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Quantification of Enterohemorrhagic and Shiga Toxin-Producing *Escherichia coli* from Retailed Meats

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Abstract: Meats are apparently found to be the crucial vehicle for the transfer of Shiga toxin-producing *E. coli* (STEC) to human. The existence of STEC including the enterohemorrhagic *Escherichia coli* (EHEC) in meats was reported worldwide. However, the information in terms of quantity of STEC and EHEC were insufficient in Thailand. In this study, the preliminary investigation of STEC and EHEC quantity in meats was carried out by most probable number-polymerase chain reaction (MPN-PCR) method. In the screening process, a total of 30 (beef, pork and chicken) meat samples purchased from various fresh market in Hat-Yai city, were investigated. The highest amount of STEC was found in beef samples as 27 MPN/g while the lowest amount of STEC was found in chicken meat sample as 3 MPN/g. Only one chicken meat sample revealed the existence of EHEC as 3 MPN/g. No EHEC was found in beef and pork samples. The presence of *E. coli* O157 was also examined by the isolation method using immunomagnetic separation specific to O157 serotype and it displayed the consistent results to the MPN-PCR method. These results exhibited the baseline data on the STEC and EHEC quantity in meats sold in Southern Thai area and may suggest the high prevalence of pathogenic *E. coli* carrying virulence genes which may be resulted in pathogenesis to human.

Key words: EHEC · STEC · Retailed Meat · Thailand · Shiga Toxin

INTRODUCTION

Diarrheal disease plays a pivotal role as the public health concern. Diarrheal disease accounts for 11 % of child deaths worldwide. It kills more than malaria, measles or even AIDS [1]. Amongst six different *Escherichia coli* pathotypes, enterohemorrhagic *E. coli* (EHEC) displays the most devastating effects to the host [2]. This resulted in part from the main virulence factors of this pathotype, Shiga toxins (Stx). Shiga toxin-producing *E. coli* (STEC) carries stx_1 and/or stx_2. EHEC is a subset of STEC [3] which carries an extra gene, *eae* coding for intimin responsible for bacterial attachment [4].

To date, several food-borne outbreaks caused by STEC have been recorded. In United States, as of September 26, 2006, a total of 183 persons from 26 States which infected by an outbreak *E. coli* O157:H7 strain, were reported to Centers for Disease Control and Prevention (CDC). Ninety-five persons (52%) were hospitalized and 29 (16%) displayed hemolytic uremic syndrome (HUS). One dead case was reported and the fresh Spinach was reported to be a source of outbreak [5]. One study from Ethelberg *et al*. [6] reported the outbreak of STEC O26:H11 isolated from organic fermented beef sausage in Denmark in 2007. The bacteria carried stx_1 and *eae* but not stx_2. No cases of HUS reported.

In the central part of Thailand in 2003, the presence of STEC infection was investigated at Bamrasnaradura Infectious Diseases hospital. In that period, 119 non-bloody and 92 bloody diarrheal patients were examined

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for STEC. Five STEC were detected from three patients. They belonged to serotype O26:H$^-$, O111:H$^-$, O128:H2, O125:H21 including one O157:H7 isolates from normal control group [7].

Based upon our previous studies, that aimed to investigate *E. coli* O157:H7 from beef, few STEC were also observed [8,9]. However, the information in term of STEC quantity is still deficient. This encouraged us to quantify the amount of EHEC and STEC in retailed meat samples in this area to gain the preliminary information that is useful for further investigation of EHEC and STEC in the future.

MATERIALS AND METHODS

Sample Collection: In order to obtain the baseline data on the EHEC and STEC quantity, a total of 62 samples comprising three types of meats, beef (Most probable number-polymerase chain reaction (MPN-PCR), *n*=10; Immunomagnetic separation (IMS), *n*= 32), pork (MPN-PCR; *n*= 10), chicken (MPN-PCR; *n*=10), were purchased from various local markets in Hat-Yai city, Songkhla, Thailand between April 2013 and October 2013. The samples were collected once a week to ensure that the meats were from different batches. All purchased samples were processed within 2 h.

