Epidemiol. Infect. (2001), **127**, 27–36. © 2001 Cambridge University Press
DOI: 10.1017/S0950268801005635 Printed in the United Kingdom

Phylogenetic diversity and similarity of active sites of Shiga toxin (Stx) in Shiga toxin-producing *Escherichia coli* (STEC) isolates from humans and animals

H. ASAKURA[1], S-I. MAKINO[1]*, H. KOBORI[1], M. WATARAI[1], T. SHIRAHATA[1], T. IKEDA[2] AND K. TAKESHI[2]

[1] *Department of Veterinary Microbiology, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido, 080-8555, Japan*
[2] *Hokkaido Institute of Public Health, Sapporo, Hokkaido 060-0819, Japan*

(*Accepted 28 February 2001*)

SUMMARY

Nucleotide sequences of Shiga toxin (Stx) genes in STEC from various origins were determined and characterized by phylogenetic analysis based on Shiga toxin (Stx) with those deposited in GenBank. The phylogenetic trees placed Stx1 and Stx2 into two and five groups respectively, and indicated that Stx1 in sheep-origin STEC were placed into a different group from those in other STEC, and that Stx2 of deer-origin STEC also belonged to the unique group and appeared to be distantly related to human-origin STEC. On the other hand, Stx of STEC isolated from cattle, seagulls and flies were closely related to those of human-origin STEC. Such a diversity of Stx suggested that STEC might be widely disseminated in many animal species, and be dependent on their host species or their habitat. In addition, the active sites in both toxins were compared; the active sites in both subunits of Stx in all the animal-origin STEC were identical to those in human-origin STEC, suggesting that all the toxin of STEC from animals might be also cytotoxic, and therefore, such animal-origin STEC might have potential pathogenicity for humans.

INTRODUCTION

A variety of Shiga toxin (Stx)-producing *Escherichia coli* (STEC) serotypes are frequently isolated from humans and animals. Although the predominant STEC seroype associated with human infection is O157: H7 [1], non-O157 STEC can also cause human disease [2–4]. Most of them are non-pathogenic for animals, which result in harbouring STEC by healthy animals asymptomatically [5–9], while several STEC serotypes cause diseases in pigs and calves [4, 10–12]. Therefore, animals harbouring STEC may constitute a major reservoir for STEC infection of humans. In addition, some STEC are also isolated from sewage [13, 14]; STEC exist as normal resident flora in environments closely associated with humans and may constitute a risk of human STEC infection. The most important virulence factors in STEC are Stx1 and Stx2, whose structural genes are mostly located on bacteriophages and may be distributed widely among various bacterial species in the environment. In this study, we determined the DNA sequences of the toxin genes of STEC from various origins and compared them by phylogenetic analysis. The amino acid sequences of the active sites in both toxins were also compared. The characterization of STEC from animals and their potential pathogenicity for humans are discussed.

MATERIALS AND METHODS

Strains used in this study (Table 1) were isolated as described previously [15–18]. Briefly, 1·0 g of faeces was incubated in 10 ml of brain heart infusion broth

* Author for correspondence.

Table 1. *STEC strains isolated in this laboratory*

Strain	Origin	Serotype	Stx type	Sampling date	Accession no.*	Reference
S-1	Deer	O111:H45	2	Jun. 1997	AB012101	16
S-2	Deer	O111:H45	2	Jun. 1997	AB048222	16
S-3	Deer	O111:H45	2	Jun. 1997	AB048223	16
S-4	Deer	Out:H45	2	Jun. 1997	AB012102	16
S-5	Deer	O93:H-	2	Jun. 1997	AB048224	16
S-6	Deer	O96:H-	2	Jun. 1997	AB048225	16
S-7	Deer	O96:H-	2	Jun. 1997	AB048226	16
S-8	Deer	Out:H25	2	Jun. 1998	AB048227	This study
S-9	Deer	O8:H7	2	Jun. 1998	AB048228	This study
S-10	Deer	O96:H-	2	Jun. 1998	AB048229	This study
S-11	Deer	O96:H-	2	Jun. 1998	AB048230	This study
HI-1	Sheep	O153:H25	1	Jun. 1997	AB048231	15
HI-2	Sheep	O165:H-	1, 2	Jun. 1997	AB048232/AB048233	15
HI-5	Sheep	Out:H16	1	Jun. 1997	AB048234	15
HI-7	Sheep	Out:H-	1	Jun. 1997	AB048235	15
HI-11	Sheep	O2:Hnt	2	Nov. 1997	AB048236	15
HI-N	Sheep	Out:H-	1, 2	Oct. 1997	AB048237/AB048238	This study
K-7	Seagull	O136:H16	2	Jul. 1998	AB030484	17
K-10	Seagull	O153:H-	1	Jul. 1998	AB030485	17
F-16	Fly	O157:H7	1, 2	Jul. 1997	AB015056/AB015057	18
C-1	Cattle	O157:H7	2	1997	AB048239	This study
C-2	Cattle	O157:H7	2	1997	AB048240	This study
Obi-1	Human	O157:H7	2	Oct. 1996	AB048835	31
A-1	Human	O157:H7	2	Oct. 1996	AB048836	This study
A-2	Human	O157:H7	2	Oct. 1996	AB048837	This study

* Accession numbers indicated above are deposited in GenBank.

