesses a number of virulence factors. Besides the *stx* genes, human pathogenic STEC strains often carry the *eae* gene, one of the genes located on LEE pathogenicity island encoding the adherence factor intimin [4] and the *astA* gene encoding a heat-stable enterotoxin EAST1 [5]. STEC strains may also be hemolytic due to the presence of the α -hemolysin or the enterohemolysin or both. The α -hemolysin gene *hlyA* is located on the chromosome [6] while the enterohemolysin (*ehxA*) is harbored by a plasmid [7]. Many adherence-related factors were found in STEC [8-13]. EHEC factor for adherence (*efa1*) was shown to be essential for the adherence of the bacteria to cultured epithelial cells [11]. The IrgA homologue adhesin (*iha*) is a STEC adherence-conferring molecule conferring the adherence phenotype upon a nonadherent laboratory *E. coli* strain [13]. *lpfA*_{O113}, *lpfA*_{O157/O1-154} and *lpfA*_{O157/O1-141} are adhesion genes in LEE-negative STEC strains [9,14]. Many STEC strains contain the heterologous 60-MDa virulence plasmid, which encodes a potential adhesin ToxB [10]. Other novel adhesion factors reported include autoagglutinating adhesin (*saa*) [12] and porcine attaching and effacing (A/E) associated protein (*paa*) [8]. Most STEC strains isolated from diarrheal pigs can produce one or more of the fimbriae, F4, F5, F6, F17, F18 and F41. Different types of fimbriae were reported to be associated with STEC diarrhea in animals of different age groups [15-18]. The *Yersinia* high-pathogenicity island (HPI) carrying *fyuA* (encoding the pesticin receptor) and *irp* (encoding the siderophore yersiniabactin) is also present in certain

non-O157 STEC lineages and was previously reported only in *stx*_{2e} carrying human isolates [19].

Domestic ruminants, especially cattle, are the major reservoirs of STEC. Other animals like sheep, goats have been confirmed as important natural reservoirs in some countries [2,20-22]. Swine also play an important role as a carrier of this pathogen. STEC strains that produce Stx_{2e} can cause edema disease in pigs [23] and can also been isolated from human stools at low frequency. STEC carried by healthy pigs may pose a potential risk to humans [24-27]. Relatively little is known about the prevalence and characteristics of STEC in pigs in China. In this study, we isolated and characterized STEC from different pig slaughter houses and pig farms from 3 geographical regions, Beijing city, Chongqing city and Guizhou province in China.

Results

Prevalence of STEC in swine samples

Out of 1003 swine samples collected in this study, 25.42% (255/1003) were *stx*-positive by PCR. A total of 93 STEC isolates was obtained from 62 samples, giving a culture positive rate of 24.31% (62/255) of all *stx*-positive samples. Different *stx*-positive rates in small intestine contents (10.83%), colon contents (47.24%) and feces (19.33%) samples were observed. The colon contents samples gave the highest *stx*-positive rate ($P < 0.05$) and also the highest culture positive rate (18.09%) ($P < 0.05$) (Table 1).

Only a single isolate was recovered from 44 *stx*-positive samples each. But 2 isolates per sample were recovered from 15 samples, 3 isolates per sample from 3 samples, 4 isolates per sample from 2 samples and 5 isolates per sample from 1 sample.

Serogroups and serotypes

The 93 STEC isolates were typed into 19 serotypes, comprising 12 O serogroups and 15 H types. Forty-four isolates were O antigen untypable and 21 isolates were non motile which were designated as [H]. Nineteen

Table 1 Prevalence of STEC in swine samples

Sample location (city/province)	No. of samples	Type of samples (N, %)	<i>stx</i> positive samples (N, %)	Samples with STEC isolates (N, %)	STEC isolates (N, %)
Beijing	523	SC (248, 24.73)	SC (30, 8.55)	SC (3, 0.85)	SC (7, 1.99)
		CC (275, 27.42)	CC (139, 42.64)	CC (36, 11.04)	CC (57, 17.48)
Chongqing	326	F (326, 32.50)	F (63, 19.33)	F (17, 5.21)	F (23, 7.06)
Guizhou	154	SC (103, 10.27)	SC (8, 2.28)	SC (4, 1.14)	SC (4, 1.14)
		CC (51, 5.08)	CC (15, 4.60)	CC (2, 0.61)	CC (2, 0.61)
Total	1003	SC (351, 35.00)	SC (38, 10.83)	SC (7, 1.99)	SC (11, 3.13)
		CC (326, 32.50)	CC (154, 47.24)	CC (38, 11.66)	CC (59, 18.09)
		F (326, 32.50)	F (63, 19.33)	F (17, 5.21)	F (23, 7.06)

Sample codes: F, fecal samples; CC, colon contents samples; SC, small intestine contents samples. The number (N) and rate (%) are showed in the parentheses.

serotypes were found including O2:H32/[H32], O9:H30/[H30], O20:H30/[H30], O20:H26, O76:H25, O86:H11, O87:H10, O100:H20/[H20], O114:[H30], O116:H11, O143:H38/[H38], O159:H16, O172:H30/[H30], ONT:H7, ONT:H17, ONT:H19/[H19], ONT:H21/[H21], ONT:H30/[H30], ONT:[H33].

The predominant serotypes were O20:H30/[H30], ONT:H30/[H30], O2:H32/[H32], O100:H20/[H20], O9:H30/

[H30], ONT:H19/[H19], O143:H38/[H38], O172:H30/[H30] which consisted of 22 (23.66%), 22 (23.66%), 11 (11.83%), 8 (8.60%), 4 (4.30%), 4 (4.30%), 3 (3.23%) and 3 (3.23%) isolates respectively. Five serotypes (O20:H26, O86:H11, ONT:H7, ONT:H17, ONT:H21/[H21]) contained 2 isolates each and 6 serotypes (O76:H25, O87:H10, O114:[H30], O116:H11, O159:H16, ONT:[H33]) contained only 1 isolate each (Table 2).

