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stx2vha Is the Dominant Genotype of Shiga Toxin-Producing *Escherichia coli* O157:H7 Isolated from Patients and Domestic Animals in Three Regions of China

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***Abstract*: Shiga toxin-producing *Escherichia coli* (STEC) O157:H7 strains were isolated from domestic animals and patients from Xuzhou City, Jiangsu Province, China and the bordering Anhui and Henan Provinces and were examined for the *stx* genotype. Of 390 strains, 277 were identified as genotype *stx2vha*; 41, *stx2*; 51, *stx2-stx1*; 1, *stx2-stx2vha-stx1*; 5, *stx2-stx2vha*; and 15 were un-typeable. Of the 277 *stx2vha*-bearing isolates, 116 were isolated from goats; 42, cattle; 38, hens, and 35 from pigs. The study shows *stx2vha* is the dominant genotype and goats are an important reservoir.**

***Key words*: Shiga toxin-producing *Escherichia coli* (STEC) O157:H7, Genotypes, Un-typeable, Goats, Cattle**

In humans, Shiga toxins (Stxs) are the major virulence factors of Shiga toxin-producing *Escherichia coli* (STEC) responsible for hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) (11). The Stxs include Stx1 and Stx2 possessing similar biological activities but distinct in their antigenicity. The association of STEC with HC and HUS implies that Stx2 is more closely related to these diseases than Stx1 (10, 15). At least 11 *stx2* variants that have been described may have direct impact on the capacity of a given STEC to cause disease (3, 4, 7, 8, 19). Recent studies show the strains carrying *stx2vha* may be less virulent and less frequently cause bloody diarrhea, one of the major symptoms of HC (14). Therefore, *stx* sub-typing is suggested to be helpful in understanding the role of the different subtypes in clinical medicine and epidemiology. *E. coli* O157:H7 was initially isolated in 1986 in Xuzhou City, Jiangsu Province, China. In 1999, a large outbreak of *E. coli* O157:H7 infections occurred in Xuzhou City and neighboring Anhui Province and resulted in 195 hospitalized HUS patients with 177 deaths (23). The majority of the patients were elderly. In 2000, 35 new cases were reported in neighboring Henan Province with 28 deaths. *E. coli* O157:H7 was isolated from 10–20% of the animals in the villages including pigs, cattle, goats and chickens. Identical pulsed-field gel electrophoresis (PFGE) patterns were shown for the human and animal isolates. Therefore, we postulate that domestic animals harboring this pathogen were responsible for these two outbreaks. In this study, we compare the genotypes of *stx* in STEC O157:H7 strains including the *stx2* sub-types recently

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Abbreviations: EHEC, entero-hemorrhagic *Escherichia coli*; HC, hemorrhagic colitis; HUS, hemolytic-uremic syndrome; PCR, polymerase chain reaction; PFGE, pulsed-field gel electrophoresis; RFLP, restriction fragment length polymorphism; STEC, Shiga toxin-producing *Escherichia coli*; Stx, Shiga toxin; VT, vero-cytotoxin.

Table 1. The *stx2* genotypes of *E. coli* O157:H7 isolates from China

Province	Genotype	Sources	No. of isolates	Year of isolation				
				1992	1999	2000	2001	2002
Jiangsu	*stx2vha*		169					
		Cattle	31		14	10	5	2
		Pig	26		23		1	2
		Chicken	25		17	5	3	
		Goat	64		53	7		4
		Beetle	3			3		
		HC patient	17		1	14	2	
		Other	2		2			
		Rabbit	1		1			
	stx2		27					
		Cattle	2		1	1		
		Pig	8		8			
		Chicken	4		4			
		Goat	13		9	4		
	Un-typeable		4					
		Cattle	2			1		1
		Chicken	1			1		
		Goat	1					1
	stx2+stx1		21					
		Pig	3		3			
		Fly	1		1			
		Goat	7		7			
		HC patient	3		3			
		HUS patient	2		2			
		Cattle	2		2			
		Chicken	3		3			
Shangdong	*stx2+stx1*		5					
		Cattle	2				2	
		HC patient	3	3				
	stx2+stx1+stx2vha	Cattle	1	1				
	stx2vha		3					
		Cattle	2	1				1
		HC patient	1	1				
	stx2		0					

isolated in Xuzhou City, Jiangsu Province and from Anhui and Henan Provinces; and compare them to other strains isolated from other parts of China.

