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Multilocus Sequence Typing and Virulence Factors Analysis of *Escherichia coli* O157 Strains in China[§]

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***Escherichia coli* O157:H7, an important food-borne pathogen, has become a major public health concern worldwide. The aim of this study was to investigate the molecular epidemiologic feature of *E. coli* O157:H7 strains in China. 105 *E. coli* O157:H7 isolates were collected from various hosts and places over 9 years. A multilocus sequence typing scheme (MLST) was applied for bacteria genotyping and polymerase chain reaction (PCR) was used for virulence factor identification. Seven new MLST sequence types (STs), namely ST836, ST837, ST838, ST839, ST840, ST841, and ST842 were identified, which grouped into two lineages. Phylogenetic analysis suggested that the most two frequent STs in China, ST837 and ST836, may be the derivatives of *E. coli* O157:H7 Sakai or *E. coli* O157:H7 EDL933. Geographical diversity and host variety of *E. coli* O157:H7 were observed in China. In addition, the different distribution of *tccp* was detected. The data presented herein provide new insights into the molecular epidemiologic feature of *E. coli* O157:H7, and aid in the investigation of the transmission regularity and evolutionary mechanism of *E. coli* O157:H7.**

***Keywords*:** *E. coli* O157: H7, *tccp*, STs, *stx*

Two separate outbreaks of *Escherichia coli* (*E. coli*) O157:H7 in Oregon and Michigan, USA, in 1982 were reportedly caused by the consumption of contaminated ground beef (Riley *et al.*, 1983), and followed by similar large outbreaks in Japan (Michino *et al.*, 1999) and Scotland (Cowden *et al.*, 2001). In 2006 an outbreak in the USA was associated with polluted spinach (Wendel *et al*., 2009) and a further outbreak in 2009 was attributed to the consumption of raw, refrigerated, pre-packaged cookie dough (http://www.cdc.gov/ecoli/2009/0622.html). During 1999 there was a large-scale epidemic in the east of China due to *E. coil* O157:H7. It is associated with several human conditions including diarrhea, hemorrhagic colitis (HC), and hemolytic uremic syndrome (HUS) (Browning *et al.*, 1990; Armstrong *et al.*, 1996; Dunn *et al.*, 2004). Results from several studies suggest that *E. coil* O157:H7 acts in different ways to produce these various human diseases/conditions, and it differs genotypically and in the prevalence of virulence genes (Baker *et al.*, 1997; Kim *et al.*, 1999; Kim *et al.*, 2001; Roe *et al.*, 2003, 2004).

The ability of *E. coli* O157:H7 strains to cause severe diseases in human is primarily related to their capacities to secrete shiga toxin (Stx) and other toxins and to induce attaching and effacing lesion (A/E lesion) (Pradel *et al.*, 2001; Lim *et al.*, 2010). Stx mainly form two subgroups, Stx1 and Stx2, both of which are toxic to humans and animals for their ability to inhibit protein synthesis (O'Brien and Holmes, 1987; Sandvig *et al.*, 2004). Enterohemolysin, encoded by *hlyA* on the large plasmid-borne (about 90 kb) gene, is associated with several severe clinical diseases in humans (Schmidt *et al.*, 1995). The induction of A/E lesion is an outcome of interplay among effector both from bacteria and human, and TCCP (Tir-cytoskeleton coupling protein) is one of the most important effectors in the crosstalk for its Nck-like coupling activity (Garmendia *et al*., 2004). Typically, TCCP consists of an 87 amino acids N-terminus, several almost identical 47 amino acids proline-rich repeats (PRRs) in the middle, and a truncated repeat containing 15 amino acids at the C-terminus (Garmendia *et al*., 2004).

The majority of human *E. coli* O157:H7 infections was caused by the consumption of contaminated food or water (Riley *et al*., 1983; Armstrong *et al.*, 1996; Muhldorfer *et al.*, 1996; Muller *et al.*, 2001; Dunn *et al.*, 2004). In China, *E. coli* O157:H7 has been detected in many different cities, however there is little information available about the prevalence and epidemiological characteristics of this pathogen (Ma *et al.*, 2009). Multilocus sequence typing (MLST) has been proposed as a nucleotide sequence-based approach that can be applied to many bacterial pathogens (Urwin and Maiden, 2003). In this study a MLST system was developed using a collection of archived *E. coli* O157:H7 isolates in China and their virulence genes were identified at the same time.

