International Food Research Journal 18: 683-688 (2011)

High concentration of Shiga toxin 1-producing *Escherichia coli* isolated from Southern Thailand

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Abstract: Shiga toxin positive (*stx*$^+$) *Escherichia coli* were isolated from beef marketed in southern Thailand using an immunomagnetic separation (IMS) technique and detection on CHROMagar. A total of 6 *stx*$^+$ *E. coli* isolates were obtained from 10 beef samples. Four strains were serotype O157 and possessed the *stx*$_2^+$ gene. The other two strains were non-O157 serotypes. Using the PCR technique, we demonstrated one strain possessed *stx*$_1$ whereas the other contained *stx*$_2$. Reverse passive latex agglutination was performed to investigate Stx production, it was found that *stx*$_1^+$ *E. coli* (designated as PSU 5023), produced high levels of Stx1 (titre = 1: 2,048), but the other five *stx*$_2^+$ strains were unable to produce Stx2 or produced it in too low amounts to detect (titre < 2). Investigation of the *Q* gene using primers specific to the *Q* gene region of bacteriophage 933W revealed that only *stx*$_1^+$ *E. coli* PSU 5023 possessed this gene. We suggest that this *Q* gene might promote high level production of Stx1. This is the first report of a high concentration of toxin from a Stx1-producing non-O157 strain of *E. coli* in Thailand and it is important for a public health warning.

Keywords: Shiga toxin, *stx*$_1$, beef, STEC, O157, *Q* gene

Introduction

Shiga toxin-producing *Escherichia coli* (STEC) are important pathogenic bacteria because some strains cause hemorrhagic colitis (HC) and the hemolytic uremic syndrome (HUS) (Riley *et al*., 1982; Karmali *et al*., 1983). These strains are defined as enterohemorrhagic *E. coli* (EHEC). The major virulence factor of EHEC is the Shiga toxin (Stx) that has 2 major types, Stx1 and Stx2. An EHEC isolate may produce Stx1 or Stx2 or both. Stx2 is more important than Stx1 in causing HUS. Stx1 and Stx2 are encoded by *stx*$_1$ and *stx*$_2$ respectively (Griffin, 1995). Most of the EHEC isolated from patients are *E. coli* O157:H7 harboring *stx*$_2$. Disease outbreaks caused by these bacteria have been reported worldwide including the United State, Europe, Asia and Australia (Paton *et al*., 1996; Ahmed and Donaghy, 1998; Michino *et al*., 1998; Rangel *et al*., 2005). However, there have been other serotypes of STEC associated with both sporadic disease and outbreaks and the incidence of disease due to other serotypes is considered to be on the rise (Johnson *et al*., 1996). Four outbreaks of non-O157 STEC were reported in Japan from 1984 to 1995 (Takeda, 1997), with the pathogen belonging to the serotypes O145: NM, O111: NM, and Out: H19. In the United States and in Italy, outbreaks of infection caused by O104:H21 (CDC, 1995) and O111: NM isolates respectively (Caprioli *et al.*, 1994) have been reported.

No outbreak of non-O157 *E. coli* that produced only the single Stx1 toxin had been reported previously in humans. However, in 1996, an outbreak caused by *E. coli* (serotype O118:H2) that produced only the Stx1 toxin occurred at a junior high school in Komatsu, Japan (Hashimoto *et al*., 1999). A total of 126 subjects (22.5%) developed a diarrhoeal illness. The pathogen was isolated from the stools of 131 subjects, 49 of which were asymptomatic. Gastrointestinal symptoms from the diarrhoeal subjects resembled those associated with previous infections of Stx-producing *E. coli*, but were mild. No cases of the hemolytic-uremic syndrome developed. One month later a small outbreak (the number of patients was 6) caused by only Stx 1-producing *E. coli* O26:H11 occurred in Toyama prefecture, Japan (Takeda, 1997). Thus, incidence of infections cause by Stx1-producing *E. coli* is becoming increasingly important.

It has been demonstrated that clinical O157 strains produced different amounts of Stx2 (Wagner *et al*., 1999). The genes encoded for *stx*$_1$ and *stx*$_2$ are found on lysogenic lambdoid bacteriophages (Schmidt, 2001). In the *stx*$_2$ phage genome, the *stx*$_2$ gene is located downstream of the phage *Q* gene which encodes for the antiterminator Q protein that plays an important role in regulating toxin production (Robert *et al*., 1998). The *Q* gene of bacteriophage 933W (Q_{933}) was demonstrated to implicate in most clinical O157:H7 isolates (LeJeune *et al*., 2004). In this study, we have isolated a non-O157 *E. coli* strain that harbors *stx*$_1$ and produced a high titre of Stx1.