Table 2 Serotypes, virulence factors and sequence types (STs) of swine STEC isolates

ST	No. of isolates	Serotype ^a	stx _{2e} ^b	hlyA	ehxA	astA	irp2	fyuA	paa	F18
ST10	2	O2:H32/[H32](1CC, 1SC)	+	-	-	-	-	-	-	-
ST88	4	ONT:H19/[H19](1SC, 3CC)	+	-	-	+	+	+	-	-
ST206	3	O143:H38/[H38](3CC)	+	-	-	-	-	-	-	-
ST361	1	O20:H30 (1CC)	+	-	-	+	-	-	-	-
	1	ONT:H30 (1CC)	+	-	-	+	-	-	-	-
ST501	2	O86:H11 (2CC)	+	+	-	+	-	-	-	+
	1	ONT:H30 (1SC)	+	-	-	-	-	-	-	-
ST540	3	ONT:[H30] (1SC, 2CC)	+	-	-	-	-	-	-	-
	1	O114:[H30] (1CC)	+	-	-	-	-	-	-	-
ST641	1	O87:H10 (1SC)	+	+	-	-	-	-	-	+
ST694	1	ONT:[H33] (1CC)	+	-	-	+	-	-	-	-
	2	O20:H26 (2 F)	+	-	-	+	-	-	-	-
	17	O20:H30/[H30](4 F, 13CC)	+	-	-	+	-	-	-	-
ST710	1	O20:[H30] (1 F)	+	-	+	+	-	-	+	-
	3	O20:[H30](1 F, 2CC)	+	-	-	+	-	-	-	-
	3	O172:H30/[H30](3CC)	+	-	-	+	-	-	-	-
ST953	2	ONT:H17 (2CC)	+	-	-	-	-	-	+	-
	10	ONT:H30 (10CC)	+	-	-	-	-	-	-	-
ST993	2	ONT:H30 (2CC)	+	-	-	+	-	-	-	-
	3	ONT:H30/[H30](2 F, 1CC)	+	-	-	-	-	-	-	-
ST1294	1	ONT:H30 (1CC)	+	-	-	-	-	-	-	-
ST1494	2	ONT:H21/[H21](2CC)	+	-	-	+	-	-	-	-
	1	O100:H20 (1 F)	+	-	-	+	-	-	-	-
ST2514	1	O100:H20 (1SC)	+	-	-	+	-	-	+	-
	5	O100:H20/[H20](1 F, 4CC)	+	-	-	-	-	-	-	-
	1	O100:[H20] (1CC)	+	-	+	-	-	-	+	-
ST3628	9	O2:H32/[H32](9 F)	+	+	-	-	-	-	-	-
ST3629	4	O9:H30/[H30](4CC)	+	-	-	+	-	-	-	-
	1	ONT:H30 (1CC)	+	-	-	+	-	-	-	-
ST3630	1	O159:H16 (1CC)	-	-	-	+	-	-	+	-
ST3633	1	O76:H25 (1 F)	+	+	-	-	-	-	-	-
ST3631	1	ONT:H7 (1SC)	+	-	-	+	-	-	+	-
ST3634	1	ONT:H7 (1SC)	+	-	-	+	-	-	-	-
ST3870	1	O116:H11(1 F)	+	+	-	+	-	-	-	+
Total	93	93	93	14	2	50	4	4	7	4

^aThe numbers and sources are showed in the parentheses. F, fecal samples; CC, colon contents samples; SC, small intestine contents samples. ONT, Not typeable with available O antisera. The H types of non-motility isolates are determined by *flhC* sequencing and indicated in the square brackets.

^bNinety-two STEC isolates were subtyped by primer-specific PCR except one isolate of O159:H16.

Sorbitol fermentation and hemolysis

Out of the 93 STEC isolates, 53 (56.99%) were sorbitol-positive, covering all three types of samples and three regions. Twelve serotypes including O2:H32/[H32], O9:H30/[H30], O20:H26, O76:H25, O86:H11, O87:H10, O114:[H30], O116:H11, ONT:H17, ONT:H19/[H19], ONT:H21/[H21], ONT:[H33] were sorbitol-positive while 6 serotypes (O20:H30/[H30], O100:H20/[H20], O143:H38/[H38], O159:H16, O172:H30/[H30], ONT:H7) were sorbitol negative. All except 1 ONT:H30/[H30] isolate was sorbitol-positive.

Fourteen isolates displayed apparent β -hemolytic activity on sheep blood agar including 9 of the 11 O2:H32/[H32] isolates and 2 of the 11 O86:H11 isolates, and the single O76:H25, O87:H10 and O116:H11 isolates, the majority of which (11 isolates) were recovered from swine feces in Chongqing city. The 2 hemolytic O86:H11 isolates were isolated from colon contents in a slaughter house in Beijing city and the single O87:H10 isolate was isolated from a small intestine content in a slaughter house in Guizhou province.

Shiga toxin genes, adhesin genes and putative virulence genes

The 93 STEC isolates were tested positive for *stx*₂ only. All except 1 isolate was *stx*_{2e} subtype by PCR subtyping. The exception was an O159:H16 isolate which was found to carry a new variant of *stx*_{2e} by sequencing. The new variant differs from the closest *stx*_{2e} (GenBank: AM904726) by 4.51% at nucleotide level.

Three virulence-related genes (*astA*, *ehxA* and *hlyA*) and 2 markers for HPI (*irp2* and *fyuA*) were screened. 53.76% (50/93) STEC isolates carried *astA*, 15.05% (14/93) isolates contained hemolysin gene *hlyA* and only 2.15% (2/93) isolates contained enterohemolysin gene *ehxA*. All *hlyA* positive STEC isolates showed hemolytic activity on standard sheep blood agar. Hemolysis was not observed in the 2 *ehxA*-positive STEC isolates. The *irp2* and *fyuA* genes were identified in 4 STEC isolates, all of which were ONT:H19/[H19] serotypes (Table 2).

Among the 15 adherence-associated genes, 13 (*eae*, *efa1*, *iha*, *lpfA*_{O113}, *lpfA*_{O157/OI-154}, *lpfA*_{O157/OI-141}, *toxB*, *saa*, F4, F5, F6, F17 or F41) were not detected in the 93 STEC isolates. *paa* was present in 7 STEC isolates. Two O86:H11 isolates, 1 O87:H10 isolate and 1 O116:H11 isolate carried F18. Eighty-two STEC isolates did not carry any of the adherence-associated genes tested (Table 2).

Antibiotic resistance in the swine STEC isolates

Antimicrobial resistance was determined against 23 antibiotics. The highest prevalence was tetracycline resistance with a rate of 79.57%. Most isolates were resistant to nalidixic acid and trimethoprim-sulfamethoxazole, followed by resistance to kanamycin with a rate of

78.49%, 73.12% and 55.91% respectively. Resistance rate to streptomycin, chloramphenicol, ampicillin and piperacillin was 48.39%, 37.63%, 25.81% and 20.43%, respectively. Lower resistance was observed for cephalothin, nitrofurantoin, ciprofloxacin, ceftriaxone, aztreonam, cefotaxime, cefuroxime, gentamicin, norfloxacin, levofloxacin, ampicillin-sulbactam with a rate ranging from 2.15% to 17.20%. All isolates were susceptible to imipenem and meropenem (Additional file 1: Table S1).