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Materials and Methods

Bacterial strains used in the study

E. coil O157:H7 isolates were obtained from 11 spots located in Jangsu province and Chongqing in China from 1999 to 2007. They were identified by a sorbitol fermentation test and MUG (4-methylumbelliferyl-beta-D-glucuronide) assay. Then latex agglutination was used to detect somatic and flagella antigens. Bacteria were stored at -80ºC until required. *E. coli* O157:H7 Sakai (GenBank: BA000007) and EDL933 (GenBank: AE005174) were used as reference strains in the study.

MLST analysis

Bacterial DNA extraction was carried out in accordance with the DNA contamination management guidelines of Millar *et al*. (2002). The purified genomic DNA were stored in TE buffer at 4ºC until required. MLST analysis was performed using sequences obtained from seven housekeeping genes, namely *aspc*, *icdA*, *uidA*, *clpX*, *mdh*, *fadD*, and *lysp*. The primers for DNA amplications are designed according to the recommendations of the *E. coli* MLST web site (http://mlst.ucc.ie/mlst/dbs/Ecoli) and are shown in Table 1. The DNA sequences were determined with ABI PRISM 310 DNA sequencer and the program BURST was used for MLST data analysis (Rozas *et al.*, 2003).

Detection of virulence genes

The presence of stx_1, stx_2, *hlyA*, and *tccp* genes were verified by PCR using the primers shown in Table 1 (De Baets *et al.*, 2004). About 5 μl of PCR product was subjected to electrophoresis on a 1.2% agarose gel, stained with ethidium bromide, and examined under a UV illuminator. The number of PRR in *tccp* was calculated according to the approximate length indicated on the agarose gel.

Results

MLST profiles of *E. coli* O157:H7

During a period of about 7 years in 11 monitoring spots, using the biochemical and immunologic assay, 105 *E. coli* O157:H7 strains were isolated from animals (sheep n=40, cattle n=31, fowl n=12, and pig n=9) and from HUS patients (n=13), as shown in Supplementary data Table 1. And these isolates were subjected to MLST analysis. Seven loci selected in this study was subjected to stabilizing selection pressure, as the dN/dS ratio (ratio of non-synonymous substitutions to synonymous substitutions) was lower than 1.0. MLST results resolved the 105 *E. coli* strains into seven new STs, namely ST837, ST841, ST840 ST836, ST838, ST839, and ST842. These STs were new combinations of known alleles except ST841 and ST842, the *clxp* allele (gene accession number: HM640979) of which was not reported before (Supplementary data Table 1). The most frequent STs was ST837 and ST836, represented by 54.3% and 38.1% of total collected isolates, which was followed by ST841 represented by 4 isolates, ST838, ST839, ST840, and ST842 were represented by single isolates (Fig. 1A).

Clonal and phylogenetic analysis of *E. coli* O157:H7

The relationships between the STs was examined using eBURST, which uses allele profiles to reconstruct the relation-