The strains used in this study were isolated from the feces of human patients and animals during 1999 and 2000 from Xuzhou City, Jiangsu Province and the bordering provinces of Anhui and Henan. Other strains isolated from other parts of China were also included (Table 1). There were a few strains without clear records indicating where they were isolated and were named as unknown sources. The strains were isolated using the immuno-magnetic separation method (9). Briefly, specimens were incubated with paramagnetic beads coated with anti-O157 specific antibody (Dynal Co., Ltd., Denmark), followed by culture on sorbitol MacConkey medium containing cefixime and tellurite (CT-SMAC). All sorbitol-negative colonies were tested for the presence of O157 antigen, and confirmed using the PCR method for virulence genes *stx1*, *stx2*, EHEC-*hlyA*, EHEC-*eaeA*, rfb_{O157} and $fliC_{H7}$ (17, 20, 21, 24).

The putative *stx2* variants were screened using PCR with primer pairs described by several laboratories (17) that include: stx2c-stx2d, stx2v1-stx2v2, stxea-stxeb, stx2e-stx2f, and stx2cm-stx2f (Table 2). The template DNA used for amplification was extracted from whole organisms by boiling. Ampli*Taq* DNA polymerase and

Table 1. Continued

Province	Genotype	Sources	No. of isolates	Year of isolation				
				1992	1999	2000	2001	2002
Henan	*stx2vha*		36					
		Cattle	1			1		
		Goat	32			32		
		HC patient	3				3	
	stx2vha+stx2		5					
		Cattle	2				2	
		Dog	1				1	
		Goat	1				1	
		Duck	1				1	
	stx2		5					
		Goat	4			4		
		Goose	1				1	
	stx2+stx1		6					
		Cattle	1					1
		Vegetable	5				4	4
	Un-typeable		11					
		Goat	11			11		
Anhui	*stx2vha*		50					
		Cattle	7		7			
		Pig	6		6			
		Chicken	13		13			
		Goat	14		14			
		Goose	1		1			
		Fly	1		1			
		Unknown	8		8			
	stx2		9					
		Cattle	1		1			
		Pig	1		1			
		Goat	4		4			
		Unknown	3		3			
	stx2+stx1		0					
Other provinces	*stx2+stx1*		19					
		Vegetable	19			19		
	stx2vha		19					
		Goat	5				5	
		Unknown	9				9	
		Patient	1				1	
		Cattle	1				1	
		Pig	3				3	

Table 2. Primers used for *stx2* typing

Primer pair	Sequence (5′-3′)	Suggested sub-types	Sizes (bp)	Reference of source
stx2c stx2d	AAG AAG ATG TTT ATG GCG GT CAC GAA TCA GGT TAT GCC TC	*stx2*, *stx2vha*, *stx2vhb*	285	Pierard et al. (17)
stx2v1 stx2v2	CAT TCA CAG TAA AAG TGG CC GGG TGC CTC CCG GTG AGT TC	*stx2vha*, *stx2vhb*, *stx2d*	385	Pierard et al. (17)
stx2e stx2f	AAT ACA TTA TGG GAA AGT ATA TAA ACT GCA CTT CAG CAA AT	*stx2vhb stx2*, *stx2vha*, *stx2d*	348	Pierard et al. (17)
stx2cm stx2f	AAG AAG ATA TTT GTA GCG G TAA ACT GCA CTT CAG CAA AT	*stx2d*	256	Pierard et al. (17)
stxea stxeb	CCT TAA CTA AAA GGA ATA TA CTG GTG GTG TAT GAT TAA TA	*stx2e*	230	Pierard et al. (17)