BHI; Difco Lab., Detroit, MI) supplemented with vancomycin at a final concentration of 6 μg/ml at 37 °C for 6 h with shaking. Then, 0·1 ml of the enrichment culture was added to 10 ml of BHI broth, followed by incubation at 37 °C for 20 h with shaking. 10 μl of the enrichment culture were diluted in 100 μl of sterile distilled water, following by heating at 95 °C for 5 min. One μl of the suspension was used for PCR by using of *stx* common primers [19]. PCR-positive cultures were spread on DHL plates to produce discrete colonies, 500 of which were examined by colony hybridization using a 518 bp DNA fragment amplified with *stx* common primers. The toxin types and cytotoxities of all strains were examined by PCR [19] and Vero cell assay [15, 16]. Serological typing was carried out by tube agglutination with rabbit antisera raised against antigenic test strains of *E. coli* O1 to O173 and H1 to H56. The DNA sequences of all toxin genes were determined by sequencing of PCR products as described previously [16], and submitted to the Genbank with the accession numbers shown in Table 1 . The amino acid sequences of Stx1 and Stx2 were compared with each other by using evolutional tree analysis with the UPGMA method by GENETYX-MAC Ver. 10.2 (Software Development Co. Ltd., Japan.). Other techniques were performed as described previously [15, 16].

RESULTS AND DISCUSSION

Analysis of phylogenetical trees of Stx1

When we defined that amino acid sequences of A-subunits in Stx1 and Stx2, which had over 99% and 97% homologous to each other, respectively, belonged in a single group, analysis of phylogenic trees of them revealed two (Fig. 1) and five clusters (Fig. 2), respectively. Although the Stx1 sequences in seagull- (strain K-10, accession no. AB030485), sheep- (HI-2, AB048232) and fly-origin STEC (F-16, AB015056) belonged in the first group, whose members were mostly human-origin STEC [20–24], the Stx1 sequences of the other four sheep-origin STEC (HI-1, AB048231; HI-5, AB048234; HI-7, AB048235; HI-N, AB048237) belonged in the second group (Fig. l). Since Z36901, which was a member of the second group, was derived from a sheep-origin STEC DG131/3 isolated in Germany [24], the Stx1 in the

https://doi.org/10.1017/S0950268801005635 Published online by Cambridge University Press

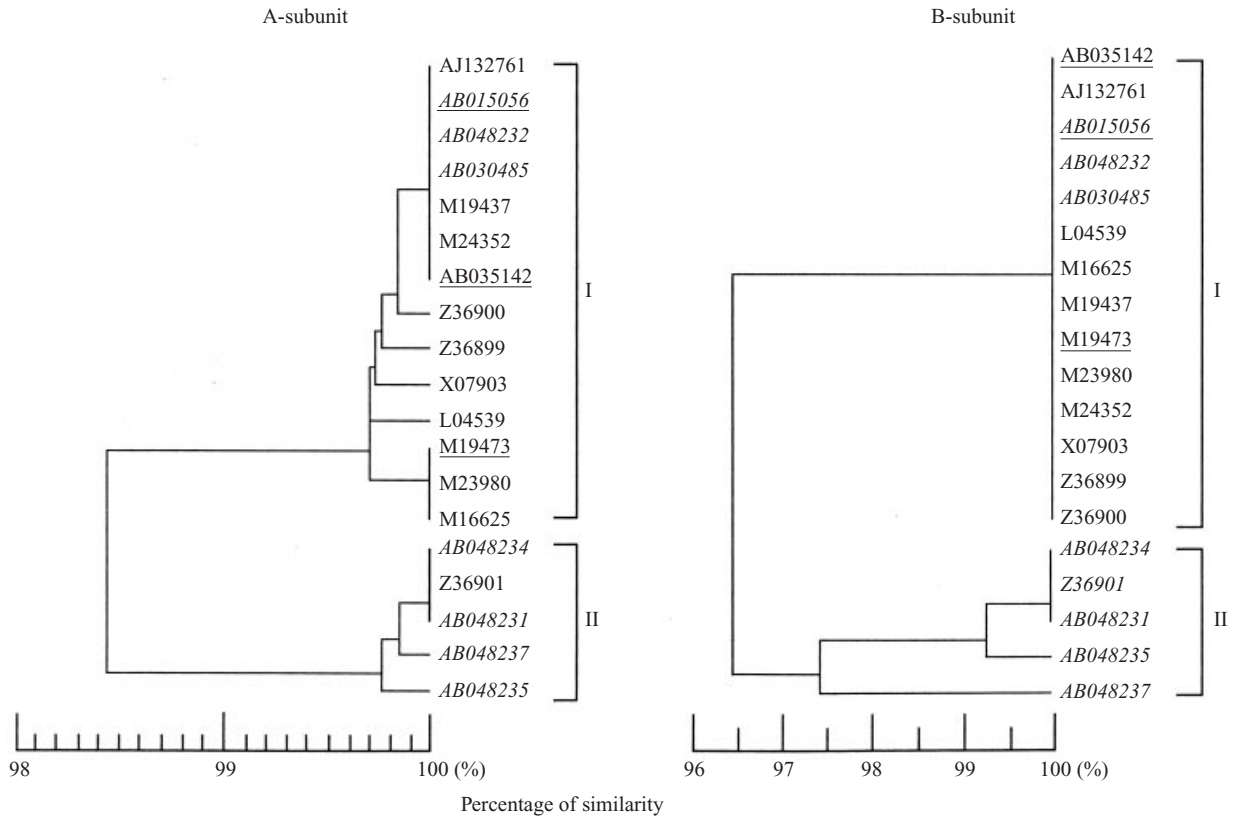

Fig. 1. Phylogenic trees of amino acid sequence of Stx1. The tree was constructed using the UPGMA method and analysis was based on Stx1 amino acid sequences. Accession numbers in SWISS-PROT and PIR databases are indicated. Strain designations were described in Tables 1 and 2. O157 strains are underlined, while animal origin strains are italicized.

second group might be a new Stx1 variant commonly distributed in sheep. On the other hand, the Stx1 sequence of HI-2 (AB048232) was a member of the first group (Fig. 1), and furthermore its Stx2 sequence (AB3048233) was also in the first group (Fig. 2), whose members were also mostly human-origin STEC [25–31]. Those findings suggested that HI-2 was closely related to human-origin STEC; this strain might cycle between sheep and humans. Alternatively, the transmission of the toxin genes might occur by phage conversion, via the close relationship of sheep to human with respect to meat and wool production. However, the amino acid sequences of the stx_1 genes were stably preserved because substantial amino acid and nucleotide sequence similarities between the two groups were mostly more than 96·8% (Fig. 1) and 96·0% (data not shown), respectively.