Table 1. Primers used for amplification of housekeeping and virulence genes

Gene locus		Primer sequence (5′→3′)	Gene product or function	Predicted length (bp)
Housekeeping genes	*uidA*	F: CATTACGGCAAAGTGTGGGTCAAT R: CCATCAGCACGTTATCGAATCCTT	beta-D-glucuronidase	658
	mdh	F: GTCGATCTGAGCCATATCCCTAC R: TACTGACCGTCGCCTTCAAC	malate dehydrogenase	650
	lysp	F: CTTACGCCGTGAATTAAAGG R: GGTTCCCTGGAAAGAGAAGC	lysine-specific permease	594
	aspc	F: GTTTCGTGCCGATGAACGTC R: AAACCCTGGTAAGCGAAGTC	aspartate aminotransferase	596
	clpx	F: CTGGCGGTCGCGGTATACAA R: GACAACCGGCAGACGACCAA	ATP-dependent Clp protease	672
	fadD	F: GCTGCCGCTGTATCACATTT R: GCGCAGGAATCCTTCTTCAT	cyl-CoA synthetase	658
	icdA	F: CTGCGCCAGGAACTGGATCT R: ACCGTGGGTGGCTTCAAACA	isocitrate dehydrogenase	669
Virulence genes	*hlyA*	F: AGCCGGAACAGTTCTCTCAG R: CCAGCATAACAGCCGATGT	enterohemolysin	527
	tccp	F: CGCCATATGATTAACAATGTTTCTTCAC R: CTCGAGTCACGAGCGCTTAGATGTATT	attaching and effacing lesion	700-1000
	stx_1	F: ACACTGGATGATCTCAGTGG R: CTGAATCCCCCTCCATTATC	Stx1	614
	stx_2	F: GGTCACTGGTTCGAATCCAGTAC R: GGGATCCTGAATTGTGACACAGATTACACTTGTTAC	Stx2	1400

F, forward primers; R, reverse primers

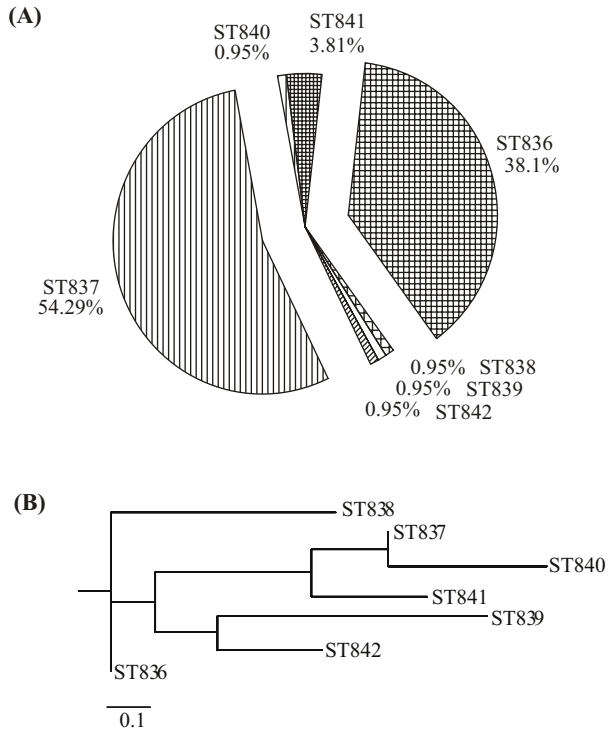

Fig. 1. MLST profiles of *E. coli* O157:H7 in China. (A) The pie chart represents the ratio of seven new STs identified in China. The number near each pie indicates the accurate percentage of different STs. (B) Dendrogram of genetic relationships among seven STs. Linkage distance is indicated by a scale at the bottom.

ships between the different clonal lineages. Results showed that the seven STs were grouped into two linkages (Fig. 2): ST837, ST841, and ST840 belong to one group, which consist of 62 (59.0%) isolates and was represented by ST837, while ST836, ST838, ST839, and ST842 belong to the other group, and it was represented by ST836 (Fig. 1B).

In order to evaluate the relationship between STs in China and that in *Ec*MLST database, the relationships among members of the different STs are demonstrated in the Fig. 2A. The minimum spanning (MS) tree was generated from the allelic profiles by using EHEC O157:H7 strain EDL933 and Sakai (ST69 and ST66 respectively, http://mlst.ucc.ie/mlst/dbs/Ecoli) as outgroup. MS tree showed that *E. coli* O157:H7 STs mainly classied into three clonal complexes, which is represented by ST66 (Sakai strains), ST836 and ST139 respectively. STs isolated in china classied into the same clonal complex, suggesting its relative conserved genetic background. Meanwhile splits tree decomposition demonstrated a similar network among EHEC STs, as indicated by MS tree (Fig. 2B).