the enzyme buffer were purchased from Sino-US Bio Company, Beijing, China. PCR was performed using a GeneAmp PCR system 400 (Perkin-Elmer). The PCR mixture was denatured for 5 min at 94 C followed by 30 cycles of amplification: denaturation for 25 sec at 94 C; annealing for 50 sec at 55 C or for *stx2e* reaction at 45 C; and primer extension for 26 sec at 72 C. Negative (*E. coli* HB101) and positive (*E. coli* O157:H7 EDL933) controls were included in each PCR protocol. The PCR products were electrophoresed in a 1.5% agarose gel and visualized using ethidium bromide staining. Restriction fragment length polymorphism (RFLP) PCR using the stx2c-stx2d primer pair was used to discriminate specific genotypes coding for the *stx2*, *stx2vha*, and *stx2vhb* sub-types. The predicted restriction patterns were identified following digestion of the amplified products using *Hae*III and *Rsa*I restriction endonucleases (18). To confirm the *stx2vha* subtype found by RFLP-PCR, chromosomal DNA from each strain was purified, digested with *Pst*I, separated in 0.7% agarose gels, and used for Southern blots. The PCR fragment generated by the primer, stx2c-stx2d, was labeled with digoxigenin-11-dUTP (Roche Applied Science, Mannheim, Germany), and used as the Southern blot probe. The Southern hybridizations were conducted under conditions of high stringency. PCR fragments of some strains were amplified using two oligonucleotide primers located downstream and upstream from the genes encoding the B subunits of the Stx2 to confirm a particular *stx* genotype. The amplified products (approximately 200 bp) were purified using the Prep-A-Gene purification system (Bio-Rad, Hercules, Calif., U.S.A.). The nucleotide sequence was determined with double-stranded DNA using the dideoxy chain termination method using a model 373A automatic DNA sequencer (Applied Biosystems, Inc.).

A standard PFGE protocol for *E. coli* O157 was used. Briefly, the cell suspension buffer was 100 mM Tris and 100 mM EDTA at pH 8 where the suspensions were adjusted to a turbidity reading of 0.48 to 0.52 using the digital output of a Dade Microscan turbidity meter (Baxter Diagnostics, Inc., McGraw Park, Ill., U.S.A.). The chromosome of the *E. coli* O157 isolates were digested with 40–50 U *Xba*I at 37 C for 2 hr. The electrophoresis conditions had an initial switch time of 2.16 sec with a final switch time of 54.17 sec using ramped switching for 18 hr. Gel images were captured on a Gel Doc 1000 or Gel Doc 2000 imaging system (Bio-Rad), converted to a tiff file, and analyzed using BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium). Strain PFGE patterns were considered clonally related if they had a similarity coefficient higher than 95%. Analysis of band patterns was performed using the Dice coefficient with a 1% tolerance for the band migration distance. Clustering of patterns was performed using an un-weighted paired group with arithmetic averaging (upGMA).