Analysis of phylogenetical trees of Stx2

Analysis of phylogenetical trees revealed that the Stx2 genes in fly- (F-16, AB015057), cattle- (C-1, AB048239; C-2, AB048240; 5, AB017524 (32)), sheep- (HI-2, AB048233) and seagull-origin STEC (K-7, AB030484) were placed into the first group with almost all of those in human-origin STEC. Only the Stx2 sequences in a sheep- (HI-11, no. AB048236) and a deer-origin STEC (S-8, AB048227) belonged in the second group. The other 10 deer-origin STEC (S-1, AB012101; S-2, AB048222; S-3, AB048223; S-4, AB012102; S-5, AB048224; S-6, AB048225; S-7, AB048226; S-9, AB048228; S-10, AB048229; S-11, AB048230) belonged in the third group, in which three human-origin non-O157 STEC strain EH250 (AF043627) [33], PH (L11078) [27] and OX3:H21 (X65949) [34], and sheep-origin STEC (HI-N, AB048238) belonged. Although all deer-origin STEC, except S-1 (AB012101), S-2 (AB048222) and S-3 (AB048223), were thought to be different to each other by plasmid profile (Fig. 3), RAPD (Fig. 4), PFGE (data not shown), the deer-origin STEC isolates commonly produced only Stx2, of which 10 were members of the third group (Fig. 2). In addition stx_2 gene of S-3 had single nucleotide difference (99·8% similarity) from those of S-1 and S-2. Furthermore, since three human-origin STEC

https://doi.org/10.1017/S0950268801005635 Published online by Cambridge University Press

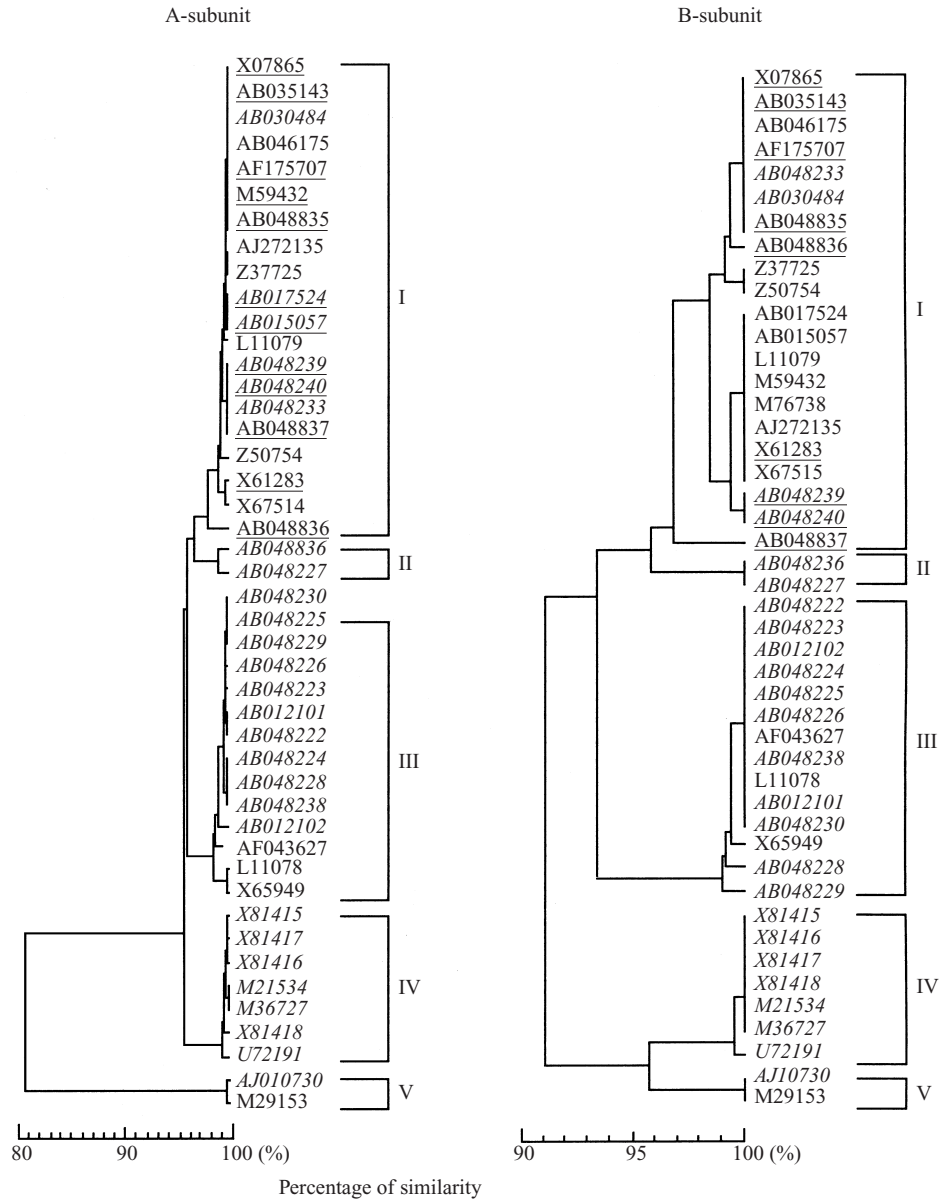

Fig. 2. Phylogenic trees of amino acid sequence of Stx2. The tree was constructed using the UPGMA method and analysis was based on Stx2 amino acid sequences. Accession numbers in SWISS-PROT and PIR databases are indicated. Strain designations were described in Tables 1 and 2. O157 strains are underlined, while animal origin strains are italicized.