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Its *Q* gene including other important characteristics were examined and its significance to current public health issues is discussed.

Materials and Methods

Isolation of STEC

Beef samples were purchased from various fresh markets in Hat-yai city, Thailand. All of them were from local Thai cattle. The samples were delivered to the laboratory within 2 h of collection and processed immediately. Fifty grams of beef were mixed by homogenizer, with 450 ml of Tryptic Soy broth (TSB; Becton Dickinson). For the first enrichment culture, a portion of the liquid was separated and mixed with 20 µg/ml of novobiocin, then incubated at 37ºC for 6 h. A second enrichment culture was performed by transferring 1 ml of the first enrichment culture to 10 ml of TSB and incubated at 42ºC for 2 h (Koitabashi *et al*., 2008).

Immunomagnetic separation technique

One milliliter of the culture was treated with 20 µl of immunomagnetic beads coated with antibody specific to the O157 antigen (Dynabeads, Oslo, Norway). The immunomagnetic beads were recovered after gently, continuously inverting the tube for 30 min at room temperature. The beads were washed with phosphate buffer saline pH 7.4 then spread on a CHROMagar O157 plate (CHROMagar microbiology, Paris, France). The plate was incubated at 37ºC for 16-18 h and 3 mauve colonies were selected from each plate for examination the presence of *stx* and *eae* genes.

Determination of stx *and* eae *genes*

The tested isolates were investigated for stx_1, stx_2, and *eae* genes by PCR method using EVT-1 and EVT-2, EVS-1 and EVS-2, and AE19 and AE20 primer pairs, respectively, as described previously (Vuddhakul *et al*., 2000). PCR amplification of the stx_1 and *eae* genes was performed in a 25 µl reaction mixture consisting of 3.0 mM $MgCl_2$, 0.1 mM dNTPs, 0.4 µM of each primer pair, 0.5 U of *Taq* DNA polymerase, 1X buffer and 1.0 µl of DNA template. Amplification was performed with a single cycle at 94ºC for 3 min, followed by 35 cycles of denaturation at 94ºC for 1 min, annealing at 55ºC for 1 min, and extension at 72ºC for 1 min, and a final extension at 72ºC for 7 min. PCR amplification of stx_2 used the same procedure as described above except the annealing temperature was 50ºC.

Investigation of Stx production

In this study, Stx production by all STEC isolates was determined by PCR targeted to the *Q* gene and its surrounding region using the toxin-non-producing PCR (TNP-PCR) technique (Koitabashi *et al*., 2006). Briefly, the 20 µl of reaction mixture contained 0.2 mM each of the dNTPs, 0.2 µM of each of the primer pair (either TNPf1 and TNPr1, TNPf2 and TNPr2, TNPf3 and TNPr2, or TNPf4 and TNPr3), 4 mM $MgCl_2$, 0.5 U *Taq* DNA polymerase (Promega, U.S.A.) and 2 µl of the 10X buffer. PCR conditions included a pre-heat step at 96ºC for 5 min followed by 30 cycles of amplification each consisting of 1 min denaturation at 94ºC for 1 min, annealing at 55ºC for 1 min, extension at 72ºC for 1 min, and then followed by one extension cycle at 72ºC for 7 min.

In addition, the production of Stx was confirmed by reverse passive latex agglutination (RPLA) using the VTEC-RPLA kit (Denka Seiken Co., Ltd, Japan). The highest dilution of the test sample, giving a positive reaction was defined as the RPLA titer. The test was carried out in duplicate.

Serotyping

Serotyping of O antigen was performed using *E. coli* O157 antiserum (Denka Seiken Co., Ltd, Japan). Briefly, the tested strain was cultured on LB for 16-18 h and was subjected to autoclaved at 121ºC for 15 min. Then the harvested cells were agglutinated with the antiserum.

Q_{933}/Q_{21} *examination*

To investigate any correlation between Stx production and the type of *Q* gene, PCR amplification of the *Q* gene was performed using two primer pairs, qEf-1 and qEr-2, and qDf-1 and qDr-2, specific to the *Q* gene detected in EDL933 (Q_{933}) and Thai-12 *E. coli* (Q_{21}) strains, respectively (Koitabashi, *et al*., 2006). PCR was performed in a 20 µl volume consisting of 20 ng of chromosomal DNA, 2.5 mM $MgCl_2$, 0.1 µM of each primer pair, 0.4 mM of each dNTP, 0.5 U of *Taq* DNA polymerase (Promega, U.S.A.), and 1X *Taq* DNA polymerase buffer (Promega, U.S.A.). Amplification was performed with a single cycle at 94ºC for 2 min, followed by 30 cycles of denaturation at 94ºC for 30 sec, annealing at 55ºC for 30 sec, and extension at 72ºC for 2 min, and a final extension at 72ºC for 7 min.