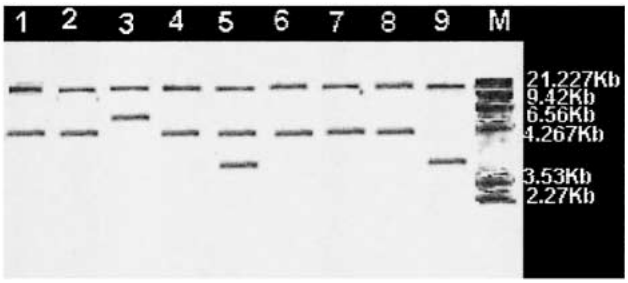

Fig. 1. Southern blot analysis of the chromosomal DNA digest using *Pst*I for sub-typing of the *stx2* gene. M, molecular weight markers; 1, strain 2154 (*stx2vha*; isolated from a goat); 2, strain 01B014 (*stx2vha*; isolated from a patient); 3, N144 (*untypeable*; isolated from cattle); 4, strain 20 (*stx2vha*; isolated from a dung beetle); 5, strain 364 (*stx2vha-stx2-stx1*; isolated from cattle); 6, strain 363 (*stx2vha*; isolated from cattle); 7, strain 367 (*stx2vha*; isolated from a patient); 8, strain 99B099 (*stx2vha*; isolated from a pig); 9, strain 882364 (*stx1-stx2*, isolated from a patient).

Table 1 shows the 390 isolates including 213 isolates from Xuzhou City and Anhui Province in 1999, 94 from Xuzhou City and Anhui and Henan Provinces in 2000, and 38 from other provinces in China in various years. Using the methods described above, 2 subtypes of *stx2* gene were identified in the 390 isolates, *stx2* and *stx2vha*, as well as 15 strains of un-typeable *stx2* genes (Fig. 1). These sub-types were further confirmed by DNA sequencing of the PCR products. No other *stx2* sub-types were detected. In 390 strains of STEC O157:H7 tested, 277 (71.0%) were genotypes *stx2vha*; 41 (10.5%), genotype *stx2*; 51 (13.1%), *stx2-stx1*; 1 (0.25%), *stx2-stx2vha-stx1*; and 5 (1.28%) strains were identified as *stx2vha-stx2* (Table 1). The *stx2* genes in 15 (3.85%) strains were un-typeable. 79.8% (170 of 213) of the strains isolated in 1999 were *stx2vha*. The *stx2vha*-bearing strains isolated from patients and animals exhibited identical PFGE patterns from the *Xba*I digested chromosomal DNA. Representative examples are shown in Fig. 2. The *stx2*-bearing strains showed PFGE patterns with a similarity coefficient >95% and are considered clonal. Both of these strains from patients and from animals were isolated in Xuzhou City of Jiangsu Province and bordering provinces where animals carrying *E. coli* O157:H7 were assumed responsible for outbreaks that occurred in 1999 and 2000.

This genotype appears to have emerged as the dominant genotype of STEC O157:H7 since 1999 following the first major outbreak identified in China (22) (Fig.