Most Probable Number-Polymerase Chain Reaction (MPN-PCR) Method: MPN-PCR was performed as described by Chang *et al*. [10] with slight modifications. Briefly, twenty five grams of meat were homogenized with 225 ml of tryptic soy broth (TSB). The liquid portion was used to perform the three-tube MPN, 100 fold and 1,000 fold dilutions from the prepared stomacher fluids.

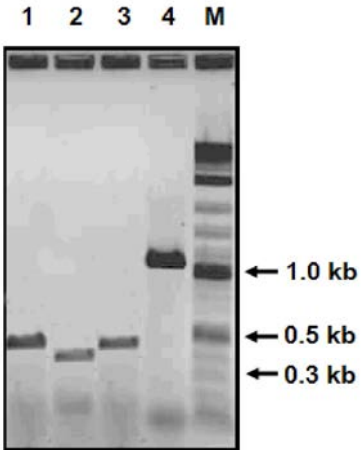

Fig. 1: PCR assay detecting 420 bp *rfb*O157, 350 bp stx_1, 404 bp stx_2 and 1,087 bp *eae* genes. Lane 1, 2, 3 and 4, *E. coli* O157:H7 strain EDL933; lane M, 2-log DNA ladder.

One milliliter from each dilution was transferred into triplicate MPN tubes and incubated at 37°C for 24 h. One milliliter of culture from the turbid tube was then subjected to PCR template prepared by boiling method [11]. The boiled bacterial culture were immersed on ice for 10 min prior to be centrifuged at 11,000 × *g* for 5 min. Decimal dilution of boiled supernatant was used as PCR template. Four genes, stx_1, stx_2, *eae* and *rfb*O157, were investigated for the detection of EHEC and STEC by simplex PCR (Go*Taq* Flexi system, Promega) (Figure 1). The oligonucleotide primers used in this study including the condition for PCR were described in Table 1 and Table 2. PCR amplification was carried out in 25 µl reaction mixture comprising 1X Go*Taq* Flexi green buffer, 3.0 mM $MgCl_2$,

Table 1: Oligonucleotide primers used in this study

Primer	Sequence (5' to 3')	Target gene	Amplicon size	Reference
EVT-1	AATGGTGCTTGCGCTTGCTGC			
EVT-2	GCCGCTTTATCCAACCTGGTA	stx_1	350 bp	[8]
EVS-1	ATCAGTCGTCACTCACTGGT			
EVS-2	CCAGTTATCTGACATTCTG	stx_2	404 bp	[8]
AE-19	CAGGTCGTCGTGTCTGCTAAA			
AE-20	TCAGCGTGGTTGGATCAACCT	*eae*	1,087 bp	[24]
O157-F	CGTGATGATGTTGAGTTG			
O157-R	AGATTGGTTGGCATTACTG	*rfb*O157	420 bp	[25]

Table 2: PCR profiles for virulence genes amplification.

	PCR conditions				
Profile name	Pre-heat	Denaturation	Annealing	Extension	Final extension
stx_1	95°C, 3 min	94°C, 1 min	55°C, 1 min	72°C, 50 sec	72°C, 5 min
stx_2	95°C, 3 min	94°C, 1 min	50°C, 1 min	72°C, 50 sec	72°C, 5 min
eae	95°C, 3 min	94°C, 1 min	55°C, 1 min	72°C, 1.15 min	72°C, 5 min
*rfb*O157	95°C, 3 min	94°C, 1 min	45°C, 1 min	72°C, 50 sec	72°C, 5 min

0.1 mM dNTPs, 0.4 µM each primer pair, 0.5 unit of Go*Taq* DNA polymerase and 2 µl of DNA template. After 35 amplification cycles, the amplicons were observed by 1% agarose gel electrophoresis and stained by ethidium bromide. For amplification of *stx*$_1$, *stx*$_2$, *eae and rfb*O157 genes, *E. coli* O157:H7 strain EDL933 was used as a positive control strain.

