ENTEROAGGREGATIVE *ESCHERICHIA COLI* O104 FROM THAI AND IMPORTED MALAYSIAN RAW BEEF

Nureesan Wameadesa[1,2], Aphisara Sae-lim[2], Fadeeya Hayeebilan[2], Pattamarat Rattanachuay[3] and Pharanai Sukhumungoon[1,2]

[1]Food Safety and Health Research Unit, [2]Department of Microbiology, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla; [3]Department of Pre-clinic, Faculty of Science and Technology, Prince of Songkla University, Pattani campus, Pattani, Thailand

Abstract. Local Thai and imported Malaysian beef in southern Thailand area carry several Shiga toxin-producing *Escherichia coli* (STEC) serotypes. STEC O104 is an important pathogen capable of causing outbreaks with considerable morbidity and mortality. This study investigated the presence of *E. coli* O104 from local Thai and imported Malaysian beef obtained from markets in Hat Yai City, Songkhla Province during August 2015 - February 2016. Thirty-one *E. coli* O104 strains were isolated from 12 beef samples (16% and 23% Thai and imported Malaysian, respectively). Thirty strains possessed *aggA* (coding for a major component of AAF/I fimbriae), a gene associated with enteroaggregative *E. coli* (EAEC) pathotype, and all strains carried *fimH* (encoding Type 1 fimbriae). Thirty strains belonged to phylogenetic group B1 and one strain (from Malaysian beef) to group A. Agglutination of yeast cells was observed among 29 *E. coli* O104 strains. Investigation of stx_2 phage occupancy loci demonstrated that *sbcB* was occupied in 12 strains. Antimicrobial susceptibility assay revealed that 7 strains were resistant to at least one antimicrobial agent and two were multi-drug resistant. One strain carried extended spectrum β-lactamase gene $bla_{\text{CTX-M}}$ and three carried bla_{TEM}. PFGE-generated DNA profiling showed identical DNA pattern between that of one EAEC O104 strain from Thai beef and another from Malaysian beef, indicating that these two strains originated from the same clone. This is the first report in Thailand describing the presence of EAEC O104 from both Thai and imported Malaysian beef and their transfer between both countries. Thorough surveillance of this pathogen in fresh meats and vegetables should help to prevent any possible outbreak of *E. coli* O104.

Keywords: *Escherichia coli*, EAEC O104, imported beef, local beef

INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC), in particular serotype O157:H7, are zoonotic pathogens causing hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) (Karmali *et al*, 1983; Riley *et al*, 1983). While STEC O157:H7 frequently causes a severe form of diarrhea and systemic diseases, a small number of STEC serotypes, *viz*. O91:H21, O113:H21, and O104:H21, are less frequently involved in bloody diarrhea (Bugarel *et al*, 2010). Although these latter STEC sero-

Correspondence: Pharanai Sukhumungoon, Department of Microbiology, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand.
Tel: +66 (0) 74 288322; Fax: +66 (0) 74 288311
E-mail: pharanai82@gmail.com

types can sporadically cause disease, a large outbreak caused by *E. coli* O104:H4 was reported from northern Germany in 2011 and is still the largest outbreak in Germany to date, ending up with 3,816 cases of gastroenteritis, 845 HUS and 54 deaths (Frank *et al*, 2011). This strain carries cardinal virulence genes from two diarrheagenic *E. coli* (DEC) pathotypes, stx_{2a} from an enterohemorrhagic *E. coli* (EHEC), and *aggA* and *aggR* coding for a major subunit of AAF/I fimbriae and a transcriptional activator of AAF/I, respectively, of an enteroaggregative *E. coli* (EAEC) (Kunsmann *et al*, 2015). Even though the source of infection of this German outbreak was suspected primarily to be related to the consumption of contaminated salad sprouts (Karch *et al*, 2012) and the outbreak-causing strain might not be related to farm cattle and their products, these sources are still considered potential vehicles of STEC. More importantly, transmission of *E. coli* from food animals to human intestinal tract has been documented (Bergeron *et al*, 2012), confirming the potential role of raw meat as STEC vehicle.