Antibiotic susceptibility tests

Antibiotic susceptibility was performed using the disk diffusion method (National committee for clinical laboratory standard 2001). Eight antibiotics (Oxoid, England) were used, ceftriaxone (30 µg), trimethoprim/sulfamethoxazole (1.25 µg), norfloxacin (10 µg), amikacin (30 µg), ciprofloxacin

Table 1. Characterization of Shiga toxin-producing *E. coli* isolated from beef samples

Sample no.	No. of positive strains	Serotype O157/non-O157	Virulence genes		Drug resistance	TNP-PCR	RPLA
			stx_1	stx_2			
5	1	0/1	+	-	S[1]	-	2,048
6	1	1/0	-	+	S	+	<2
7	3	3/0	-	+	S	+	<2
9	1	0/1	-	+	S	-	<2

[1] Susceptible to all antibiotics tested

(5 µg), ceftazidime (30 µg), gentamicin (10 µg) and imipenem (10 µg). *E. coli* ATCC 25922 was used as a control strain.

AP-PCR analysis

Previously we had isolated stx_1^+ stx_2^+ *E. coli* non O157 from beef exported from Malaysia sold in the Hat-yai market (Sukhumungoon *et al*., 2011). Thus to determine if this strain shared the same origin of the stx_1^+ *E. coli* strain isolated in this study, DNA fingerprint of the two strains was analyzed by the AP-PCR technique. DNA was extracted using a standard phenol/chloroform extraction method. AP-PCR was carried out using primer 2 as described previously (Vuddhakul *et al*., 2000). EDL933 and Thai-12 *E. coli* O157 were used as control strains.

Results and Discussion

Ten samples of beef were investigated and 6 strains of STEC were detected in 4 samples (Table 1). Serotyping and virulence genes examination indicated that 4 strains were O157 and harbored stx_2. For the other two non-O157 strains, one possessed stx_1 (designated as PSU 5023) and another possessed stx_2 (designated as PSU 5030). All the stx_2^+ strains except PSU 5030 were positive for the TNP-PCR assay.

In Thailand, infection due to STEC is very rare (Bettelheim *et al*., 1990; Kalnauwakul *et al*., 2007). Therefore, the *Q* gene and the surrounding DNA sequences of the stx_2-positive Thai O157 (Thai-12) strain were determined (Koitabashi *et al*., 2006). It was found that the original sequence of typical stx_2 phage in this region was replaced by the *Q* gene (Q_{21}) and flanking sequences similar to those of the prophage Φ21 and the TNP-PCR has been developed to detect this region. The positive TNP-PCR indicates that the tested strains are unable to produce Stx2 (Koitabashi *et al*., 2006). In this study, all stx_2^+ O157 and non-O157 (PSU 5030) strains were confirmed for Stx2 production by RPLA and none of them produced any toxin (titre < 2).

Interestingly, one strain of *E. coli* containing stx_1 (PSU 5023) was non-O157 and produced high concentration of Stx1 (titer = 2,048) as determined by RPLA. This strain was TNP-PCR negative which indicated that its *Q* gene was not replaced by Q_{21}. Therefore, an investigation of the *Q* gene of PSU 5023 was performed by PCR. This strain produced a positive Q_{933} with a 567 bp amplicon size but was negative for Q_{21} (Figure 1). Katsushi *et al*., (2000) demonstrated that the stx_1 gene of prophage VT1-Sakai was located downstream of the *Q* gene, suggesting that its expression was regulated by the Q protein. In addition, the *Q* gene sequences of prophageVT1-Sakai showed a 97.4% homology to the *Q* gene of the 933W prophage (Q_{933}). It was found that the presence of Q_{933} in EHEC was correlated to the high production of Stx as determined by ELISA (LeJeune *et al*., 2004). In addition, Neely and Friedman (1998) demonstrated that *Q* gene upstream of stx_1 gene of prophage H-19B was high homology (>90%) to *Q* gene of 933W prophage and could activate Stx2 production from 933W prophage. Thus, the high production of Stx1 by the PSU 5023 strain in this study may be due to the presence of Q_{933}. We also found that the TNP-PCR negative strain (PSU 5030) was negative for the presence of Q_{933} (Figure 1). This confirms the important role of Q_{933} in toxin production. Further study of deletion Q_{933} gene and determine Stx1 production in this PSU 5023 strain will confirm this.