(EH250, AF043627; PH, L11078; OX3:H21, X65949) belonged in the third group commonly produced Stx2d (Fig. 2) [27, 33, 34], those stx_{2d} gene might be transmitted by strains from animals like deer. It was not clear why deer-origin STEC shown in this study commonly produced only Stx2. However, those phenotypes might be geographically restricted to Hokkaido, because deer-origin STEC producing either or both of Stx1 and Stx2 have been isolated [35]. Moreover, the stx_2 gene in deer-origin S-8 (AB048227) was closely related to human-origin STEC rather than other deer-origin STEC. The results suggest that the stx_2 gene might be transmitted between human and wild deer strains.

The stx_{2e} genes in the fourth group were common to swine- and rabbit-origin STEC [10, 12, 36, 37] (Fig. 2). Furthermore, their types of *stx* variants were not detected in human-origin STEC. O-serotypes such as O139 commonly found in swine-origin STEC have not been isolated from human *E. coli* infections though serotype O26 in strain R107 and serotype O101:H- in strain E-D42 were isolated from humans [2, 11], suggesting that the stx_{2e} genes in this group are host-specific and unable to transfer to humans

https://doi.org/10.1017/S0950268801005635 Published online by Cambridge University Press

Table 2. *Characteristics of STEC strains refered from database*

Species	Strain	Origin	Serotype	Stx	Accession no.	Reference*
Stx1						
Shigella dysenteriae	60R	Human	—	Shiga toxin	M24352	22
	—	Human	—	Shiga toxin	M19437	52
	—	Human	—	Shiga toxin	X07903	53
S. sonnei	CB7888	Human	—	Shiga toxin	AJ132761	54
E. coli	EDL933	Human	O157:H7	1, 2	M19473	21
	H-19B	Human	O26:H11	1	M16625	20
	H30	Human	O26:H11	1	M23980	22
	GPU96MM	Human	O157:H7	1, 2	AB035142	Unpublished
	O111:H-	Human	O111:H-	1	L04539	23
	94C	Human	O48:H21	1, 2	Z36899	24
	CB168	Human	O111:H-	1	Z36900	24
	DG131/3	Sheep	OX3:H8	1	Z36901	24
Stx2						
E. coli	EDL933	Human	O157:H7	2	X07865	25
	GPU936MM	Human	O157:H7	1, 2	AB035143	Unpublished
	5	Cattle	O157:H7	1, 2	AB017524	32
	KNIH317	Human	O157:H7	2	AF175707	26
	EH250	Human	Out:H12	2d	AF043627	33
	T4/97	Pigeon	O128:H2	2f	AJ010730	38
	PH	Human	O111:H-	2d	L11078	27
	O31	Human	OX3:H21	2c	L11079	27
	94C	Human	O48:H21	1, 2	Z37725	30
	H.I.8	Human	O128:H12	2f	M29153	39
	O86	Human	O86	2	AB046175	Unpublished
	126814	Human	O26:H11	1, 2	AJ272135	Unpublished
	E32511	Human	O157:H-	2c	M59432	28
	R107	Rabbit	O26	2e	U72191	37
	7279	Human	O157:H7	2	X61283	29
	OX3:H21	Human	OX3:H21	2d	X65949	34
	E-D68	Swine	O101:H14	2e	X81415	36
	E-D53	Swine	O101:H14	2e	X81416	36
	E-D43	Swine	O101:H14	2e	X81417	36
	E-D42	Swine	O101:H-	2e	X81418	36
	S1191	Swine	O139	2e	M21534	12
	412	Swine	O139:K82	2e	M36727	10
Enterobacter cloacae		Human	—	2	Z50754	55
Citrobacter freundii		Human	—	2c	X67514	56

* Unpublished means that only DNA sequences were submitted to the database.

regardless of their serotypes. On the other hand, two stx_{2f} genes in the fifth group were unique, because both DNA sequences in the A-subunit showed only 70% identity to other sequences, whereas other sequences were more than 92·9% identical to each other (data not shown). Although pigeon-origin STEC (AJ010730) [38] and human-origin STEC H.I.8 (M29153) [39] belonged in the fifth group, it was unrelated to either human-origin STEC or also seagull-origin STEC, suggesting that bird-origin STEC might show more variability and also transfer to humans because they had broader living spaces with humans than other animals. In this study, although we phylogenetically analysed various origin O157 strains, all genes shown as underlined numbers belonged in the first group (Figs 1, 2). Therefore, the toxin genes in O157 STEC appear to be more highly conserved. Simultaneously, since several toxin genes highly similar to those in O157 STEC were isolated from non-O157 STEC (Figs 1, 2), the toxin genes might be transmitted between O157 and non-O157 by phage conversion. However, since we reported two different types of phages, 933W and Stx2 ϕ -K7 [17], whose Stx2 sequences were 100% identical (see X07865 and AB030484 in Fig. 2) [17, 25], various types of phages would have chances to encode toxin

https://doi.org/10.1017/S0950268801005635 Published online by Cambridge University Press

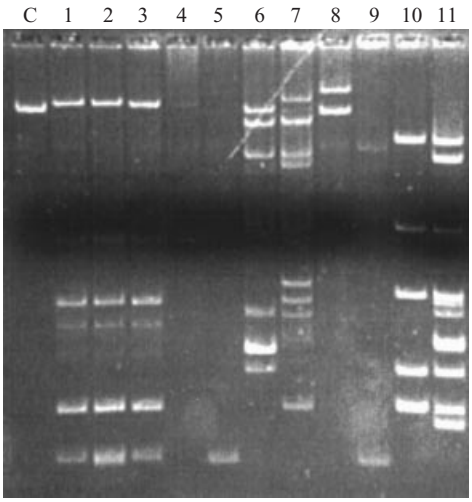

Fig. 3. Plasmid profiles of STEC isolates from wild deer. Lanes: C, STEC O157:H7 EDL931 as control strain; 1, S-1; 2, S-2; 3, S-3; 4, S-4; 5, S-5; 6, S-6; 7, S-7; 8, S-8; 9, S-9; 10, S-10; 11, S-11.