Geographic and host distribution of *E. coli* O157:H7 STs

A diverse geographic distribution of STs among different monitoring spots was observed. As shown in Table 2, The majority of *E. coli* O157 was isolated in the spot E (30 isolates, 28.6%) and spot F(27 isolates, 25.7%). ST836 and ST837 were the most universal tpye in all monistoring spots. The 4 ST841 stains were only obtained from the spot E. The rare STs, ST 840, ST838, ST839, and ST842, which consist of only one isolated, was observed at the spot B, D, E, and K, respectively.

Host diversity of *E. coli* O157 was also observed. Sheep and cattle were the main natural reservoir of *E. coli* O157, isolates from which consist of 38.1% (40 isolates) and 29.5% (31 isolates) of total collections respectively (Table 3). STs from human containing ST836, ST837, ST841, and ST842. An interesting finding was that the majority of isolates from sheep, cattle fowls and pigs were ST837 and ST836. However, in human the frequency of ST841 was as high as close to that of ST836 and ST837. In addition, the prevalence of allele *clpX* gene in human isolates was significantly different that in animal isolates, while analyzing the seven housekeeping loci (data not shown).

Virulence gene prevalence of *E. coli* O157:H7

E. coli O157 isolates was subjected to PCR assays and four genes, namely stx_1, stx_2, *hylA*, and *tccp* were investigated. Results showed that all *E. coli* O157 isolates tested carried stx_2 and *hylA*. And only one isolate proved to carry both stx_1 and stx_2, which was isolated from human and belonged to ST841

Table 2. Geographic distribution of *E. coli* O157:H7 STs in 11 monitoring spot

Monitoring spot (address)	Group 1			Group 2				
	ST837	ST 840	ST841	ST836	ST838	ST839	ST842	Total
A (Dongtai)	3	0	0	1	0	0	0	4
B (Taichang)	8	1	0	2	0	0	0	11
C (Fengxian)	6	0	0	7	0	0	0	13
D (Muyang)	6	0	0	3	1	0	0	10
E (Tongshan)	13	0	4	12	0	1	0	30
F (Suzhou)	15	0	0	12	0	0	0	27
G (Donghai)	0	0	0	1	0	0	0	1
H (Binhai)	1	0	0	1	0	0	0	2
I (Guanyun)	1	0	0	0	0	0	0	1
J (Chongqing)	4	0	0	1	0	0	0	5
K (Xuzhou)	0	0	0	0	0	0	1	1
Total	57	1	4	40	1	1	1	105

The number of isolates was shown in the Table.