In southern Thailand, there has been large scale transport of beef across the Thai-Malaysian border and distribution of these meat products throughout Hat Yai City during at least for the past decade, and at least two STEC serotypes, O157 and O26, were detected from such raw meats in this area (Sukhumungoon *et al*, 2011; Sirikaew *et al*, 2015). However, the presence of *E. coli* O104 has not been investigated. Hence, the current study determined prevalence of *E. coli* O104 from both domestic Thai beef and imported beef from Malaysia. Molecular characterization of virulence factors and DNA and antibiogram profiling of isolated strains also were performed.

MATERIALS AND METHODS

Immunomagnetic separation (IMS) of *E. coli* O104 from beef

Beef samples (56 domestic Thai and 13 imported Malaysian) were collected from open-markets throughout Hat Yai City, Songkhla Province, Thailand during August 2015 - February 2016. Samples were processed within two hours after collection. Raw beef (50 g) was mixed with 450 ml of tryptic soy broth (TSB) (Becton Dickinson, Sparks, MD) and homogenized for 1 minute using stomacher (Seward, London, UK). Liquid portion was statically incubated at 37°C for 6 hours, then 1 ml aliquot of culture solution was mixed with 20 µl of anti-O104 antibody-coated magnetic beads (Captivate O104, Lab M, Lancashire, UK) and incubated with occasional mixing (inverting tube) for 30 minutes. Magnetic beads were washed twice with phosphate-buffered saline pH 7.4 (PBS) and after harvesting by a magnetic concentrator, magnetic bead-bacteria complexes were streaked on eosin methylene blue (EMB) agar (Beckton Dickinson) and incubated at 37°C for 18 hours. Ten to 20 green metallic sheen colonies were selected for identification.

PCR-based identification of *E. coli* O104

PCR template preparation was performed as described previously (Sirikaew *et al*, 2015). In short, an individual colony from EMB agar was inoculated into 3 ml of TSB and incubated at 37°C for 3 hours. One ml aliquot of bacterial culture was boiled for 10 minutes and supernatant was diluted 10 folds with sterile deionized water. PCR amplification of *rfb*O104 was conducted in a 25-µl reaction mixture containing 1X Go*Taq* DNA polymerase buffer, 0.4 µM of the gene-specific primer pair (Table 1), 0.1 mM dNTPs, 3.0 mM $MgCl_2$, 0.5 U Go*Taq* DNA polymerase (Promega,

Madison, WI) and 2 μl of DNA template. Thermocycling was carried out in a T100™ Bio-rad thermal cycler (Bio-rad, Hercules, CA) as follows: 95ºC for 3 minutes; followed by 35 cycles of 94ºC for 1 minute, 48ºC for 1 minute, and 72ºC for 1 minute; with a final heating at 72ºC for 5 minutes. Amplicons were resolved by 1.0% agarose gel-electrophoresis, stained with ethidium bromide staining and documented using a WSE5200 Printgraph 2M gel imaging system (ATTO, Tokyo, Japan). *E. coli* identification was confirmed by the presence of *uidA* (Table 1).