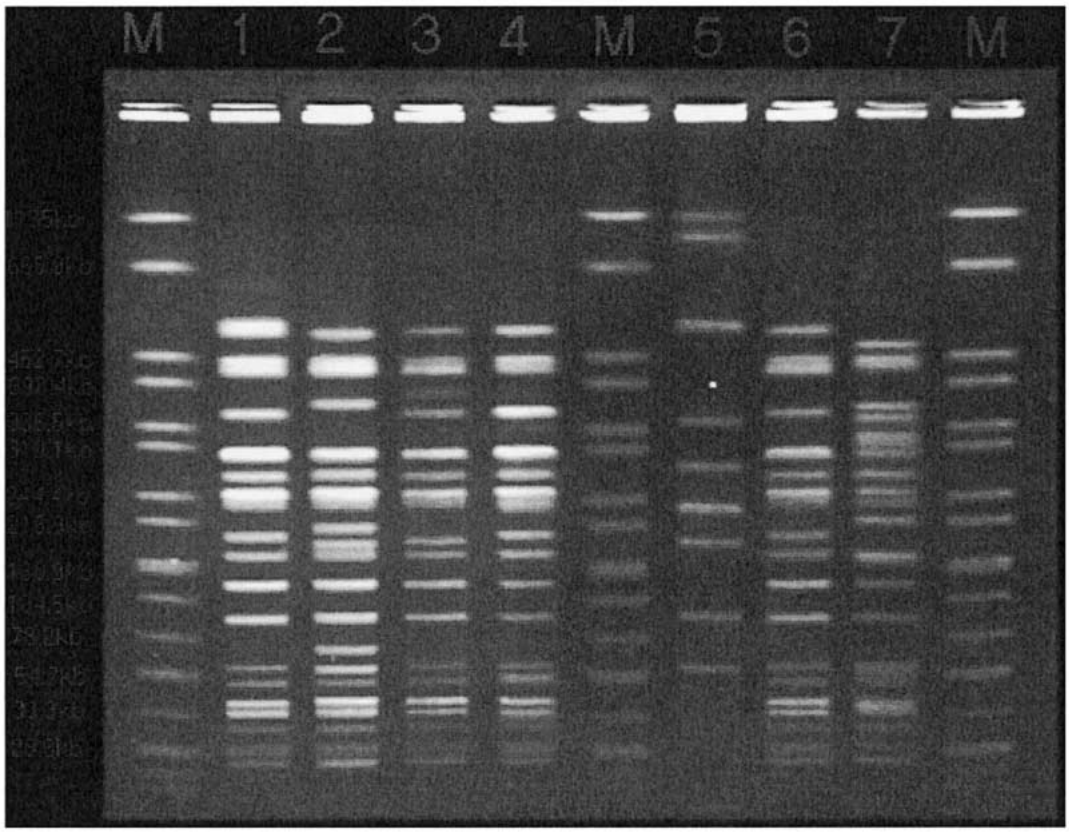

Fig. 2. PFGE patterns of *Xba*I digest of chromosomal DNA. M, molecular weight markers; 1, strain 1124 (*stx2vha*; isolated from a goat); 2, strain 99E168 (*stx2vha*; isolated from a chicken); 3, strain 99B049 (*stx2vha*; isolated from a chicken); 4, strain 61 (*stx2vha*; isolated from a HC patient); 5, 99D207 (*non-stx-producer*; isolated from a chicken); 6, strain 99C168 (*stx2vha*; isolated from a goat); 7, strain 99C151 (*stx2vha*; isolated from cattle).

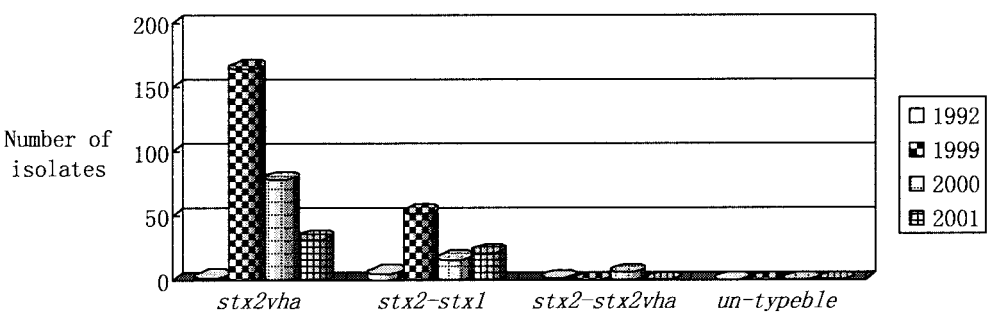

Fig. 3. Yearly isolation of STEC O157:H7 genotypes.

3). Bertin et al. (1) reported among the *stx2*-positive bovine isolates in France that 39.5% *stx2vhb*, 39% *stx2*, 25.5% *stx2vha*, and 8.5% *stx2d* sub-types were detected alone or in combination with other *stx2* subtypes. *stx2* and *stx2* along with either *stx2vha* or *stx2c* are prevalent in Belgium and Germany. Friedrich et al. (6) found that 68 (25%) of 268 STEC isolates from HUS patients harbored *stx2* along with *stx2c*. The frequency of isolates harboring the *stx2c* genotype was similar among isolates from patients with HUS (3.7%) and diarrhea (5.0%). Nishikawa et al. (14) report that 36 of 168 strains isolated from patients were *stx2vha*. The risk of developing HUS following infection with STEC harboring the *stx2* genotype was higher than that following infection with strains harboring the *stx2c* genotype. From the limited number of reports available on the *stx* genotype isolation frequency, as far as we can determine this is the first report that indicates the *stx2vha* subtype has become the dominant genotype isolated from both animals and HC patients.