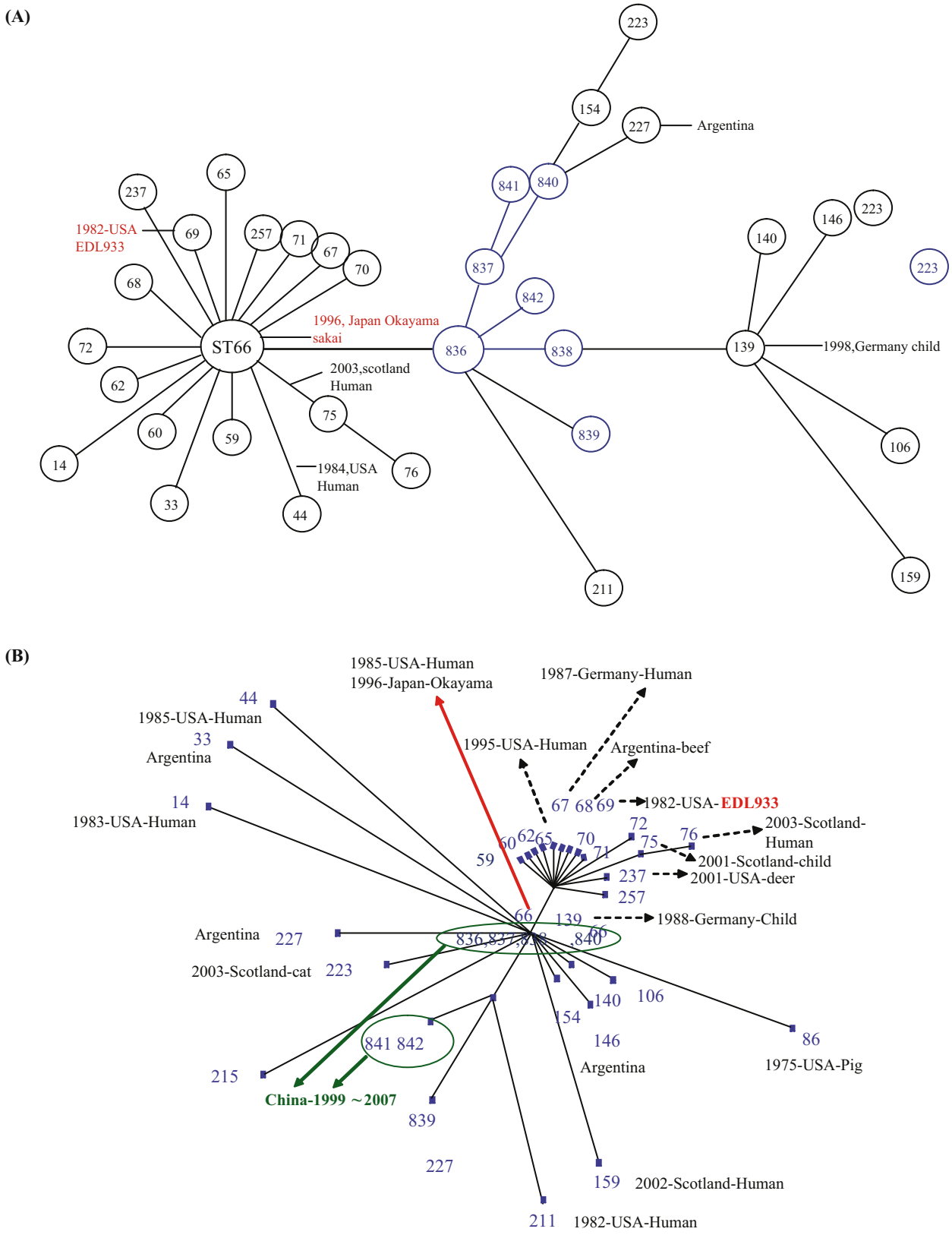

Fig. 2. Phylogenetic analysis of *E. coli* O157:H7. (A) Minimum-spanning tree was generated from the allelic profiles by using EHEC O157:H7 strain EDL933 and Sakai (ST66 and ST69 respectively, http://mlst.ucc.ie/mlst/dbs/Ecoli) as outgroup. Each ST is represented by a circle named with its ST. The STs identified in China were shown in blue. (B) SplitsTree decomposition analysis of MLST data of *E. coli* O157:H7. SplitsTree decomposition network was obtained using distance matrix obtained from allelelic profiles using a web version of Splits-Tree.

Table 3. Host distribution of *E. coli* O157:H7 STs

Host	Group 1			Group 2				
	ST837	ST840	ST841	ST836	ST838	ST839	ST842	Total
Sheep	20	0	0	19	1	0	0	40
Cattle	22	0	1	7	0	1	0	31
Fowl	6	1	0	5	0	0	0	12
Pigs	5	0	0	4	0	0	0	9
Human	4	0	3	5	0	0	1	13
Total	57	1	4	40	1	1	1	105

The number of isolates was shown in the Table.

(Table 4). Interesting findings were obtained in *tccp* gene distribution. Three different *tccp* were found in our isolates, namely *tccp*3, *tccp*4 and *tccp*5, which contains 3, 4, and 5 PRRs respectively. The *tccp*5 was the most frequent gene type, which consist of 81.9% (86 isolates) of total collections. And it was followed by *tccp*3 and *tccp*4, This distribution pattern was in consistant with that in ST836 and ST837. The *tccp*5 appeared in most *E. coli* O157 isolates from human, but in only approximately 70% (73 isolates) of animal isolates.

Discussion

The MLST method provides a scalable typing system that reflects the population and evolutionary biology of the bacterium, and makes valid comparisons between results from different laboratories possible. It applies neutral or slowly accumulating genetic variations in housekeeping genes, which are not affected by the rapid evolution detected within genes encoding proteins that influence survival in a particular niche (Selander *et al.*, 1986; Urwin and Maiden, 2003; Cooper and Feil, 2004). Thus in this study the MLST approach was applied to investigate the clone relationships of *E. coli* O157:H7 isolates from China.