Pathotype classification

PCR amplification of indicator genes of each of the 6 DEC pathotype was performed as previously described (Sirikaew *et al*, 2015): *stx* + *eae* for EHEC, *bfp* + *eae* for EPEC, *est*/*elt* for ETEC, *aggR* for EAEC, *ipaH* for EIEC, and *daaE* for diffusely adherent *E. coli* (DAEC). PCR reaction was conducted as described above using gene-specific primer pairs (Table 1). Thermocycling conditions were as follows: 95ºC for 3 minutes; followed by 35 cycles of 94ºC for 1 minute, 40ºC (for *est*), 50ºC (*elt*, *aggR*, stx_2), 55ºC (stx_1, *eae*, *bfp*, *daaE*), or 60ºC (*ipaH*) for 1 minute, and 72ºC for 1 minute except *eae* for 1.15 minutes; with a final step of 72ºC for 5 minutes. Amplicons were analyzed as described above. Other EAEC- and *E. coli*-associated virulence genes were similarly investigated using primers listed in Table 1 and the following annealing temperatures: 47ºC for *aggA* and *aafA*, 52ºC for *aap*, 52ºC for *astA*, 50ºC for *pet*, 55ºC for *lpf*, 67ºC for *agn43*, 58ºC for *iutA*, 45ºC for *escV*, and 56ºC for *fimH*.

Phylogenetic group analysis

Phylogenetic group analysis was carried out by uniplex-PCR targeting *chuA*, *yjaA* and TspE4.C2 gene fragment (Clermont *et al*, 2000) using specific primers (Table 1). PCR was conducted in a 25-μl mixture consisting of 0.4 μM each primer pair, 0.1 mM dNTPs, 1X Go*Taq* DNA polymerase buffer, 0.5 U Go*Taq* Flexi DNA polymerase (Promega, Madison, WI), and 2 μl of DNA template. Thermocycling conditions were as follows: 95ºC for 3 minutes; followed by 35 cycles of 94ºC for 50 seconds, 54ºC for 50 seconds, 72ºC for 30 seconds; and a final heating at 72ºC for 5 minutes. Amplicons were analyzed as described above.

Clump formation assay

EAEC pathotype has the ability to produce bacterial scum on surface of broth culture (Albert *et al*, 1993). Clump formation was determined as previously described (Albert *et al*, 1993). In brief, a single colony was inoculated into 5 ml of Mueller-Hinton broth (MHB) (Becton Dickinson), incubated at 37ºC for 20 hours with shaking and scrutinized for presence of clumps, visible as scum at the surface of broth culture. EAEC O44 strain PSU280 and methicillin-resistant *Staphylococcus aureus* strain PSU20 was used as positive and negative control strains, respectively.

Yeast agglutination assay

Adherence of *E. coli* to host tissues often is in concordance with agglutination to erythrocytes and yeast cells (Schembri *et al*, 2000). Agglutination to yeast cells was performed as described by Schembri *et al* (2001) with slight modifications. In short, a 30 μl aliquot of an 18-hour bacterial culture in Luria Bertani (LB) broth (Becton Dickinson) was mixed with 30 μl of 5% (w/v) *Saccharomyces cerevisiae* (stock culture) in normal saline solution on a glass slide. Agglutination was monitored by eye and considered positive if agglutination occurred within 1 minute after mixing. A suspension of yeast cells in LB broth was used as negative control.

Table 1
Primers used in the study.