Four isolates (4.3%) were susceptible to all 23 antimicrobial agents tested. Thirteen isolates (13.98%) were only resistant to 1 antimicrobial substance, while 76 isolates (81.72%) exhibited resistance to 2 or more antimicrobials tested. The STEC isolated from pig farms in Chongqing city showed resistance to a larger number of antimicrobial agents, and at a significantly higher rate than those isolated from slaughter houses in Beijing city ($P < 0.05$) (Figure 1 and Additional file 1: Table S1). An O116:H11 isolate exhibited multi-drug resistant phenotype against 19 of all 23 antimicrobial agents (excluding imipenem, meropenem, gentamicin and levofloxacin).

Pulsed-field gel electrophoresis (PFGE)

All 93 STEC isolates were analyzed by PFGE but only 88 isolates produced clear bands to give a PFGE profile which were divided into 63 PFGE patterns (EZKX01001 to EZKX01063). The most prevalent serotype O20:H30/[H30] with 22 isolates were typed into 16 PFGE patterns and the 11 O2:H32/[H32] isolates were typed into 8 PFGE patterns. An UPGMA dendrogram was constructed (Figure 2). The 88 STEC isolates could be divided into six clusters, A to F, at a similarity of 75% or greater. Cluster A contains all 4 O9:H30/[H30] and all 3 O100:H20/[H20] isolates. Cluster B contained the majority of O20:H30 isolates which were grouped into 3 subclusters. All the 11 of O2:H32/[H32] isolates also fell into cluster B as one subcluster. Cluster C was heterogeneous containing 6 serotypes. Clusters D to F contained mostly one serotype: O143:H38/[H38], ONT:H19/[H19], ONT:H30/[H30] respectively. Although isolates were largely grouped together by serotypes, identical PFGE profiles were also found among isolates of different serotypes (O20:H30/[H30] and O172:H30/[H30]) which were not from the same sample but from the same sampling point.

Multi-locus sequence typing (MLST)

The 93 STEC isolates were typed into 21 sequence types (STs) with 7 novel STs (Table 2). Four new STs (ST3628, ST3629, ST3633 and ST3634) were resulted from a novel allele in *fumC* (allele 470), *gyrB* (allele 351), *icd* (allele 396) and *recA* (allele 267) respectively. Three new STs (ST3630, ST3631 and ST3870) were due to new combinations of previously known alleles. The predominant STs were

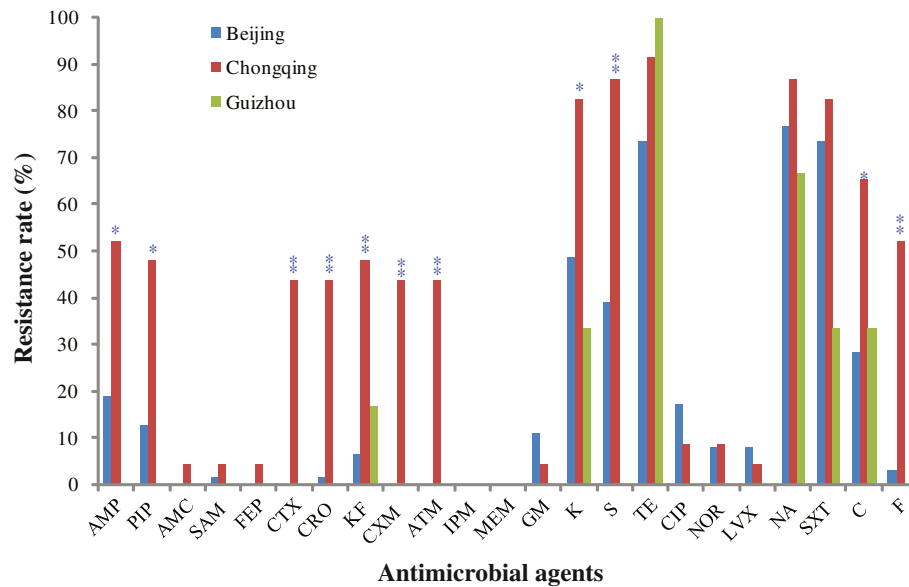


Figure 1 Antimicrobial resistance profiles of the STEC isolates. Three regions sampled are Beijing city (in blue), Chongqing city (in red), Guizhou province (in green). Statistical test was only performed between Chongqing and Beijing. A *and** were placed above the histogram for Chongqing samples if $P < 0.05$ and $P < 0.001$ respectively. Antibiotics abbreviations are: AMP, Ampicillin; PIP, Piperacillin; AMC, Amoxicillin-clavulanic acid; SAM, Ampicillin-sulbactam; FEP, Cefepime; CTX, Cefotaxime; CRO, Ceftriaxone; KF, Cephalothin; CXM, Cefuroxime; ATM, Aztreonam; IPM, Imipenem; MEM, Meropenem; GM, Gentamicin; K, Kanamycin; S, Streptomycin; TE, Tetracycline; CIP, Ciprofloxacin; NOR, Norfloxacin; LVX, Levofloxacin; NA, Nalidixic acid; SXT, Trimethoprim-sulfamethoxazole; C, Chloramphenicol; F, Nitrofurantoin.

ST710 and ST993 containing 25 (26.88%) and 15 (16.13%) isolates respectively. Six STs contained 3 or more isolates with ST3628, ST2514, ST540, ST3629, ST88 and ST206 comprising 9 (9.68%), 8 (8.60%), 6 (6.45%), 5 (5.38%), 4 (4.30%) and 3 (3.23%) isolates respectively. Five STs (ST10, ST361, ST1494, ST953 and ST501) contained 2 isolates each. Eight STs (ST641, ST691, ST1294, ST3630, ST3631, ST3633, ST3634 and ST3870) had only 1 isolate each. STEC isolates from Beijing, Chongqing and Guizhou were typed into 14, 6 and 5 STs respectively. ST2514 were recovered from all 3 regions and ST710 and ST993 were recovered from 2 regions, while other STs was only found in one region.

A minimum spanning tree was constructed (Figure 3A). Most STs differed from each other by 2 or more alleles while three pairs of STs (ST10 and ST3628, ST540 and ST3629, and ST88 and ST3870) and one set of 3 STs (ST3630, ST3631 and ST3634) differed from each other by only 1 allele. There is good concordance between STs and serotype. One ST consisted of solely or predominantly one serotype. However ST710, the most frequent ST, contained 3 serotypes, O20:H30/[H30], O172:H30/[H30] and O20:H26 with the first serotype being predominant. PFGE and MLST were also largely consistent in the clustering of the isolates (Figure 2). ST540 and ST3629 with 1 SNP difference in *icd* allele were grouped together with ST2514 in PFGE cluster A. All ST710 isolates were grouped into 2 subclusters within PFGE cluster B which

were separated by ST3628, ST10 and ST1294. ST10 and ST3628 isolates were grouped together which differed by 1 SNP difference in *gyrB*. PFGE clusters D and F were inclusive of all ST206 isolates and ST993 isolates respectively. However, the 5 STs (ST361, ST501, ST953, ST1494 and ST3633) within PFGE cluster C and the 3 STs (ST88, ST3631 and ST694) within PFGE cluster E were not closely related to each other by MLST (Figure 3A). On the other hand, ST88 was not grouped together with ST3870 by PFGE, which differed by 1 SNP difference in *gyrB*. The sole ST3870 isolate C09 also differed from the 4 ST88 isolates by serotype, hemolysis and antibiotic resistance profile.