A total of seven new STs were identified, which were not present in the database of EcMLST systems (http://www.shigatox.net/). The BURST analysis indicates a relative conserved genetic background of *E. coli* O157:H7 isolates from China, and it revealed that the ST836 may be the ancestor of the 105 strains studied. ST836, ST837 and ST841 appeared in 1999, when an outbreak of *E. coli* O157 occurred in the east of China. The other STs identified in this study may be the offspring of those originating from the 1999 outbreak. While comparing isolates in china with that from EcMLST database and reference strains Sakai and EDL933, we found a close relationship betweent ST69 (EDL933) and ST66 (Sakai). Thus we proposed that the *E. coli* Sakai strain (ST66), which is responsible for the outbreak in Japan (ST69), is a descendant of EDL933 with DNA insertion/deletion and recombination. In addition, ST836, which represents the STs in China, is also closely associated with ST66. For ST836 were isolated from Chinese patient in the large outbreak in 1997, we speculate that it may be transferred from Japan after genetic mutations.

Table 4. Virulence genes prevalence of *E. coli* O157:H7 STs

Lineages	STs	*tccp3*	*tccp4*	*tccp5*	stx_1	stx_2	Total
Group 1	837	6	2	49	0	57	57
	840	0	0	1	0	1	1
	841	0	0	4	1	4	4
Group 2	836	6	4	30	0	40	40
	838	0	0	1	0	1	1
	839	1	0	0	0	1	1
	842	0	0	1	0	1	1
Total		13	6	86	1	105	105

The number of isolates was shown in the Table. The *tccp*3, *tccp*4, and *tccp*5 stands for *tccp* gene contains 3, 4, and 5 prolin rich repeats respectively.

In this study, we found an extensive genomic diversity within populations of *E. coli* O157, even among different monitoring spots. This variety is inconsistent with that in previous reports (Miao and Miller, 1999; Ohnishi *et al.*, 2001; Allison, 2007). It may because bacteriophage-mediated lateral gene transfer induces numbers of DNA insertion/deletion and recombination events. Meanwhile genetic diversity was also observed between isolates from human and animals. As animals, especially cattle are the natural host for *E. coli* O157:H7, the bacteria may suffer less evolutionary pressure while colonizing in animals than in human (Blanco *et al.*, 2004a, 2004b; Zweifel *et al.*, 2004). This may explain the reason for the genetic variation of *E. coli* 157:H7 isolated from human.

The production of Stx and induction of A/E lesion was considered as the predominant two ways of EHEC to cause infectious diseases, especially HUS. As observed in our study, All 105 isolates investigated carry the *stx* (stx_1 or stx_2) gene, however just a part of them is pathogenic to human. This finding suggests an indispensable contribution of A/E lesion to *E. coli* associated diseases. The different distribution of *tccp* gene in *E. coli* O157:H7 isolates from human and animals provideadditional evidence. TCCP is an important bacterial effector and stimulates actin polymerization during the formation of A/E lesion (Campellone *et al.*, 2004; Garmendia *et al*., 2004). Previous *in vitro* and *in vivo* studies have demonstrated that A/E lesion form more efficiently with the increase of PRRs number (Cheng *et al.*, 2008). The percentage of bacteria carrying *tccp*5 (*tccp* gene with 5 PRRs), *tccp*4, and *tccp*3 in human isolates is significant higher than in animal isolates, respectively. That may be the reason for Stx-producing *E. coli* O157:H7 was unable to cause diseases in human.

To summarize, our findings provided novel insights into the

molecular epidemiologic feature of *E. coli* O157:H7 in China. By MLST method, seven new STs which grouped into two clonal complex were identified and the connection between *E. coli* O157:H7 isolates in China and that in other places of the world was also established. In further studies, we plan to collect and analyze a larger number of isolates to systematically investigate the molecular epidemiology and transmission regularity of *E. coli* O157:H7.

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