Gene	Virulence factor	Primer name	Sequence (5′ - 3′)	Amplicon size (bp)	Reference
rfb_{O104}	O104 antigen	O104rfbO-f	TGAACTGATTTTTAGGATGG	351	Bielaszewska *et al*, 2011
		O104rfbO-r	AGAACCTCACTCAAATTATG		
bfpA	Bundle forming pili	EP-1	AATGGTGCTTGCGCTTGCTGC	326	Gunzburg *et al*, 1995
		EP-2	GCCGCTTTATCCAACCTGGTA		
eae	Intimin	AE-19	CAGGTCGTCGTGTCTGCTAAA	1,087	Gannon *et al*, 1993
		AE-20	TCAGCGTGGTTGGATCAACCT		
stx1	Shiga toxin 1	EVT-1	CAACACTGGATGATCTCAG	350	Sukhumungoon *et al*, 2011
		EVT-2	CCCCCTCAACTGCTAATA		
stx2	Shiga toxin 2	EVS-1	ATCAGTCGTCACTCACTGGT	404	Sukhumungoon *et al*, 2011
		EVS-2	CCAGTTATCTGACATTCTG		
aggR	AggR, a transcriptional activator of AAF/I	AggR-1	CAGAATACATCAGTACACTG	433	Tsukamoto, 1996
		AggR-2	GAAGCTTACAGCCGATATAT		
ipaH	Enteroinvasive mechanism	ipaIII	GTTCCTTGACCGCCTTTCCGATACCGTC	603,619	Sethabutr *et al*, 1993
		ipaIV	GCCGGTCAGCCACCCTCTGAGAGTAC		
daaE	F1845 fimbriae	daaF-F	GAACGTTGGTTAATGTGGGGTAA	542	Vidal *et al*, 2005
		daaF-R	TATTCACCGGTCGGTTATCAGT		
elt	Heat-labile enterotoxin	TW20	GGCGACAGATTATACCGTGC	450	Stacy-Phipps *et al*, 1995
		JW11	CGGTCTCTATATTCCCTGTT		
est	Heat-stable enterotoxin	JW14	ATTTTTACTTTCTGTATTAGTCTT	190	Stacy-Phipps *et al*, 1995
		JW7	CACCCGGTACAAGGCAGGATT		
astA	EAST1	EAST11a	CCATCAACACAGTATATCCGA	111	Yamamoto and Echeverria, 1996
		EAST11b	GGTCGCGAGTGACGGCTTTGT		
aggA	Fimbriae AAF/I	aggA-F	TTAGTCTTCTATCTAGGG	457	Vila *et al*, 2000
		aggA-R	AAATTAATTCCGGCATGG		
pet	Plasmid encoded toxin	pet-F	ACTGGCGGACTCATTGCTGT	832	Vila *et al*, 2000
		pet-R	GCGTTTTTCCGTTCCCTATT		
iutA	Aerobactin	AerJ f	GGCTGGACATCATGGGAACTGG	300	Johnson and Brown, 1998
		AerJ r	CGTCGGGAACGGGTAGAATCG		
fimH	Type 1 fimbrial tip	fimH-F	TGCAGAACGGATAAGCCGTGG	508	Johnson and Stell, 2000
		fimH-R	GCAGTCACCTGCCCTCCGGTA		