Goats were the dominant isolation source for the STEC O157:H7 strains from animal origin in our survey

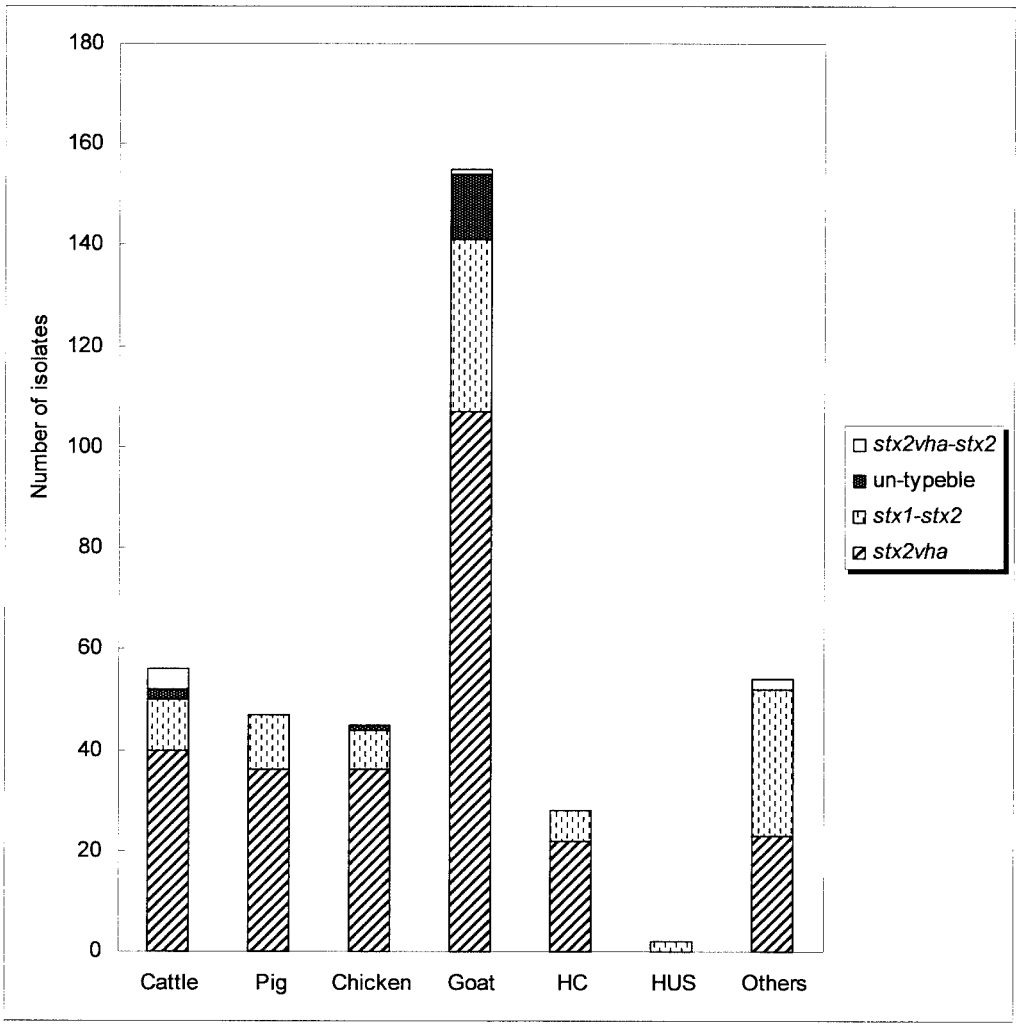

Fig. 4. Isolation of STEC O157:H7 *stx2* genotypes from animals, patients with HC and patients with HUS.

and *stx2vha* was the dominant genotype for all animal species examined. The frequency of detection of *E. coli* O157:H7 was not due to efforts to examine goats more intensively than other animals. Of 390 strains analyzed, 213 *E. coli* O157:H7 were isolated in 1999: 16.93% (N=189) from cattle, 8.26% (N=605) from pigs, 15.42% (N=590) from goats and 8.61% (N=604) from chickens (Xu, J., et al. unpublished data). Among them, 161 were identified as genotype *stx2vha*. This suggests that cattle and goats are important reservoirs for STEC O157:H7.