Isolates of the same STs generally showed the same or similar drug resistance patterns (Figure 2). All ST3628 isolates showed the same multi-drug resistance to 14 antibiotics. Similarly, isolates of ST206, ST953 and ST1494 showed respective identical resistance profiles. All ST3629 isolates were resistant to tetracycline. However there existed variations of drug resistance within an ST. ST710 showed the most variability with resistance to 1 to 11 drugs. ST2514 which was isolated from all 3 regions also showed varied resistance profiles.

Discussion

Different prevalence of STEC in pigs were reported previously [24,25,27-29]. Kaufmann *et al.* [24] compared the STEC shedding rate in pigs at slaughter, which varied

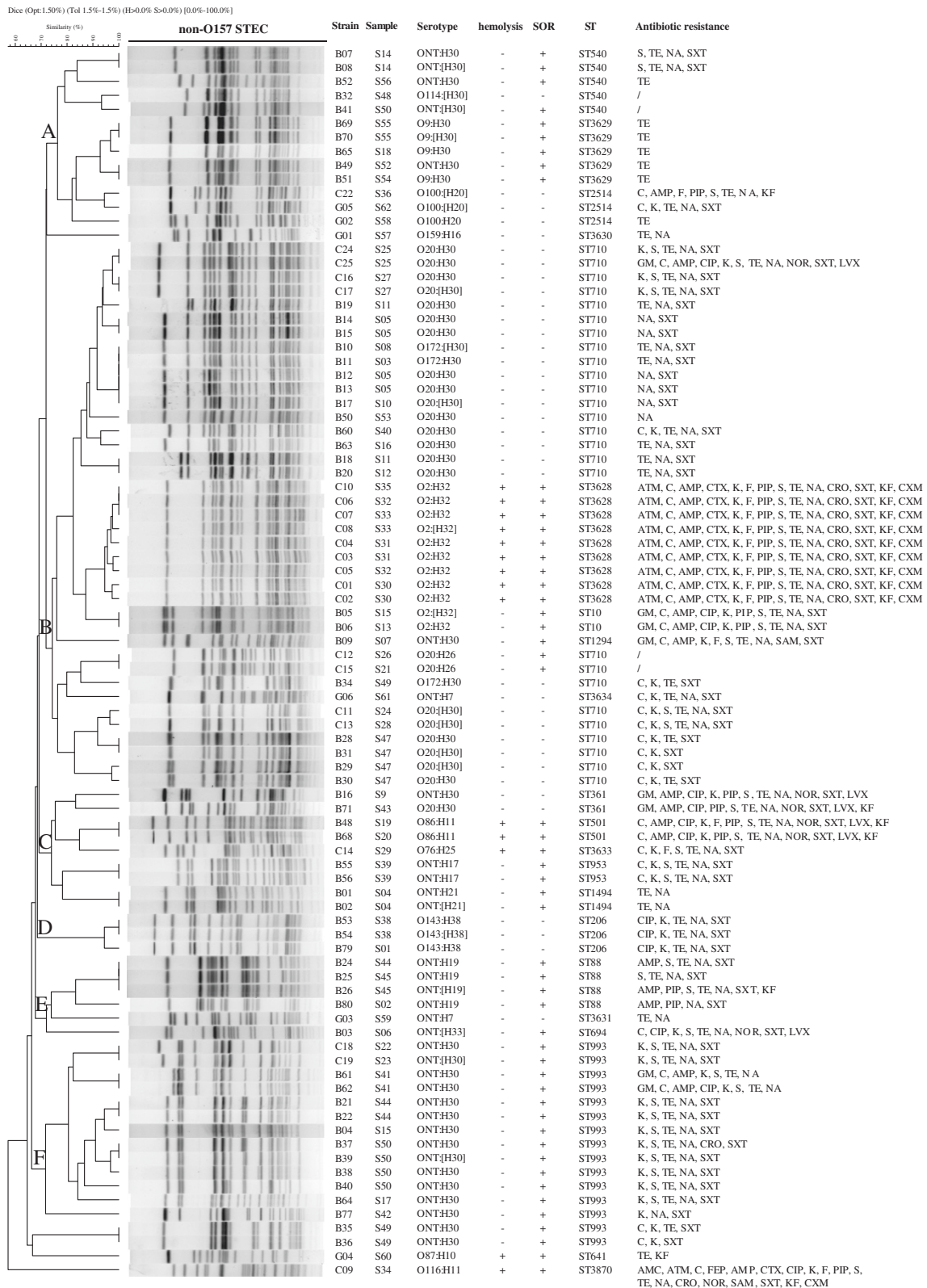


Figure 2 (See legend on next page.)

(See figure on previous page.)

Figure 2 Dendrogram of PFGE profiles of 88 STEC isolates from pigs in farms and slaughter houses. The 6 PFGE clusters were marked on the node as **A** to **F**. Non-typeable with available O antisera was marked ONT and non-motile isolates were labeled with the H types in square brackets. Displayed on the right hand side are strain name, sample name, serotype, hemolysis, sorbitol fermentation (SOR), sequence type (ST) and antibiotic resistance. Abbreviations for antibiotics are: AMP, Ampicillin; PIP, Piperacillin; AMC, Amoxicillin-clavulanic acid; SAM, Ampicillin-sulbactam; FEP, Cefepime; CTX, Cefotaxime; CRO, Ceftriaxone; KF, Cephalothin; CXM, Cefuroxime; ATM, Aztreonam; IPM, Imipenem; MEM, Meropenem; GM, Gentamicin; K, Kanamycin; S, Streptomycin; TE, Tetracycline; CIP, Ciprofloxacin; NOR, Norfloxacin; LVX, Levofloxacin; NA, Nalidixic acid; SXT, Trimethoprim-sulfamethoxazole; C, Chloramphenicol; F, Nitrofurantoin. Place of isolates were contained in the first letter of strain names: B means Beijing city, C means Chongqing city and G means Guizhou province.

widely and ranged from 2.1% to 70% depending on the health conditions of the pigs and the detection method used. As shown in this study the anatomic sites sampled also affected the rate of isolation and consequently affected the prevalence in the population reported. Fecal samples were commonly used [24-26]. In our study we sampled the small intestinal content, the colon content and the feces. The prevalence of STEC in the colon (47.24%) was almost 2.5 times higher than in feces (19.33%) ($P < 0.05$) and 4.4 times higher than in the small intestine (10.83%) ($P < 0.05$). STEC strains are thought to mostly colonize the colons of humans [30] and it is likely to be the same for pigs.