aap	Dispersin	F	CTTGGGTATCAGCCTGAATG	310	Cerna *et al*, 2003
		R	AACCCATTCGGTTAGAGCAC		
lpf	Long polar fimbriae	*lpfA1*-F	GGTCGTTTTTGCCTTAACCGC	≈500	Torres *et al*, 2004
		lpfA1-R	AGGTTGAAATCGACCTGCGC		
agn43	Antigen 43	1-Kpn	GAACCTGTCGGTACCGATGCCCTCCC	≈900	Danese *et al*, 2000
		2-Bam	CGGGATCCGTTGCCACTGTACCGGGCTTGACGACC		
wrbA	Quinone oxidoreductase	*wrb*A1	ATGGCTAAAGTTCTGGTG	600	Toth *et al*, 2003
		*wrb*A2	CTCCTGTTGAAGATTAGC		
yecE	Unknown	EC10	GCCAGCGCCGAGCAGCACAATA	400	DeGreve *et al*, 2002
		EC11	GGCAGGCAGTTGCAGCCAGTAT		
sbcB	Exonuclease I	*sbc*B1	CATGATCTGTTGCCACTCG	1,800	Ohnishi *et al*, 2002
		*sbc*B2	AGGTCTGTCCGTTTCCACTC		
yehV	Transcriptional regulator	Primer A	AAGTGGCGTTGCTTTGTGAT	340	Shaikh and Tarr, 2003
		Primer B	AACAGATGTGTGGTGAGTGTCTG		
Z2577	Oxidoreductase	Z2577F	AACCCCATTGATGCTCAGGCTC	909	Koch *et al*, 2003
		Z2577R	TTCCCATTTTACACTTCCTCCG		
aafA	Fimbriae AAF/II	aafA-F	TGCGATTGCTACTTTATTAT	242	Vila *et al*, 2000
		aafA-R	ATTGACCGTGATTGGCTTCC		
chuA	Heme transport	chuA1	GACGAACCAACGGTCAGGAT	279	Clermont *et al*, 2000
		chuA2	TGCCGCCAGTACCAAAGACA		
yjaA	Unknown	yjaA1	TGAAGTGTCAGGAGACGCTG	211	Clermont *et al*, 2000
		yjaA2	ATGGAGAATGCGTTCCTCAAC		
TspE4.C2	Unknown	TspE4.C2-1	GAGTAATGTCGGGGCATTC A	152	Clermont *et al*, 2000
		TspE4.C2-2	CGCGCCAACAAAGTATTACG		
escV	LEE	escV-F	GGCTCTCTTCTTCTTTATGGCTG	534	Müller *et al*, 2006
		escV-R	CCTTTTACAAACTTCATCGCC		
uidA	β-Glucoronidase	uidA-F	ATCACCGTGGTGACGCATGTCGC	486	Heninger *et al*, 1999
		uidA-R	CACCACGATGCCATGTTCATCTGC		
bla_{SHV}	β -lactamase	bla-SHV.SE	ATGCGTTATATTCGCCTGTG	747	Paterson *et al*, 2003
		bla-SHV.AS	TGCTTTGTTATTCGGGCCAA		
bla_{CTX-M}	β -lactamase	CTX-M-U1	ATGTGCAGYACCAGTAARGTKATGGC	593	Boyd *et al*, 2004
		CTX-M-U2	TGGGTRAARTARGTSACCAGAAYCAGCGG		
bla_{TEM}	β -lactamase	TEM-164.SE	TCGCCGCATACACTATTCTCAGAATGA	445	Monstein *et al*, 2007
		TEM-164.AS	ACGCTCACCGGCTCCAGATTTAT		

[a]Repetitive sequence primer.

Evaluation of intactness of stx_2 phage insertion site

Bacterial virulence is increased by stx_2 phage integration (Brüssow *et al*, 2004). Intactness of five *E. coli* specific integration sites for stx_2 phage (DeGreve *et al*, 2002; Ohnishi *et al*, 2002; Koch *et al*, 2003; Shaikh and Tarr, 2003; Toth *et al*, 2003) was investigated by PCR, based on the notion that stx_2 phage integration into a particular locus results in an inability to PCR-amplify that locus due to the large (42-63 kb depended on each phage strain) stx_2 phage genome insertion (Serra-Moreno *et al*, 2007). In short, PCR amplifications of the insertion loci were carried out as described above using primers specific to each integration locus (Table 1) and the following annealing temperature: 60ºC for *yecE*, 47ºC for *wrbA*, 50ºC for *yehV*, 50ºC for *sbcB*, and 53ºC for Z2577 locus. Amplicons were analyzed as mentioned above.

Antibiogram determination

Antimicrobial susceptibility of *E. coli* O104 strains was evaluated using a disk diffusion method (CLSI, 2014) using 10 common antimicrobial agents: amikacin (30 μg), cefoxitin (30 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), cotrimoxazole (25 μg), fosfomycin (200 μg), gentamicin (10 μg), imipenem (10 μg), streptomycin (10 μg), and tetracycline (30 μg) (Oxoid, Hampshire, UK).

Identification of extended spectrum β-lactamase (ESBL) genes

The presence of three ESBL genes, bla_{SHV}, bla_{CTX-M}, and bla_{TEM}, were identified using PCR as described by Monstein *et al* (2007). In brief, uniplex-PCR was performed as described above using specific primer pairs (Table 1) and the following annealing temperatures: 50ºC for bla_{SHV}, 55ºC for bla_{CTX-M} and 55ºC for bla_{TEM}. Amplicons were analyzed as described above.

