

# MOST PROBABLE NUMBER-POLYMERASE CHAIN REACTION-BASED QUANTIFICATION OF ENTEROTOXIGENIC *ESCHERICHIA COLI* FROM RAW MEATS IN SOUTHERN THAILAND

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**Abstract.** The detection of enterotoxigenic *Escherichia coli* (ETEC) in food, especially raw meat, has rarely been documented in Thailand, although the presence of this bacterial pathogen is considered of important public health concern. The quantity of ETEC in 150 meat samples collected from fresh food markets in southern Thailand were determined using a most probable number (MPN)-PCR-based quantification approach. ETEC contamination of raw chicken, pork and beef samples was 42%, 25% and 12%, respectively (a significant difference between chicken and beef,  $p < 0.05$ ). The maximum MPN/g value for enterotoxin gene *est*-positive ETEC from pork and *elt*-positive ETEC from chicken were  $> 1,100$  MPN/g, but the range of MPN/g values was greater for ETEC from chicken than from pork or beef. ETEC from raw chicken meat contained significantly more *elt*- than *est*-positives ( $p < 0.05$ ). Thus, a significant proportion of raw meat, in particular chicken, sold in fresh food markets in southern Thailand harbors ETEC and poses a potential threat to consumer health.

**Keywords:** enterotoxigenic *Escherichia coli*, meat, most probable number PCR, Thailand

## INTRODUCTION

Diarrheagenic *Escherichia coli* plays a role as one of the causative agents of gastrointestinal illnesses. Among the six categories of *E. coli*, viz enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli*

(EHEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC) and diffusely adherent *E. coli* (DAEC), ETEC is the most common especially in developing countries (Anonymous, 1999). ETEC is associated with morbidity and mortality in children under five years of age, as well as travelers to the endemic areas (Subekti *et al*, 2003; Anonymous, 2006). After the first report of ETEC in Calcutta, India, in 1956 (De *et al*, 1956), a gradual increase of ETEC cases has been documented, with approximately 800,000 cases of death attributed to ETEC annually (Turner *et al*, 2006).

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Pathology caused by ETEC is due to the heat-labile (LT) or heat-stable enterotoxin (ST) or both (Stacy-Phipps *et al*, 1995). Genes encoding LT (*elt*) and ST (*est*) are located on a transmissible plasmid. LT is a 84 kDa oligomeric protein composed of one A-subunit and five B-subunits (Spangler, 1992), and is structurally, functionally and immunologically similar to the closely related cholera toxin (CT) produced by *Vibrio cholerae* O1. LT induces a watery diarrhea via irreversibly binding to GM1 ganglioside, resulting in an increase of intracellular cyclic AMP level followed by an imbalance of the bowel's absorptive capacity. On the other hand, ST is a low molecular weight, non-antigenic protein of 72 amino acids. Its cysteine-rich pre-propeptide is processed during export to form a mature active toxin of 18 to 19 amino acids (So and McCarthy, 1980). ST binds reversibly to guanylate cyclase, leading to increased levels of cyclic GMP and, as with LT, ST engenders outpouring of diarrheal stool through a similar mechanism as LT (Qadri *et al*, 2005).

In addition to the enterotoxins, the presence of colonization factors (CFs) is also important for ETEC pathogenesis. To effectively engender illness, ETEC establishes adherence to the epithelium of the small intestine by means of CFs prior to attacking the cells by secreting enterotoxins. More than 23 different human ETEC CFs are currently known (Gaastra and Svennerholm, 1996), but additional CFs may exist (Nazarian *et al*, 2014). A wide variety of CF antigens (CFAs), coli surface antigens (CSs) and putative CFs (PCFs) have been reported (all nomenclatures except CFAs are currently designated as CS) (Qadri *et al*, 2005), the most common are CFA/I, CS1-6 and CS21 (Subekti *et al*, 2003; Isidean *et al*, 2011; Rodas *et al*, 2011; Svennerholm and Lundgren, 2012).

The presence of enterotoxins and various CFs of ETEC are crucial for human illness. In southern Thailand, Kalnauwakul *et al* (2007) have reported the presence of ETEC in 13 of 530 (2.5%) diarrheal stool samples collected at Songklanagarind Hospital, Hat Yai City, Songkhla Province. Of these 13 isolates, 7 are *est*-positive and 5 *elt*-positive ETEC, and one carries both genes. In addition, of 9 isolates subjected for susceptibility to antimicrobial agents, all isolates exhibited resistance to 6 of 10 common antimicrobial agents tested. The diarrheal patients undoubtedly obtained ETEC by consumption of contaminated water or food (Blanco *et al*, 1995; Daniel *et al*, 2000; Huerta *et al*, 2000), and one of the potential sources of ETEC is raw meat (Lee *et al*, 2009; Kagambèga *et al*, 2012).

However, data regarding ETEC quantity in raw meat in Thailand are insufficient. Thus, this study quantified the natural contamination of ETEC in meat sold in retail outlets in southern Thailand.