In this study, 93 isolates were recovered from 62 of the 255 *stx*-positive samples, giving a culture positive rate of 24.31%, this result is similar to that of Botteldoorn *et al.* [28], in which STEC isolates were obtained from 31% of the *stx* PCR-positive pig samples. Failure to isolate STEC from the *stx*-positive samples may due to the perturbation of high levels of background microflora, the loss of *Stx* prophages during subculture, the presence of other bacteria carrying *stx* or low levels of STEC in the samples.

In the present study, 12 serogroups and 19 serotypes were identified. The majority of these serotypes have been isolated from swine, sheep, cattle, food, and water in other countries [24,31-36]. The most prevalent serotype is O20:H30/[H30], which was also reported in cattle and sheep in different countries [31,32]. Six serotypes (O100:H20/[H20], O143:H38/[H38], O87:H10, O172:H30/[H30], O159:H16, O9:H30/[H30]) were rarely found in STEC isolates isolated from swine and other ruminants, implying that these serotypes may be restricted to the swine populations in these regions and their environments. Serotypes O86:H11, O20:NM, O100:NM, O9:NM, O172:NM and O114:NM have previously been described among STEC isolated from human patients [37-42]. Serotype O157:H7, which is common serotype causing human disease in some countries, was not detected.

A possible reason for no isolation of O157:H7 might be the method used. Isolation of O157 STEC often requires more targeted methods, such as the use of O157 immunomagnetic beads to capture the bacteria from enrichment broth and then culture on selective media [43].

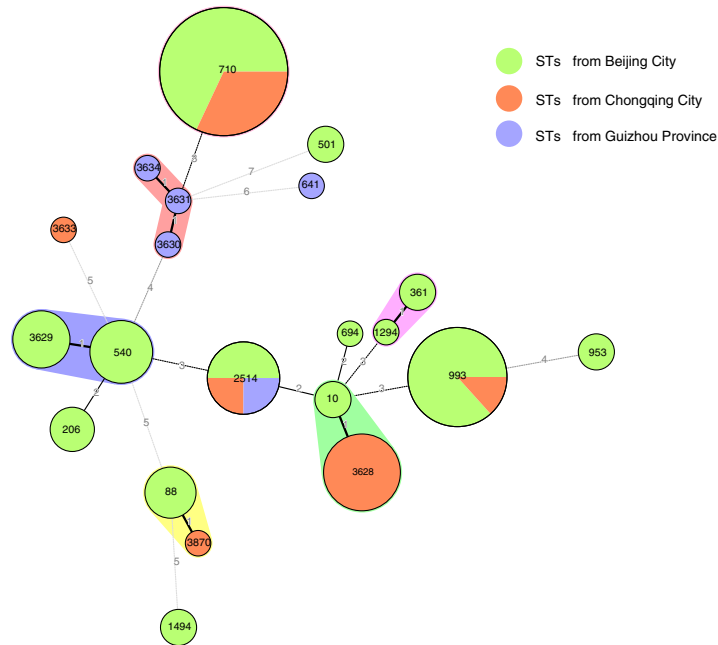
We previously used immunomagnetic separation to successfully isolate O157 STEC from pigs, although that was in an outbreak setting and was in a different geographic region [44]. In this study we used CHROMagar™ ECC only and didn't specifically target O157 STEC. CHROMagar™ ECC has been used by others for isolation of STEC from pigs [45]. However, that study did not isolate O157 STEC either. Therefore, the CHROMagar™ ECC may not be an ideal media for O157 STEC isolation.

We used sorbitol-MacConkey agar as a quick method to pick potential O157 colonies since sorbitol fermentation is a traditional feature for differentiating O157:H7 which is sorbitol-negative although there are sorbitol-positive O157 STEC [46]. In this study, a fair proportion (43%) of non-O157 STEC is actually sorbitol-negative. Therefore sorbitol fermentation is not a good indicator for O157:H7.

We analyzed multiple colonies from 21 samples to determine diversity within a sample (Figure 2). Two samples contained isolates with identical properties, suggesting they are the same strain, while the majority of the samples contain isolates belonging to the same sequence type but differing by one or more of the phenotypic or genetic properties tested, indicating that they are variants of the same clone. The most common variations are non-expression of the H antigen, variation of antibiotic resistance and/or variation in PFGE patterns. However 4 samples contained 2 different STs. Samples S15, S41, S49 and S50 all contain the prevalent ST993 and an additional ST, being ST10, ST88, ST710 and ST540 respectively, suggesting 2 different clones infecting the same pig.

Many studies have underlined the potential key role of the *Stx2* subtypes in the severity of disease. Although *Stx2e* is not a potent subtype [47], strains harboring *Stx2e* have been isolated from patients with diarrhea [48]. Intimin contributes to the development of A/E lesions and is a key virulence for some STEC serotypes [49], while *ehxA* can be found in many STEC serotypes, such as O157:H7 and O26:H11 that are associated with diarrheal disease and HUS [7,50]. However, Sonntag *et al.* reported that the *stx_{2e}*-positive *E. coli* isolated from healthy pigs rarely contains genes for intimin and enterohemolysin [19]. The prevalence of *ehxA* is very low in our samples at 2.15%, consistent with the findings of Sonntag *et al.* [19].