DNA profiling of EAEC O104 strains

Pulsed-field gel electrophoresis (PFGE) was performed for DNA profiling as described by Themphachana *et al* (2014). In short, each bacterial sample (approximately 10^8 cells) was embedded in low melting temperature agarose (Bio-Rad, Hercules, CA) to produce a plug. EAEC O104 genome was digested with *Xba*I (Promega, Madison, WI) at 37ºC for 3 hours in 1X buffer D (Promega). DNA fragments were separated by 1.0% agarose gel-electrophoresis in 0.5X Tris-Borate EDTA (TBE) buffer using CHEF DR III system (Bio-Rad) at 14ºC, 6 V/cm, and a field angle of 120º. Initial and final switch times were 2.2 and 54.2 seconds, respectively. Overall run time was 19 hours. Gel was analyzed as described above. EHEC O157:H7 strain EDL933 was used as a DNA size marker.

RESULTS

***E. coli* O104:H4 isolates from beef and pathotype classification**

Of the 1,054 green-metallic sheen isolates from 69 beef samples investigated for presence of *E. coli* O104, 31 isolates from 12 beef samples [3/13 (23%) Malaysian and 9/56 (16%) Thai beef samples] showed the presence of rfb_{O104} and were also positive for *uidA* (data not shown), indicating that they were *E. coli* O104 strains. Pathotype classification using PCR targeting indicator genes of all DEC pathotypes demonstrated that 30/31 carried *aggA* (encoding the major component of AAF/I adhesion of EAEC) but all did not carry *aggR* (encoding transcriptional activator of AAF/I, defining these *E. coli* O104 strains as atypical EAEC O104 (Table 2). Further virulence genes characterization revealed that *fimH* (encoding type 1 fimbriae) was carried in all

Table 2
Characteristics of *E. coli* O104 isolates from raw beef samples obtained from markets in Hat Yai City, Songkhla Province, Thailand during August 2015 - February 2016.

Sample no. (no. of isolates)	Strain name	Virulence trait	Phylogenetic group[a]	ESBL gene	Yeast agglutination
4 (1)[M]	NSP-1	*aggA, fimH*	B1	bla_{CTX-M}	+
18 (1)[T]	NSP-2	*aggA, fimH*	B1	-	+
26 (1)[T]	NSP-3	*aggA, fimH*	B1	bla_{TEM}	-
28 (1)[T]	NSP-4	*aggA, fimH*	B1	-	+
36 (1)[T]	NSP-5	*aggA, fimH*	B1	-	+
39 (1)[T]	NSP-6	*aggA, fimH*	B1	-	+
43 (1)[T]	NSP-7	*aggA, fimH*	B1	bla_{TEM}	+
45 (3)[T]	NSP-8	*aggA, fimH*	B1	-	+
	NSP-9	*aggA, fimH*	B1	-	+
	NSP-10	*aggA, fimH*	B1	-	+
53 (1)[M]	NSP-11	*aggA, fimH*	B1	-	+
58 (8)[T]	NSP-12	*aggA, fimH*	B1	-	+
	NSP-13	*aggA, fimH*	B1	-	+
	NSP-14	*aggA, fimH*	B1	-	+
	NSP-15	*aggA, fimH*	B1	-	+
	NSP-16	*aggA, fimH*	B1	-	+
	NSP-17	*aggA, fimH*	B1	-	+
	NSP-18	*aggA, fimH*	B1	-	+
	NSP-19	*aggA, fimH*	B1	-	+
59 (10)[T]	NSP-20	*aggA, fimH*	B1	-	+
	NSP-21	*aggA, fimH*	B1	-	+
	NSP-22	*aggA, fimH*	B1	-	+
	NSP-23	*aggA, fimH*	B1	-	+
	NSP-24	*aggA, fimH*	B1	-	+
	NSP-25	*aggA, fimH*	B1	-	+
	NSP-26	*aggA, fimH*	B1	-	+
	NSP-27	*aggA, fimH*	B1	-	+
	NSP-28	*aggA, fimH*	B1	-	+
	NSP-29	*aggA, fimH*	B1	-	+
62 (2)[M]	NSP-30	*fimH*	A	bla_{TEM}	-
	NSP-31	*aggA, fimH, iutA*	B1	-	+