Isolation of E. coli O157:H7 from Meat Samples by Immunomagnetic Separation (IMS) Technique: Fifty grams of beef were homogenized with 450 ml of TSB. The liquid portion was separated and incubated at 37°C for 6 h as a first enrichment step. Second enrichment process was performed by transferring 1 ml from first enrichment process into 10 ml of TSB and incubated at 42°C for 2 h [12]. One milliliter of culture was treated with 20 µl of immunomagnetic beads coated with antibody specific to O157 antigen (Dynabeads, Oslo, Norway). After gentle mixing, the beads were recovered through washing steps using phosphate buffer saline pH 7.4 and spread on CHROMagar O157 (CHROMagar Microbiology, Paris, France). The plates were incubated at 37°C for 18 h. Mauve colonies were selected for virulence genes examination as described above.

Antimicrobial Susceptibility Test: All *E. coli* strains were determined for the antimicrobial susceptibility by disk diffusion method [13]. The six common antimicrobial agents used in this experiment were as followed: ceftriaxone (30 µg), trimethoprim/sulfamethoxazole (1.25+23.75 µg), norfloxacin (10 µg), ciprofloxacin (5 µg), ceftazidime (30 µg) and imipenem (10 µg). Clear zone was measured by Vernier caliper. Antimicrobial disks were purchased from Oxoid (UK). *E. coli* ATCC 25922 was used as a control strain.

RESULTS AND DISCUSSION

The estimation of STEC in raw food samples especially meats is important for the public health. Several studies reported the virulence characteristics of STEC isolated from raw food materials in many areas throughout the world [14-17]. Moreover, certain reports described the infections and outbreaks resulted from the consumption of foods contaminated with many STEC serotypes including serotype O157:H7 [6,14,17].

In this study, in order to quantify the amount of EHEC and STEC in meat samples, MPN-PCR method was performed. Although the presence of *stx*$_1$ and/or *stx*$_2$ simultaneously with the presence of *eae* in the same turbid tube, could not be interpreted directly that there were any *E. coli* cells which carry these genes together, in this study, it was attributed that the samples which exhibited these genes pattern, were EHEC and the samples that exhibited only *stx*$_1$ and/or *stx*$_2$ gene only without *eae* gene were considered as STEC. In this investigation, the results revealed that the highest amount of EHEC in meats was 3 MPN/g, which appeared in one chicken meat sample. No EHEC was found in beef and pork samples. The highest quantity of STEC was found in beef samples as 27 MPN/g (*stx*$_2$-habouring *E. coli*) (Table 3). The lowest amount of STEC was found in chicken meat sample as 3 MPN/g (*stx*$_2$-habouring *E. coli*) (Table 3).

Poultry has been shown to be a potential reservoir for EHEC. In Turkey, 190 fresh chicken carcasses were examined for *E. coli* O157:H7 and showed that 2 of 190 (1.05%) carried O157:H7. Those two isolates were capable of producing Stx1 and Stx2 [18]. Additionally, one study from Dipineto *et al*. [19] revealed that 26 of 720 (3.61%) cloacal swab samples from layer hens farms in Italy, carried EHEC serotype O157:H7. In the present study,

Table 3: Quantity (MPN/g) of EHEC and STEC in retailed meats.

			MPN/g	
Type of meat	Pathotype	No. of positive sample	[a]min	max
Beef	EHEC	0	<3	<3
	stx$_1$-habouring *E. coli*	7	<3	16
	stx$_2$-habouring *E. coli*	8	<3	27
Pork	EHEC	0	<3	<3
	stx$_1$-habouring *E. coli*	6	<3	11
	stx$_2$-habouring *E. coli*	3	<3	15
Chicken	EHEC	1	<3	3
	stx$_1$-habouring *E. coli*	3	3	9.3
	stx$_2$-habouring *E. coli*	1	<3	3

[a]Min, Minimum MPN value; Max, Maximum MPN value

EHEC was observed in only one chicken meat sample (3 MPN/g) which was considered very low. The low quantity of EHEC was also observed in Thailand in the past decade. There was only 1 out of 107 (0.93%) chicken samples, exhibited the possession of Stx [20]. Although poultry has been clearly shown to be the natural reservoir of EHEC in many areas of the world, poultry may not be the potential reservoir of EHEC in Thailand.