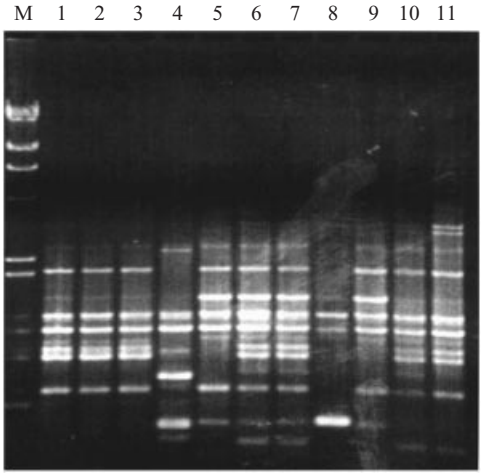

Fig. 4. RAPD analysis of STEC isolates from wild deer. RAPD analysis using total DNA from S-1 (lane 1), S-2 (lane 2), S-3 (lane 3), S-4 (lane 4), S-5 (lane 5), S-6 (lane 6), S-7 (lane 7), S-8 (lane 8), S-9 (lane 9), S-10 (lane 10) and S-11 (lane 11) were performed. λ *Hin*dIII, λDNA digested with *Hin*dIII as molecular size markers.

genes in the first groups. Since chromosomal O157 DNA contained many phage sequences (Hayashi et al., personal communication), it might be necessary to consider another mechanism for explaining why limited toxin types were identical in O157.

Comparison of the active sites in the toxin genes

In a recent study, Y_{77} [40], Y_{114} [41], E_{167} [42] and R_{170} [43, 44], in Stx1 and E_{167} and R_{170} [45] in Stx2 were identified as their active sites. In addition, three conserved regions (A, B and C) associated with toxin activities were reported [42]; Region B contained E_{167} and R_{170} of the active sites (Fig. 5). Therefore, the amino acid sequences of active sites in toxin genes were compared to investigate whether such active sites were conserved among various origin STEC and associated with human STEC infection (Fig. 5). Although all four active site sequences and Region C were conserved among all Stx, X07903, AJ010730 and M29153 showed single amino acid differences from others in Region B (Fig. 5). However, those substitutions would likely have no influence on the toxin activities because these amino acids were related to each other, meaning that all Stx would have toxin activities regardless of their origin. However, in the Region A of the Stx2, all sequences in the second, fourth and fifth groups were different from the general Stx2 sequences [25]. These substitutions might have significant influences on the toxin activities and reflect difference depending on the origin.

Stx toxins specifically recognize the glycolipid receptor via their B-subunits [46, 47]. The amino acid residues involved in recognition were also analysed by site-directed mutagenesis techniques; D_{18} in Stx1 (48), Q_{64} and K_{66} in Stx2e (48), C_{32}, A_{42} and G_{59} in Stx2 [49]. From the homology search among the B-subunits of all Stx amino acid sequences, D_{18} was conserved in all Stx1, and Q_{64} and K_{66} were also conserved in all Stx2e of the fourth group. Although the 64th residue in other Stx2 of the first, second, third and fifth groups differed from Stx2e, the Stx2f in AJ010730 and M29153 of the fifth group were the same as Stx2e of the fourth group (Fig. 6), suggesting that all members of the fourth and fifth groups might utilize a common mechanism to recognize the receptor. Actually, in the phylogenic tree analysis of the B-subunits (Fig. 2), both groups were derived from one branch.

In this paper, we propose that the cytotoxities of all STEC strains do not vary greatly because their active sites in the A-subunits are highly conserved (Fig. 5), and therefore probably retain Vero-cell cytotoxity. In fact, all STEC strains isolated in this laboratory (Table 1) were cytotoxic to Vero cells (data not shown). Their host-specificities might be different from each other because the amino acid residues involved in host–cell recognition fell into two groups (Fig. 1). Although the replacement of the active sites in the A-subunits with other amino acid residues resulted in loss of toxin activity [40–45], the replacement of the recognition sequences in the B-subunits caused only changing of the mode of the

https://doi.org/10.1017/S0950268801005635 Published online by Cambridge University Press