Cattle have long been considered the principal reservoir of STEC in many countries. However, epidemiological surveys show STEC strains are also prevalent in the gastrointestinal tracts of other domestic animals including pigs, goats, dogs, and cats. This suggests an association between endemic STEC and agricultural activities such as manure spreading, animal density, and elevated fecal bacterial counts in local streams. Fegan et al. (5) isolated STEC in 45% of 144 goat feces collected from farms and 36% of 72 lamb feces from abattoir yards, showing STEC are widely distributed in eastern Australian goat and lambs and are shed in their feces prior to slaughter. This provides a potential means for the contamination of carcasses and entry of STEC into the human food chain. Bielaszewska et al. (2) suggest goats may be a reservoir of STEC O157:H7 and a source of the infection for humans since a cluster of patients with HUS were related with ingestion of raw goat's milk. However, Bertin et al. (1) identified *stx2vha* only in cattle and not from goat isolates. *stx2vha* has been frequently detected in cattle in France. We found here STEC O157 *stx2vha* is the most frequently isolated sub-type from goats.

Of the geographical regions where the 390 strains originated, 343 were from Xuzhou City, Jiangsu Province and bordering Anhui and Henan Provinces. Outbreaks caused by *E. coli* O157:H7 were identified in Xuzhou City and bordering Anhui Province in 1999 and in Xuzhou City and bordering Henan Province in

2000. Of the 221 isolates from Jiangsu Province, 182 were from Xuzhou City. The strains from Anhui Province were all isolated in Xiaoxian County, which borders Xuzhou City, where there was a cluster of HUS cases in 1999. In 277 strains of STEC O157:H7 carrying *stx2vha*, 137 were from Xuzhou City, 51 Xiaoxian County, and 36 were from Henan Province (13). Some strains from other parts of China were also identified as bearing genotype *stx2vha* or *stx1-stx2* where sporadic cases have been observed (22). At present, we have no evidence that links the isolates from Xuzhou City, Xiaoxian County, Henan Province to other regions of China. However, our data indicate isolation of most STEC O157:H7 strains were clustered in Xuzhou City and its bordering areas. For the regions outside of Xuzhou City and its bordering areas, the detection frequency of *E. coli* O157:H7 in animals was usually less than 1% and no isolations were obtained in many provinces.

The nucleotide sequence of the un-typeable *stx2* gene was 100% identical to the *stx2* variant containing an *IS1203V* variant (*IS1203V*, Genebank accession number AB017524) (12, 16). It is possible that *IS1203V* insertion in the *stx2* gene accounted for the differences in the Southern-blot patterns. Of 390 STEC O157:H7 strains, 15 strains (3.85%) carried the un-typeable *stx2* gene. Okitsu et al. (16) proposed that this *IS1203V* was inserted in the regions encoding the amino-terminus of the B subunit with a duplication of 3 bp at the target site and results in inactivation of the Shiga toxin 2 gene. It is conceivable that strains possessing the insertion-inactivated Shiga toxin genes are likely to have a wide distribution (16). Further investigations are required to examine these possibilities and are now in progress in our laboratory.

In conclusion, we investigated the *stx2* genotypes of 390 strains of STEC O157:H7 isolated from animals and patients with HUS or diarrhea, and demonstrated the *stx2vha* is the dominant genotype with goats being an important reservoir.

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