## MATERIALS AND METHODS

### Sample collection

A total of 182 meat samples comprising of chicken (MPN-PCR,  $n=64$ ; isolation process,  $n=16$ ), pork (MPN-PCR,  $n=36$ ; isolation process,  $n=8$ ) and beef (MPN-PCR,  $n=50$ ; isolation process,  $n=8$ ), were purchased from various fresh food markets in Hat Yai City, Songkhla Province, Thailand during March to June 2014. All samples were processed within 2 hours after purchased.

### Isolation of ETEC from meat samples

Ten grams of meat from each sample were homogenized with 90 ml of tryptic soy broth (TSB) (Difco, Detroit, MI) and sedimented. The supernatant was incubated at 37°C for 6 hours without shaking

and 1 ml aliquot was centrifuged at 5,000g for 1 minute. The pellet (bacterial cells) was suspended in 100  $\mu$ l of 0.1 M sodium phosphate buffer pH 7.4 (PBS). One loopful of 10<sup>-2</sup>-diluted (in PBS) suspension was streaked on eosin-methylene blue agar (EMB) and incubated at 37°C for 18 hours. Ten colonies (with metallic sheen character) were selected randomly for detection of virulence genes as described below.

#### Most probable number (MPN)-PCR-based quantification of ETEC

MPN-PCR was performed as described previously (Pannuch *et al*, 2014). In brief, 1 ml aliquots of 10-fold, 100-fold and 1,000-fold diluted homogenized meat supernatant were incubated at 37°C for 24 hours, then boiled for 10 minutes and placed on ice for 10 minutes prior to centrifugation at 11,000g for 5 minutes. A 10-fold diluted (in sterile deionized water) aliquot of the supernatant was used as PCR template. Oligonucleotide primers used for amplification of *elt* and *est* and their annealing temperatures are shown in Table 1. PCR amplification was carried out in 25  $\mu$ l reaction mixture comprising 1X GoTaq Flexi Green buffer, 3.0 mM MgCl<sub>2</sub>, 0.1 mM dNTPs, 0.4  $\mu$ M each primer pair, 0.5 U GoTaq DNA polymerase (Promega, Madison, WI) and 2  $\mu$ l of DNA template. After 35 amplification cycles (Table 1), amplicons were analyzed by 1.0% agarose gel-electrophoresis and staining with ethidium bromide and visualized in an ATTO darkroom WSE-5200 Printgraph 2M gel imaging system (Tokyo, Japan). Each experiment was conducted in triplicate. ETEC strains PSU192 and PSU237 was used as positive strain for amplification of *est* (190 bp) and *elt* (450 bp) respectively. MPN value was computerized by MPN software, VB6 version (<http://www.i2workout.com/mcuriale/mpr/>).

#### Statistical analysis

Data were analyzed using SPSS for Windows software, version 11.0 (SPSS, Chicago, IL). Pearson chi-square ( $X^2$ ) was used to compare the relationship between the presence of ETEC and type of meat, and Mann-Whitney *U* test was employed to determine the difference in ETEC existence between groups. Student's *t*-test was applied to determine the difference between *est*- and *elt*-positive ETEC. Level of significance is set at a  $p < 0.05$ .

## RESULTS

In order to quantify the amounts of ETEC in raw meat, the MPN-PCR approach was applied to 150 meat samples (chicken, pork and beef). ETEC was found in 27/64 (42%) of chicken meat samples, which is insignificantly higher than that in pork (9/36, 25%) ( $p > 0.05$ ). However, it was found significantly higher than in beef which exhibited only 12.0% (6 of 50 samples) ( $p < 0.05$ ) (Table 2).

Focusing on each type of meat, in chicken, 7/64 (11%) of ETEC-positive samples carried *est*, with the highest MPN/g value of 93, while *elt*-positive ETEC was detected in 20/64 (31%) samples, with the highest MPN/g value of >1,100 (Table 2). For pork samples, *est*-positive ETEC was found in 4/36 (11%) samples, with the highest MPN/g value of > 1,100 (found in one sample), and 5/36 (14%) *elt*-positive ETEC were detected, with maximum MPN/g value of 9.2. As for the beef samples, *est*-positive ETEC was not found, but *elt*-positive ETEC was present in 6/50 samples (12%), with the highest MPN/g value of 150. However, for ETEC isolation performed in late June, 2014, additional 320 isolates were recovered from 32 meat samples (chicken,  $n = 16$ ; pork,  $n = 8$ ; and beef,  $n = 8$ ). Among 320 ETEC isolates

Table 1  
Oligonucleotide primers used in the study.