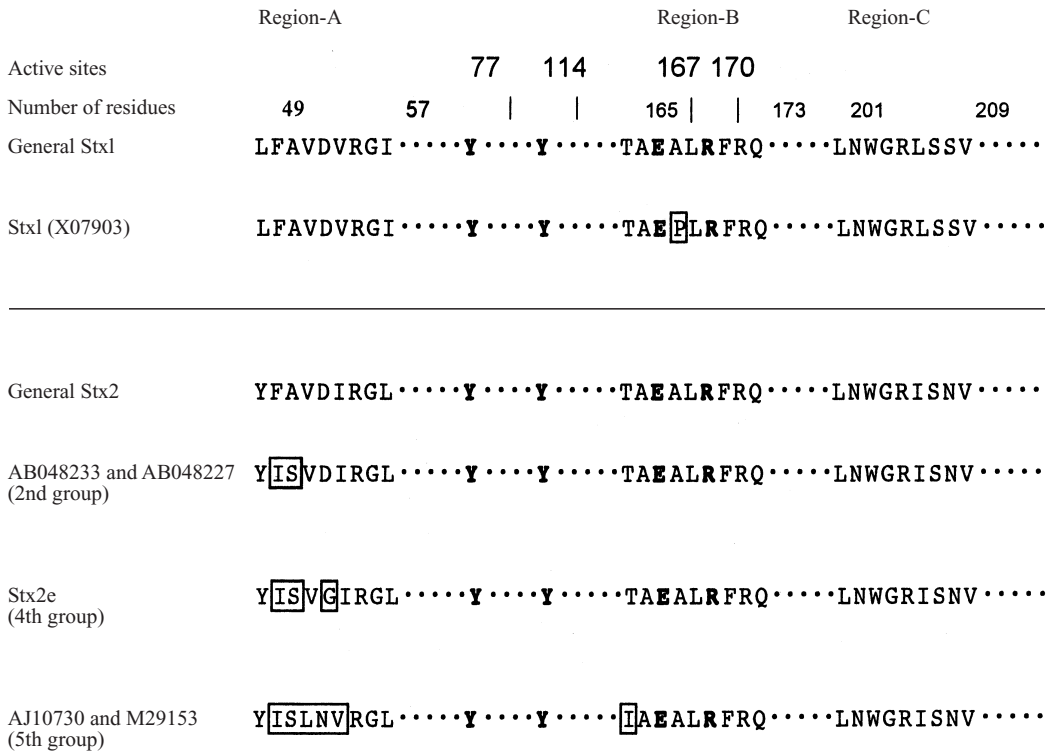

Fig. 5. Comparison of the active sites in A-subunit of Shiga toxin. Varied residues compared with those of prototype toxin are indicated as boxed. The active sites are shown as bold. The three conserved regions (A, B and C) associated with toxin activities are shown at the top (16).

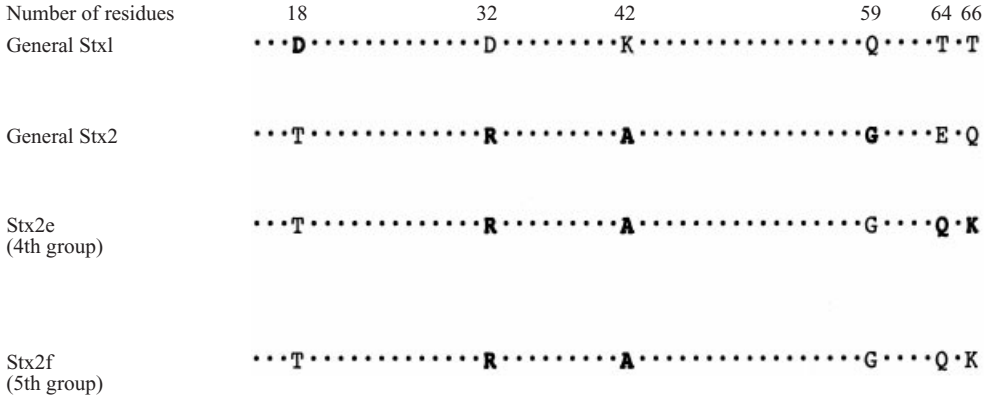

Fig. 6. Comparison of the active sites in B-subunit of Shiga toxin. Active sites residues were shown as bold.

glycolipid-recognition [45], suggesting that Stx toxin might be toxic to all animal species. However, we identified groups which appeared to be specific for particular animal species (Figs 1, 2). In fact, O-antigen types of STEC from human infection coincided with ones of the human-pathogenic *E. coli* as previously reported [2, 4]. For instance, O157, O26, O55, O111, O113, O117, O128 STEC were isolated from the human STEC infection and categorized as pathogenic *E. coli* [2, 4]. On the other hand, O139 STEC, which was most frequent serotype in swine edema disease, has not been detected in human infection [2, 4]. In addition, O93 and O96 STEC reported in this study (Table 1) have not been isolated in human *E. coli* infection [2, 4]. Moreover, O128 STEC was placed uniquely into the fifth group in the phylogenic tree (Fig. 2), suggesting that serotype O128 STEC might be related to Stx2f and that O128 STEC might have diverged evolutionarily from other STEC.

https://doi.org/10.1017/S0950268801005635 Published online by Cambridge University Press

Finally, O157 STEC appears to be the most common serotype, although the reasons are not clear. Recently, most of the STEC O157 genome sequence are reported [50], showing a genome approximately 17% larger than that of *E. coli* K-12. In addition, many virulence-related regions were identified. For example, a type III secretion gene cluster was recently identified, which appears to be associated with pathogenicity [51]. In conclusion, gene acquisition in the STEC chromosome might be associated with STEC infection, and those additional genes therefore would have to be examined for further molecular epidemiological study of STEC.

ACKNOWLEDGEMENTS

This work was supported, in part, by a grant for International Health Cooperation Research (9C1) from the Ministry of Health and Welfare. We wish to thank Professor Ben Adler for critical suggestions on the manuscript.