A



B

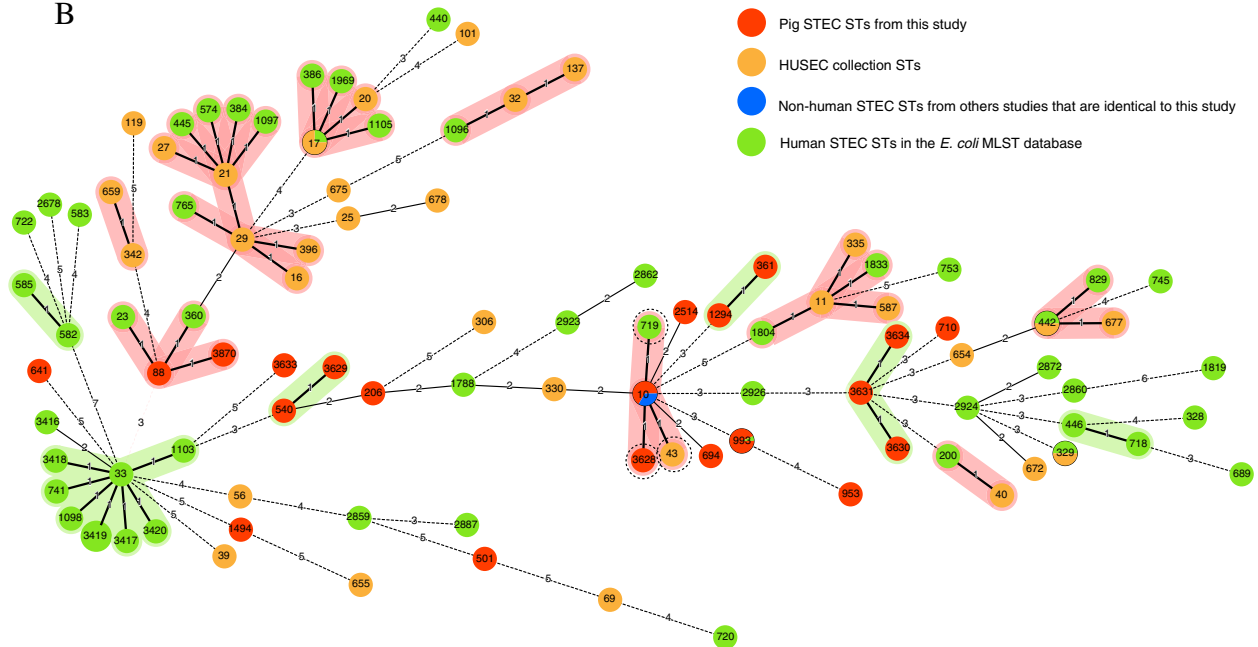


Figure 3 Genetic relationships of STEC isolates based on MLST. A) Genetic relationships of STEC sequence types (STs) from this study. Each circle represents a given ST with size proportional to the number of isolates. The colors for the slices of the pie represent places of isolates: Beijing city in green, Chongqing city in red and Guizhou province in purple. The numbers on connecting lines show the number of allelic difference between two STs. The number in a circle is the ST number. **B)** Minimal spanning tree of STs from this study, STs from the HUSEC collection and other human STEC STs. Ninety-three pig STEC isolates (in red) were compared to STs of HUSEC collection (in orange), human STEC STs (in green) and STs from other source that are identical to STs in our study (in blue) in *E. coli* MLST database. Each circle represents a given ST with the pie proportional to number of isolates in a given ST from different sources. The numbers on connecting lines show the number of allelic difference between two STs. The number in a circle is the ST number.

Other virulence factors may contribute to the pathogenicity of STEC. Although the role of EAST1 toxin in virulence to pigs has not been clearly determined, several studies have shown that *astA* gene is widely present among STEC isolates from both diarrheal and healthy pigs [15,24,26]. *astA* gene was also the most prevalent virulent gene (53.76%) among the 20 virulence genes tested in our study.

HPI was originally identified in *Yersinia* and now found in a range of pathogens [51], including the HUS-associated *E. coli* HUSEC041 [52] and the 2011 German HUS outbreak strain O104:H4 [53]. HPI had previously been detected in *Stx2e*-producing STEC strains from humans only [19]. In this study we found 4 *stx2e* STEC isolates, all ONT:H19/[H19], harbored the 2 HPI genes *fyuA* and *irp* although the frequency is low at 4.3%.

Fimbrial adhesins play an important role in colonization of the pig intestine and STEC strains may express up to 5 antigenically distinct fimbrial adhesins, F4, F5, F6, F18 and F41 [18]. Different types of fimbriae can be associated with STEC diarrhea in animals of different ages [15-18]. In this study, only 4 isolates contained a fimbrial adhesin (F18). None of the other fimbrial adhesins (F4, F5, F6, F17 and F41) was detected. Of the nonfimbrial adhesin-encoding genes, *paa* was found in 7 isolates (7.5%), but *efa1*, *toxB*, *lpfA*_{O157/O1-154}, *lpfA*_{O157/O1-141}, *lpfA*_{O113} and *saa* were not detected in any of the 93 STEC isolates. Eighty-two STEC isolates did not carry any of the adherence-associated genes tested.

Coombes *et al.* [54] reported that non-LEE encoded T3SS effector (*nle*) genes of non-O157 STEC strains are correlated with outbreak and HUS potential in humans. It will be interesting to examine our STEC isolates for the presence of the *nle* genes in future studies.

Many non-O157 STEC isolated from humans and animals have shown resistance to multiple antimicrobials [26,55,56], including resistance to trimethoprim-sulfamethoxazole and β -lactams [56,57]. STEC isolates from swine feces in the United States show high resistance rates (>38%) to tetracycline, sulfamethoxazole and kanamycin but susceptible to nalidixic acid (resistance rate 0.5%) [26]. In our study, we found that only 1 of the 12 categories of antimicrobial resistance types (carbapenems) and 2 of the 23 antimicrobial agents (imipenem and meropenem) were active against all the STEC isolates. The high prevalence (>50%) of resistance to tetracycline, trimethoprim-sulfamethoxazole, nalidixic acid and kanamycin is similar to that of other studies in China [55,58]. In a study [55] of STEC from diseased pigs in Guangdong province, China, the majority of the isolates (95%) were resistant to more than 3 antimicrobials and the resistance rates to chloramphenicol (89%) and streptomycin (83%) were far higher than that of our study (37.63% and 48.39%, respectively). We also found that isolates from

Chongqing showed a higher rate than those from the other 2 cities in this study. It should be noted that all samples collected from Chongqing were fecal samples while those from Beijing and Guizhou were small intestinal contents and colon contents samples, which may affect resistance profiles if different *E. coli* strains have a preference for the anatomic sites. However, it is more likely that the difference reflected the presence of resistant *E. coli* strains in different regions. Chongqing was dominated by the multidrug resistant ST3628. The differences in drug resistance rates between cities may be related to the differences in the prevalence of drug resistant STs.

Comparison with STs observed in human infections gives an indication of the potential risk for human infection of the swine STEC. We constructed an MST containing our STs, the 32 STs of the HUSEC collection and 52 human STEC STs from the *E. coli* MLST database (Figure 3B). None of the 21 STs in this study was identical to any of the 32 STs of HUSEC collection [52]. We only found one ST, ST993, which was observed in human infections. When comparison was made at clonal complex level, some of our STs fell into the same clonal complex as the human STs (Figure 3B). ST10 clonal complex contained 2 of our STs (ST10 and ST3628), 1 HUSEC ST (ST43) and 1 human STEC ST (ST719) from the MLST database. However, Hauser *et al.* found that 8 of the 35 STEC STs they isolated from foods shared the same STs with HUSEC strains and were similar in their virulence gene composition [59]. Since the STECs from foods and HUSEC collection were from the same geographical region, it is likely some of the HUSEC STECs were from local sources and not globally distributed. Our STECs from pigs may cause local human infections but there is no surveillance of human STECs in the regions where we sampled the swine STECs.