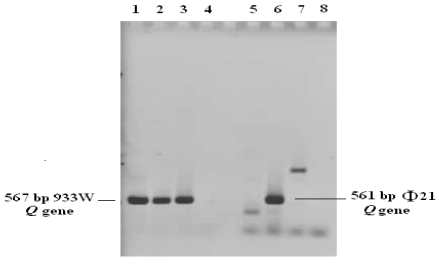

Figure 1. PCR amplification of *Q* gene using 933W *Q* gene (Q_{933}) primer pair, lane 1-4 and Φ21 gene (Q_{21}) primer pair, lanes 5-8. Lane 1, 5: *E. coli* O157:H7 EDL933; lane 2, 6: *E. coli* O157:H7 Thai-12; lane 3, 7: Stx1-producing *E. coli* PSU 5023; lane 4, 8: stx_2^+ *E. coli* PSU 5030

Investigation of stx_2^+ *E. coli* isolated from bovine and ill persons origins demonstrated that 5 out of 66 isolates and 8 out of 91 isolates from bovine and human origin respectively harbored both of Q_{933} and Q_{21} (LeJeune *et al*., 2004). In this study, *Q* gene of Thai-12 also possessed Q_{933} and Q_{21} in the same strain. It is possible that this strain was polylysogenic strain and the presence of Q_{21} in this strain caused no production of Stx2 (Koitabashi *et al*., 2006).

It has been demonstrated that Stx1 acts on renal cells. Perfusion of Stx1 into rat kidneys caused glomerular platelet aggregation, tubular damage, and acute deterioration of renal function (Yamamoto *et al*., 2005). An investigation of Stx1 on cytokine production, found that Stx1 stimulated the inflammatory cytokines, interleukin-1 (IL-1), interleukin-6 (IL-6), and the tumor necrosis factor (TNF), produced by human proximal tubule cells (Hughes *et al*., 1998). Thus these cytokines may contribute to renal dysfunction. In addition, IL-1 and TNF have been demonstrated to induce expression of Gb3 on the surface of human endothelial cells and up-regulate cell sensitivity to Stx cytotoxicity (van de Kar *et al*., 1992).

Strain PSU 5023 obtained in this study was negative for *eae* (data not shown). However, many non-O157 STEC that lacked *eae* have been associated with outbreaks or sporadic human infection (Willshaw *et al*., 1993; Bokete *et al*., 1992). Therefore, detection of the high production of the Stx1 *E. coli* in this study is important for public health and may be an underestimated threat because the strain can utilize sorbitol (data not shown). Most of the non-O157 strains are often overlooked in clinical microbiology laboratories because most laboratories use sorbitol MacConkey agar and serotyping to identify *E. coli* O157:H7. In this study, we employed an immunomagnetic technique using anti-O157 antiserum to separate EHEC O157 and accidentally obtained this non-O157 strain from beef because the colony of this non-O157 on CHROMagar was not different from other EHEC O157 strains. Thus, non-O157 STEC pathogenic strains may be present in the Thai environment but as they produce only a mild infection (Hashimoto *et al*., 1999), they are overlooked by microbiologists, and are therefore, not well recognized.

To determine whether the stx_1^+ PSU 5023 strain originated from a neighboring country, the DNA profile of this strain was compared to $stx_1^+stx_2^+$ *E. coli* derived from Malaysia (Sukhumungoon *et al*., 2011) using the AP-PCR technique. However the DNA profile of PSU 5023 was different from the DNA profile of the Malaysian strain and both of them were different from the DNA profiles of *E. coli* EDL933 and Thai-12 strain (Figure 2). This indicates that their origins are different. The diversity of non-O157 STEC in causing disease has been reported (Caprioli *et al*., 1994; CDC, 1995; Takeda, 1997). Thus serotyping of PSU 5023 appears to be of limited use for the detection of Stx-producing *E. coli*.

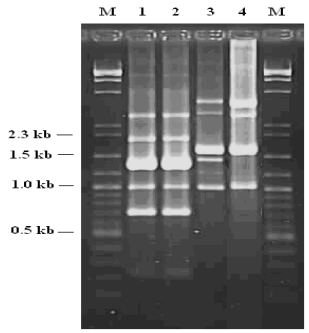

Figure 2. AP-PCR analysis of Thai stx_1^+ *E. coli* PSU 5023 and Malaysian, $stx_1^+stx_2^+$ *E. coli* Lane M, λ/ *Hind* III + 100 bp ladders; lane 1: *E. coli* O157:H7 EDL933; lane 2: *E. coli* O157:H7 Thai-12; Lane 3: PSU 5023; lane 4: Malaysian strain

In this study, all STEC isolates showed susceptible to all antibiotics employed, so it is unlikely that they have a human origin (Table 1). In conclusion, this study demonstrates the presence of stx_1^+ *E. coli* that harbors Q_{933} and produces a high concentration of Stx1 in Thailand and this strain is important for public health concerns.

Acknowledgement

This work was supported by Prince of Songkla Graduate Studies Scholarship.

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