REFERENCES

1. Griffin PM, Tauxe RV. The epidemiology of infections caused by *Escherichia coli*, other enterohemorrhagic *E. coli*, and the associated hemolytic uremic syndrome. Epidemiol Rev 1991; **13**: 60–98.
2 Zoonotic Non-O157 Shiga Toxin-Producing *Escherichia coli* (STEC). Report of a WHO Scientific Working Group Meeting, Germany, 1998.
3. Acheson DWK, Keusch GT. Which Shiga toxin-producing types of *E. coli* are important? ASM News 1996; **62**: 302–6.
4. Karmali MA. Infection by verocytotoxin-producing *Escherichia coli*. Clin Microbiol Rev 1989; **2**: 15–38.
5. Beutin L, Geier D, Zimmermann S, Karch H. Virulence markers of Shiga-like toxin-producing *Escherichia coli* strains originating from healthy domestic animals of different species. J Clin Microbiol 1995; **33**: 631–5.
6. Kudva IT, Hatfield PG, Hovde CJ. Characterization of *Escherichia coli* O157:H7 and other shiga toxin-producing *E. coli* serotypes isolated from sheep. J Clin Microbiol 1997; **35**: 892–9.
7. Montenegro MA, Bute M, Trumpf T, et al. Detection and characterization of fecal Verocytotoxin-producing *Escherichia coli* from healthy cattle. J Clin Microbiol 1990; **28**: 1417–21.
8. Rice DH, Hancock DD, Besser TE. Verotoxigenic *E. coli* O157 colonization of wild deer and range cattle. Vet Rec 1995; **137**: 524.
9. Tanaka H, Kondo R, Nishiuchi C, Matsuda J, Kimura M. Isolation of Verocytotoxin-producing *Escherichia coli* from cattle and pets. J Japn Assoc Infect Dis 1991; **66**: 448–55.
10. Gyles CL, De Grandis SA, MacKenzie C, Brunton JL. Cloning and nucleotide sequence analysis of the genes determining verocytotoxin production in a porcine edema disease isolate of *Escherichia coli*. Microb Pathog 1988; **5**: 419–26.
11. Paton JC, Paton AW. Pathogenesis and diagnosis of Shiga toxin-producing *Escherichia coli* infections. Clin Microbiol Rev 1998; **11**: 450–79.
12. Weinstein DL, Jackson MP, Samuel JE, et al. Cloning and sequencing of a shiga-like toxin type II variant from an *Escherichia coli* strain responsible for edema disease of swine. J Bacteriol 1988; **170**: 4223–30.
13. Muniesa M, Jofre J. Abundance in sewage of bacteriophages that infect *Escherichia coli* O157:H7 and that carry the Shiga toxin 2 gene. Appl Environ Microbiol 1998; **64**: 2443–8.
14. Muniesa M, Jofre J. Occurrence of phages *infecting Escherichia coli* O157:H7 carrying the Stx2 gene in sewage from different countries. FEMS Microbiol Lett 2000; **183**: 197–200.
15. Asakura H, Makino S-I, Shirahata T, et al. Detection and long-term existence of Shiga toxin (Stx)-producing *Escherichia coli* in sheep. Microbiol Immunol 1998; **42**: 683–8.
16. Asakura H, Makino S, Shirahata T, et al. Detection and genetical characterization of Shiga toxin-producing *Escherichia coli* from wild deer. Microbiol Immunol 1998; **42**: 815–22.
17. Makino S-I, Kobori H, Asakura H, et al. Detection and characterization of Shiga toxin-producing *Escherichia coli* from seagulls. Epidemiol Infect 2000; **125**: 55–61.
18. Iwasa M, Makino S-I, Asakura H, Kobori H, Morimoto Y. Detection of *Escherichia coli* O157:H7 from *Musca domestica* (Diptera: Muscidae) at a cattle farm in Japan. J Med Ent 1999; **36**: 108–12.
19. Yamasaki S, Lin Z, Shirai H, et al. Typing of verotoxins by DNA colony hybridization with poly- and oligonucleotide probes, a bead-enzyme-linked immunoorbent assay, and polymerase chain reaction. Microbiol Immunol 1996; **40**: 345–52.
20. Calderwood SB, Auclair F, Donohue-Rolfe A, Keusch GT, Mekalanos JJ. Nucleotide sequence of the Shiga-like toxin genes of *Escherichia coli*. Proc Natl Acad Sci USA 1987; **84**: 4364–8.
21. Jackson MP, Newland JW, Holmes RK, O'Brien AD. Nucleotide sequence analysis of the structural genes for Shiga-like toxin I encoded by bacteriophage 933J from *Escherichia coli*. Microb Pathog 1987; **2**: 147–53.
22. Kozlov YV, Kabishev AA, Lukyanov EV, Bayev AA. The primary structure of the operons coding for *Shigella dysenteriae* toxin and temperate phage H30 shiga-like toxin. Gene 1988; **67**: 213–21.
23. Paton AW, Paton JC, Goldwater PN, Heuzenroeder MW, Manning PA. Sequence of a variant Shiga-like toxin type-I operon of *Escherichia coli* O111:H-. Gene 1993; **129**: 87–92.
24. Paton AW, Beutin L, Paton JC. Heterogeneity of the