Beef and beef products have been known to be contaminated by STEC serotype O157:H7 and non-O157 with different contamination rates. Hussein [21] described the global assessment of STEC on beef throughout the past three decades. The prevalence rates of STEC non-O157 in ground beef, sausage, various retail cuts and whole carcasses were ranged from 2.4 to 30%, 17 to 49.2%, 11.4 to 49.6% and 1.7 to 58%, respectively. In addition, the corresponding prevalence rates of O157 serotype were 0.1 to 54.2%, 0.1 to 4.4%, 1.1 to 36.0% and 0.01 to 43.4%, respectively. In the current study, the beef retail cuts were examined as the surrogate reflecting the prevalence rate of STEC in beef in this area. Seven of 10 (70%) and 8 of 10 (80%) beef samples were shown to carry stx_1 and stx_2-habouring *E. coli*, respectively, by MPN-PCR (Table 3). The maximal amount of stx_1-habouring *E. coli* was 16 MPN/g and the maximal amount of stx_2-habouring *E. coli* was 27 MPN/g. The similar trend of the results was observed in porcine group as 11 MPN/g and 15 MPN/g for stx_1 and stx_2-habouring *E. coli*, respectively (Table 3).

In Thailand, early report from Suthienkul *et al*. [20] described the presence of STEC in vegetables, meats, cattle and farm animals. Beef samples also reflected the most prevalence of STEC as 9% (8 of 93 samples).

Based upon the quantification of STEC and EHEC by MPN-PCR in the current study, no *E. coli* O157:H7 was found. The lack of O157:H7 in early 30 meat samples was questioned whether there was no *E. coli* O157:H7 in those meat samples or the sensitivity of MPN-PCR approach was below the detection limit. Thus, additional 32 beef samples were further investigated for *E. coli* O157:H7 by IMS. Of 32 beef samples, *E. coli* O157:H7 was not found in early 31 samples but *E. coli* O157:H7 isolates were eventually found in the last sample (6 isolates) marketed on late October 2013. All isolates represented the same *stx* and *eae* genotypic pattern as shown in the former strains isolated in this area in last decade [8,9,11,22]. In addition, antimicrobial susceptibility test revealed that these six isolates were 100% susceptible to all antimicrobial agents tested. Estimation of the incidence of STEC carriage is complicated. Fecal shedding may be transient and is almost certainly affected by several factors including diet, stress, population density, geographical region and season [23]. In 2008 and 2010, 24 *E. coli* O157:H7 were isolated from 14 beef samples [8,9]. Contrary, the attempt in 2012 and 2013 revealed that 13 *E. coli* O157:H7 were isolated from only three beef samples. In this study, a range of approximately six months was applied for isolation and quantification of STEC including *E. coli* O157. However, in this six months interval, only one isolate from one beef sample was obtained. Thus, in this study, we also observed the similar phenomenon as Kudva *et al*. [23] and both MPN-PCR and IMS approaches were considered to demonstrate the corresponding results.

CONCLUSION

Retailed meat represents a risky source for EHEC and STEC in Hat-Yai city, Songkhla, Thailand that threaten human health through the food chain. Low hygienic performance of the butchers and cookers may transfer these types of bacteria to the consumers. Thus, the campaign for good practice in cooking should be announced to the public. Meanwhile, the high surveillance frequency of EHEC and STEC in common fresh meat types is encouraged to be performed to prevent the outbreaks by these *E. coli* pathotypes in this area.

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