Conclusions

In conclusion, the prevalence of STEC in healthy pigs is high (25.42%) by PCR screening although only 6.18% of the swine samples yielded an STEC isolate by microbiological culture. The vast majority of isolates belonged to a limited number of serogroups and serotypes, with O20:H30/[H30] being the predominant serotype. The majority of the STEC serotypes found in this study were also reported in other countries. All 93 STEC isolates carried the pig associated *stx2e* subtype. Only a small proportion of the STEC isolates harbored *hlyA*, *ehxA* and adhesin genes. Based on comparison by serotypes and sequence types with human strains and presence of virulence genes, the STEC isolated from pigs may have a low potential to cause human disease. However,

further investigations are needed to assess their public health significance in causing human disease in China.

Methods

Sample collection

A total of 1003 samples were collected from May 2011 to August 2012, of which 326 were fecal samples collected in pig farms in Chongqing city, 351 were small intestinal contents and 326 were colon contents collected in pig slaughter houses in Beijing city and Guizhou province. Samples were transported as soon as possible to the laboratory in the National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention in ice-bags cold conditions for the isolation of STEC.

Isolation of STEC

One gram of each sample was enriched in 5 ml of modified Tryptone Soya Broth (mTSB) supplemented with novobiocin (10 mg/liter) (Oxoid, UK) and incubated at 37°C for 18 to 24 h with shaking at 200 rpm. Briefly, 150 µl of the lysis buffer (100 mM NaCl, 10 mM Tris-HCl [pH 8.3], 1 mM EDTA [pH 9.0], 1% Triton X-100) were added to the centrifuged enrichment sample, boiled for 10 min and centrifuged. The supernatant was used as template to test for the presence of *stx*₁ and *stx*₂ by TaqMan duplex real time PCR assay developed by Bai *et al.* [60]. One loopful of the *stx*-positive enrichment culture was directly streaked onto CHROMagar™ ECC plate (CHROMagar, Microbiology, Paris, France). After overnight incubation at 37°C, 10 blue or colorless, round moist presumptive colonies on each plate were initially picked randomly to test for the presence of *stx*₁ and *stx*₂ by conventional duplex PCR assay (primers listed in Table 3) and another 10 colonies were picked if the initial 10 were negative for any of the *stx* genes. The *stx*-positive colonies were plated onto Luria-Bertani (LB) plates and incubated overnight for further identification. One to 5 *stx*-positive isolates from each sample were collected for further investigation.

Biochemical tests and serotyping of STEC isolates

All *stx*-containing isolates were confirmed to be *E. coli* by using API 20E biochemical test strips (bioMérieux, Lyon, France). Sorbitol fermentation characteristic was examined by using sorbitol-MacConkey agar (SMAC) (Oxoid, UK).

The hemolytic activity was tested by using sheep blood agar (Oxoid, UK). The presence of transparent zones around the colonies was interpreted as positive hemolytic activity [71].

The determination of O antigens was firstly carried out by testing for specific *E. coli* O groups of interest, targeting group specific genes within the O-antigen gene

cluster described by DebRoy *et al.* [72]. The entire coding sequence of the *fliC* gene was amplified by PCR with the primers *fliC*-F (5'-ATGGCACAAGTCATTAATA CCAAC-3') and *fliC*-R (5'-CTAACCTGCAGCAGACA-3') reported by Fields *et al.* [73], and then sequenced to determine the H type of each isolate. *In vitro* motility was determined by inoculation of each isolate in the center of motility agar plates (0.3% LB agar) at 37°C for up to 48 h [74]. Bacterial motility was assessed by examining the swimming ring. The O:H serotype was confirmed by the O antisera and the H antisera obtained from the Statens Serum Institut (Copenhagen, Denmark).

stx subtyping

E. coli isolates were cultured in LB broth at 37°C for 18–24 h. DNA was extracted using Wizard Genomic DNA Purification kits (Promega, USA). The presence of Shiga toxin genes were assessed in all isolates by PCR using primers targeting the *stx*₁ and *stx*₂ genes (Table 3) as described by Brian *et al.* [61]. The *stx*₂ subtypes were determined by the PCR-based subtyping method devised by Scheutz *et al.* [62]. The complete *stx*₂ gene from a selected set of STEC isolates was amplified using primers GK1 and GK2 from Gunzer *et al.* [63] and sequenced to verify the PCR-based subtyping results. The neighbor-joining cluster analysis was employed to assign new subtypes or variants as mentioned by Scheutz *et al.* [62].

Identification of virulence and adherence factors

All STEC isolates were tested by PCR to investigate the presence of *astA*, hemolysis related genes (*ehxA* and *hlyA*), HPI genes (*fyuA* and *irp*) and adhesion-related genes (*eae*, *paa*, *efa1*, *toxB*, *lpfA*_{O157/O1-154}, *lpfA*_{O157/O1-141}, *lpfA*_{O113}, *saa*, F4, F5, F6, F17, F18 and F41) using the primers listed in Table 3.

Antimicrobial susceptibility testing

Antimicrobial resistance was determined by the disc diffusion method [75]. Twelve antimicrobial groups covering 23 antimicrobial agents including penicillins (ampicillin and piperacillin), β-lactam/β-lactamase inhibitor combinations (amoxicillin-clavulanic acid and ampicillin-sulbactam), cepheims (parenteral) (cephalosporins I, II, III, and IV, cefepime, cefotaxime, ceftriaxone, cephalothin and cefuroxime), monobactams (aztreonam), carbapenems (imipenem and meropenem), aminoglycosides (gentamicin, kanamycin and streptomycin), tetracyclines (tetracycline), fluoroquinolones (ciprofloxacin, norfloxacin and levofloxacin), quinolones (nalidixic acid), folate pathway inhibitors (trimethoprim-sulfamethoxazole), phenicols (chloramphenicol) and nitrofurans (nitrofurantoinz) were tested. Results were interpreted using the Clinical and Laboratory Standards Institute (CLSI, 2012)