Target gene <sup>a</sup>	Name	Sequence (5' to 3')	PCR condition Temp <sup>o</sup> C (time in minute)				Amplicon size (bp)	
			Pre-heat	Denaturation	Annealing	Extension Finalization		
<i>elt</i>	TW20	GGCGACAGATTATACCGTGC	95 (3)	94 (1)	50 (1)	72 (1)	72 (5)	450
	JW11	CGGTCTATATCCCTGTT						
<i>est</i>	JW14	ATTTTMTTTCGTATTRCIT	95 (3)	94 (1)	40 (1)	72 (0.5)	72 (5)	190
	JW7	CACCCGGTACARGCAGGATT						

<sup>a</sup>Stacy-Phipps *et al*, 1995.

(from 32 samples), no enterotoxin genes were detected (data not shown).

## DISCUSSION

Although ETEC, one of diarrheagenic pathogens, has been studied in humans in Thailand for at least three decades (Rasrinaul *et al*, 1988; Echeverria *et al*, 1994; Ratchrachenchai *et al*, 2004), nonetheless, the presence of ETEC in food samples were less reported particularly in raw meat. In this study, we found that over 25% of raw meat sold in fresh food markets in southern Thailand were contaminated with ETEC. This high rate of ETEC detection in our survey is at odds with previous reports from other regions of Thailand. Of 390 raw meat samples collected in Bangkok, located in central Thailand, using Y-1 adrenal cell and suckling mouse assay, ETEC was detected in 2% and 8% of pork and beef, respectively (Rasrinaul *et al*, 1988). Echeverria *et al* (1994), using the same procedure, reported the presence of ETEC in 0.7% of 135 beef samples and none in 133 pork or 131 chicken samples from Ratchaburi Province, western Thailand. In these two studies, only 1 g of each meat sample (compared with 10 g in our study) was analyzed and thus may provide an under estimation of the actual level of ETEC contamination in those meat samples.

Even though raw meat is a potential source of ETEC infection (Lee *et al*, 2009; Bonyadian *et al*, 2011), reports of the presence in such a source have varied depending on countries where the investigations were conducted. A study from Korea reported 14% prevalence of pathogenic *E. coli* in fresh meat collected in several provinces, with ETEC constituting 43.6% (Lee *et al*, 2009). On the other hand, in Bogota, Colombia, no ETEC was detected in

Table 2  
MPN/g of enterotoxigenic *E. coli* in retail meats from southern Thailand, March-June, 2014.

Type of meat	Pathotype	No. of positive sample/total sample (%)	Percentage ETEC positive sample	MPN/g		
				min <sup>a</sup>	med	max
Beef ( <i>n</i> =50)	<i>est</i> -positive ETEC	0/50 (0)	12 <sup>b</sup>	<3	<3	<3
	<i>elt</i> -positive ETEC	6/50 (12.0)		<3	3 to 36	150
Pork ( <i>n</i> =36)	<i>est</i> -positive ETEC	4/36 (11) <sup>b</sup>	25 <sup>B,b</sup>	<3	3 to 15	>1,100
	<i>elt</i> -positive ETEC	5/36 (14) <sup>b</sup>		<3	3 to 9.2	9.2
Chicken ( <i>n</i> =64)	<i>est</i> -positive ETEC	7/64 (11) <sup>b</sup>	42 <sup>B</sup>	<3	3 to 15	93
	<i>elt</i> -positive ETEC	20/64 (31) <sup>B</sup>		<3	3 to 460	>1,100
Total ( <i>n</i> =150)		42 <sup>c</sup> /150 (28)	28			

<sup>a</sup>min, minimum MPN/g value; med, MPN/g values between min and max; max, maximum MPN/g value. <sup>B,b</sup>*p*<0.05 compared to beef sample. <sup>c</sup>Two samples (1 pork and 1 chicken) carried both *est* and *elt*.

76 food samples (Rúgeles *et al*, 2010). This could be due to the relatively cold climate in that city and that the samples were collected from supermarkets where it is more hygienic than open-air fresh food markets. The occurrence of ETEC infection has been reported to depend on the seasonality, with the peaks of detection in the warmer periods of the year (Qadri *et al*, 2000; Rao *et al*, 2003). Temperatures in southern Thailand ranged from 31.7°C to 36.8°C in the middle of April, 2014 (TMD, 2014).

In the ETEC isolation processes, although we could not isolate ETEC from 32 meat samples collected in late June, 2014 when the environmental temperature was dramatically decreased, possibly because of frequent rains in that time period, this current study clearly demonstrated the existence of the core ETEC virulence genes, *elt* and *est*, belonging to STI type, which is toxic to humans (Moseley *et al*, 1983). Subsequent clinical characteristics of ETEC ingestion may involve abdominal

pain, fever, nausea, vomiting and a sudden watery diarrhea as striking features, leading to a dehydration and shock in the most severe forms (Qadri *et al*, 2005).

One of the recommendations issued by the Centers for Disease Control and Prevention, USA (CDC, 2005) to travelers entering ETEC endemic areas is to avoid the consumption of undercooked meat, including poultry as the latter is more prone to ETEC contamination, shown in this and other studies (Lee *et al*, 2009; Bonyadian *et al*, 2011). The presence of ETEC in raw or undercooked meat remains an important public health issue.

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