amino-acid sequences of *Escherichia coli* Shiga-like toxin type-I operons. Gene 1995; **153**: 71–4.
25. Jackson MP, Neill RJ, O'Brien AD, Holmes RK, Newland JW. Nucleotide sequence analysis and comparison of the structural genes for Shiga-like toxin I and Shiga-like toxin II encoded by bacteriophages from *Escherichia coli*. FEMS Microbiol Lett 1987; **44**: 109–14.
26. Park YC, Shin HJ, Kim YC. Cloning and nucleotide sequence analysis of verotoxin gene from *Escherichia coli* O157 KNIH317 isolated in Korea. J Microbiol 1999; **37**: 168–74.
27. Paton AW, Paton JC, Manning PA. Polymerase chain reaction amplification, cloning and sequencing of variant *Escherichia coli* Shiga-like toxin type II operons. Microb Pathog 1993; **15**: 77–82.
28. Schmitt CK, McKee ML, O'Brien AD. Two copies of Shiga-like toxin II-related genes common in enterohemorrhagic *Escherichia coli* strains are responsible for the antigenic heterogeneity of the O157:H- strain E32511. Infect Immun 1991; **59**: 1065–73.
29. Meyer TH, Karch H, Hacker J, Bocklage H, Heesemann J. Cloning and sequencing of a Shiga-like toxin II-related gene from *Escherichia coli* O157:H7 strain 7279. Int J Med Microbiol Virol Parasitol Infect Dis 1992; **276**: 176–88.
30. Paton AW, Bourne AJ, Manning PA, Paton JC. Comparative toxicity and virulence of *Escherichia coli* clones expressing variant and chimeric Shiga-like toxin type II operons. Infect Immun 1995; **63**: 2450–8.
31. Makino S-I, Asakura H, Shirahata T, et al. Enterohemorrhagic *Escherichia coli* O157 outbreak in Obihiro city. Biological and molecular characteristics among O157 isolates. J Jpn Assoc Infect Dis 1997; **71**: 1131–6.
32. Kusumoto M, Nishiya Y, Kawamura Y, Shinagawa K. Identification of an insertion sequence, IS1203 variant, in a Shiga toxin 2 gene of *Escherichia coli* O157:H7. J Biosci Bioeng 1999; **87**: 93–6.
33. Pierard D, Muyldermans G, Moriau L, Stevens D, Lauwers S. Identification of new verocytotoxin type 2 variant B-subunit genes in human and animal *Escherichia coli* isolates. J Clin Microbiol 1998; **36**: 3317–22.
34. Paton AW, Paton JC, Heuzenroeder MW, Goldwater PN, Manning PA. Cloning and nucleotide sequence of a variant Shiga-like toxin II gene from *Escherichia coli* OX3:H21 isolated from a case of sudden infant death syndrome. Microb Pathog 1993; **13**: 225–36.
35. Fukuyama M, Yokoyama R, Sakata S, et al. Study on the verotoxin-producing *Escherichia coli*-isolation of the bacteria from deer dung. J Japn Assoc Infect Dis 1999; **73**: 1140–4.
36. Franke S, Harmsen D, Caprioli A, et al. Clonal relatedness of Shiga-like toxin producing *Escherichia coli* O101 strains of human and porcine origin. J Clin Microbiol 1995; **33**: 3174–8.
37. Kim SH, Cha IH, Kim KS, Kim YH, Lee YC. Cloning and sequence analysis of another Shiga-like toxin IIe variant gene (slt-IIera) from an *Escherichia coli* R107 strain isolated from rabbit. Microbiol Immunol 1997; **41**: 805–8.
38. Schmidt H, Scheef J, Morabito S, et al. A new shiga toxin 2 variant (Stx2f) from *Escherichia coli* isolated from pigeons. Appl Environ Microbiol 2000; **66**: 1205–8.
39. Gannon VPJ, Teerling C, Masri SA, Gyles CL. Molecular cloning and nucleotide sequence of another variant of the *Escherichia coli* Shiga-like toxin II family. J Gen Microbiol 1990; **136**: 1125–35.
40. Deresiewicz R, Calderwood SB, Robertus JD, Collier J. Mutations affecting the activity of the Shiga-like toxin IA-chain. Biochemistry 1992; **31**: 3272–80.
41. Deresiewicz RL, Austin PR, Hovde CJ. The role of tyrosine-114 in the enzymatic activity of Shiga-like toxin 1A-chain. Mol Gen Genet 1993; **241**: 467–73.
42. Hovde CJ, Carlderwood SB, Mekalanos JJ, Collier RJ. Evidence that glutamic acid 167 is an active-site residue of Shiga-like toxin I. Proc Natl Acad Sci USA 1988; **85**: 2568–72.
43. Yamasaki S, Furutani M, Ito K, et al. Importance of arginine at position 170 of the A subunit of Vero toxin produced by enterohemorrhagic *Escherichia coli* for toxin activity. Microb Pathog 1991; **11**: 1–9.
44. Ohmura M, Yamasaki S, Kurazono H, et al. Characterization of non-toxic mutant toxins of Verotoxin I that were constructed by replacing amino acids in the A subunit. Microbiol Pathog 1993; **15**: 169–76.
45. Cao C, Kurazono H, Yamasaki S, et al. Construction of mutant genes for a non-toxic verotoxin 2 variant (VT2vp1) of *Escherichia coli* and characterization of purified mutant toxins. Microbiol Immunol 1994; **38**: 441–7.
46. Lingwood CA. Verotoxins and their glycolipid receptors. Adv Lipid Res 1993; **25**: 189–211.
47. Lingwood CA. Role of verotoxin receptors in pathogenesis. Trend Microbiol 1996; **4**: 147–53.
48. Tyrrell GJ, Ramotar K, Toye B, et al. Alteration of the carbohydrate binding specificity of verotoxins from Gal 1-4Gal to GalNAc 1-3Gal 1-4Gal and vice versa by site-directed mutagenesis of the binding subunit. Proc Natl Acad Sci USA 1992; **89**: 524–8.
49. Perera LP, Samuel JE, Holmes RK, O'Brien AD. Identification of three amino acid residues in the B subunit of Shiga toxin and Shiga-like toxin type that are essential for holotoxin activity. J Bacteriol 1991; **173**: 1151–60.
50. Ohnishi M, Tanaka C, Kuhara S, et al. Chromosome of the enterohemorrhagic *Escherichia coli* O157:H7; comparative analysis with K-12 MG1655 revealed the acquisition of a large amount of foreign DNAs. DNA Research 1999; **6**: 361–8.
51. Vallance BA, Finlay BB. Exploitation of host cells by enteropathogenic *Escherichia coli*. Proc Natl Acad Sci USA 2000; **97**: 8799–806.
52. Strockbine NA, Jackson MP, Sung LM, Holmes RK, O'Brien AD. Cloning and sequencing of the genes for Shiga toxin from *Shigella dysenteriae* type 1. J Bacteriol 1988; **170**: 1116–22.

53. Kozlov YV, Kabishev AA, Fedchenko VI, Baev AA. Cloning and primary structure of Shigella toxin genes. Dokl Biochem 1987; **295**: 740–4.
54. Beutin L, Strauch E, Fischer I. Isolation of *Shigella sonnei* lysogenic for bacteriophage encoding gene for production of Shiga toxin. Lancet 1999; **353**: 1498.
55. Paton AW, Paton JC. *Enterobacter cloacae* producing a shiga-like toxin II-related cytotoxin associated with a case of hemolytic uremic syndrome. J Clin Microbiol 1996; **34**: 463–5.
56. Schmidt H, Montag M, Bockemuhl J, Heesemann J, Karch H. Shiga-like toxin II-related cytotoxins in *Citrobacter freundii* strains from humans and beef samples. Infect Immun 1993; **61**: 534–43.

https://doi.org/10.1017/S0950268801005635 Published online by Cambridge University Press