Table 3 PCR primers used for the detection of STEC virulence or adherence genes

Targets	Primer	Oligonucleotide sequence (5'-3')	Amplicon size (bp)	Reference
<i>stx</i> ₁	<i>stx</i> ₁ -F	AAATCGCCATTCGTTGACTACTTCT	370	[61]
	<i>stx</i> ₁ -R	TGCCATTCTGGCAACTCGCGATGCA		
<i>stx</i> ₂	<i>stx</i> ₂ -F	CAGTCGTCCTACTGTTTCATCA	283	[61]
	<i>stx</i> ₂ -R	GGATATTCTCCCACTCTGACACC		
<i>stx</i> _{2e}	<i>stx</i> _{2e} -F	CGGAGTATCGGGGAGAGGC	411	[62]
	<i>stx</i> _{2e} -R	CTTCCTGACACCTTCACAGTAAAGGT		
SLT-II	GK1	ATGAAGTGTATATTATTTAAATGG	1241	[63]
	GK4	TCAGTCATTATTAACCTGCAC		
<i>ehxA</i>	<i>ehxA</i> -F	GGTGCAGCAGAAAAAGTTGTAG	1551	[64]
	<i>ehxA</i> -R	TCTCGCTGATAGTGTTGGTA		
<i>hlyA</i>	<i>hlyA</i> 1-F	GACAAAGCACGAAAGATG	2930	[6]
	<i>hlyA</i> 2-R	CAACTGCAATAAAGAAGC		
<i>astA</i>	EAST11a	CCATCAACACAGTATATCCGA	111	[65]
	EAST11b	GGTCGCGAGTGACGGCTTTGT		
<i>irp2</i>	<i>irp2</i> -F	AAGGATTTCGCTGTACCGGAC	280	[66]
	<i>irp2</i> -R	TCGTCGGGCAGCGTTTCTTCT		
<i>fyuA</i>	<i>fyuA</i> -F	TGATTAACCCCGCAGCGGAA	880	[66]
	<i>fyuA</i> -R	CGCAGTAGGCACGATGTTGTA		
<i>eae</i>	<i>eae</i> -F	ACGTTGCAGCATGGTAACTC	815	[36]
	<i>eae</i> -R	GATCGGCAACAGTTTCACCTG		
<i>paa</i>	M155-F1	ATGAGGAAACATAATGGCAGG	350	[67]
	M155-R1	TCTGGTCAGGTCGCAATAC		
<i>iha</i>	<i>iha</i> -F	CAGTTCAGTTTCGATTACCC	1305	[68]
	<i>iha</i> -R	GTATGGCTCTGATGCGATG		
<i>saa</i>	<i>saa</i> -F	CGTGATGAACAGGCTATTGC	119	[14]
	<i>saa</i> -R	ATGGACATGCCTGTGGCAAC		
<i>toxB</i>	<i>toxB</i> -F	ATACCTACCTGCTCTGGATTGA	602	[69]
	<i>toxB</i> -R	TTCTTACCTGATCTGATGCAGC		
<i>efa1</i>	<i>efa1</i> -F	GAGACTGCCAGAGAAAG	479	[11]
	<i>efa1</i> -R	GGTATTGTTGCATGTTGAG		
<i>lpfA</i> _{O157/O1-154}	<i>lpfA</i> _{O157/O1-154} -F	GCAGGTACCTACAGGCGGC	525	[14]
	<i>lpfA</i> _{O157/O1-154} -R	CTGCGAGTCGGCGTTAGCTG		
<i>lpfA</i> _{O157/O1-141}	<i>lpfA</i> _{O157/O1-141} -F	CTGCGCATTGCCGTAAC	412	[70]
	<i>lpfA</i> _{O157/O1-141} -R	ATTTACAGGCGAGATCGTG		
<i>lpfA</i> _{O113}	<i>lpfA</i> _{O113} -F	ATGAAGCGTAATATTATAG	573	[9]
	<i>lpfA</i> _{O113} -R	TTATTTCTTATATTGAC		
F4(K88)	F4-F	GCTGCATCTGCTGCATCTGGTATGG	792	[15]
	F4-R	CCACTGAGTGCTGGTAGTTACAGCC		
F5(K99)	F5-F	TGCGACTACCAATGCTTCTG	450	[15]
	F5-R	TATCCACCATTAGACGGAGC		
F6(P987)	F6-F	TCTGCTCTTAAAGCTACTGG	333	[15]
	F6-R	AACTCCACCGTTTGTATCAG		
F17	F17-F	GGGCTGACAGAGGAGGTGGGGC	411	[15]
	F17-R	CCCGGCGACAACCTTCATCACC		

Table 3 PCR primers used for the detection of STEC virulence or adherence genes (Continued)

F18	F18-F	GTGAAAAGACTAGTGTTTATTTTC	510	[15]
	F18-R	CTTGTAAGTAACCGCGTAAGC		
F41	F41-F	GAGGGACTTTTCATCTTTTAG	431	[15]
	F41-R	AGTCCATTCATTATAGGC		

breakpoints, when available. *E. coli* ATCC^R 25922 was used as quality control.

PFGE and MLST

STEC isolates were digested with *Xba*I and separated by PFGE using the non-O157 STEC PulseNet protocol (www.pulsenetinternational.org). Gel images were converted to Tiff files and then analyzed using BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium).

MLST was performed according to the recommendations of the *E. coli* MLST website (<http://mlst.ucc.ie/mlst/dbs/Ecoli>) using 7 housekeeping genes (*adhA*, *fumC*, *gyrB*, *icd*, *mdh*, *purA* and *recA*). Alleles and sequence types (STs) were determined following the website instructions [76]. MLST data for the HUS-associated enterohemorrhagic *E. coli* (HUSEC) collection were obtained from www.ehec.org [52]. All human STEC STs from the *E. coli* MLST databases were downloaded for comparison. A minimum spanning tree based on these STs was generated with BioNumerics software.

Four novel alleles, *fumC470*, *gyrB351*, *icd396* and *recA267* were submitted to *E. coli* MLST website. The sequences obtained in this study have been deposited in GenBank: KC924398 (*icd396*), KC924399 (*gyrB351*), KC924400 (*fumC470*), KC924401 (*recA267*) and KC339670 (a new variant of *stx_{2e}*).

Statistical analysis

Statistical tests were performed using SAS, Version 9.1 (SAS Institute Inc., Cary, NC., USA). Statistically significant differences were calculated using a χ^2 test where appropriate. *P* values of <0.05 were considered statistically significant.

Ethics statement

Samples of pig feces, small intestinal contents and colon contents of finished pig were acquired with the oral consent from the pig owners. This study was reviewed and approved by the ethics committee of the National Institute for Communicable Disease Control and Prevention, China CDC, according to the medical research regulations of the National Health and Family Planning Commission of People's Republic of China (permit number 2011-10-4).

Additional file

Additional file 1: Table S1. Antibiotic resistances of swine STEC isolates.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

QM carried out the sample collection, isolation of STEC, biochemical tests and serotyping of STEC isolates, identification of virulence and adherence factors, antimicrobial susceptibility testing, MLST, *stx* subtyping, data analysis and drafting of the manuscript. YX and RL carried out study design, overseeing the study, and editing of the manuscript. The rest of the authors contributed sample collection, strains isolation, biochemical tests and serotyping of STEC isolates, MLST, or PFGE. All authors read and approved the final manuscript.

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