[a]GroupA, *chuA* and TSPE4.C2 negative; group B1, *chuA* negative and TSPE4.C2 positive. [M]Imported from Malaysia. [T]Thai.

EAEC O104 strains but *iutA* (encoding aerobactin) was found in only one strain (NSP-31 from Malaysian beef) (Table 2). The *aafA* and *aap* coding for fimbriae AAF/II and dispersin, respectively were also absent in all strains.

Phylogenetic grouping

All but one EAEC O104 belonged to phylogenetic group B1 (*chuA*$^-$, TspE4.C2$^+$), the exception being EAEC O104 strain NSP-30 that belonged to group A (*chuA*$^-$, TspE4.C2$^-$) (Clermont *et al*, 2000) (Table 2).

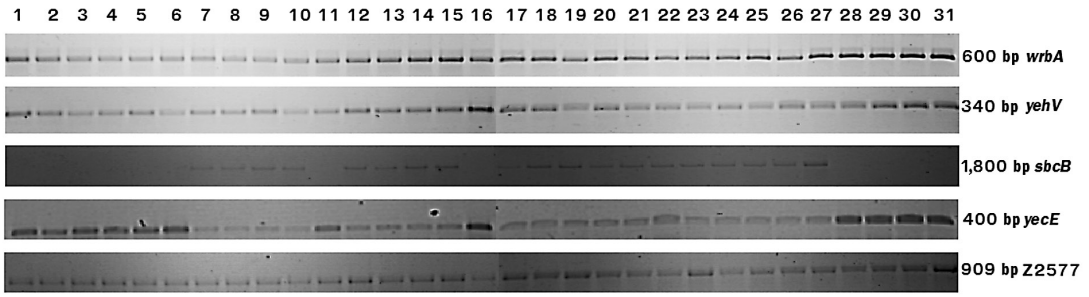

Fig 1–PCR amplification of stx_2 phage insertion-associated genes in *E. coli* O104 strains isolated from raw beef samples obtained from markets in Hat Yai City, Songkhla Province, Thailand during August 2015 - February 2016. PCR was carried out using primer pairs listed in Table 1. Lanes 1-31, strains NSP-1 - 31.

Yeast agglutination and clump formation

Agglutination of *S. cerevisiae* was demonstrated by 29 EAEC strains, with the exception of NSP-3 from Thai and NSP-30 from Malaysian beef samples (Table 2). On the other hand, weak production of bacterial scum was observed at the surface of MHB broth for all EAEC O104 strains (data not shown).

Intactness of stx_2 phage insertion sites

Intactness of five genes frequently occupied by stx_2 phage was examined by PCR, which revealed presence of intact *wrbA*, *yecE*, *yehV*, and Z2577 locus for all strains but no *sbcB* amplification for 12 strains (NSP-1 - 6, NSP-11, NSP-16, and NSP-28 – 31) (Fig 1).

Antibiogram profiles and presence of ESBL genes

Seven of 31 EAEC O104 strains were resistant to at least one antimicrobial agent and two were multi-drug resistant, namely, NSP-3 from Thai beef resistant to fosfomycin, streptomycin, tetracycline, and trimethoprim/sulfamethoxazole and NSP-30 from Malaysian beef resistant to ciprofloxacin, gentamicin, and streptomycin (Fig 2). Four strains possessed ESBL genes: NSP-1 carrying bla_{CTX-M} and NSP-3, NSP-7 and NSP-30 carrying bla_{TEM} (Table 2).

DNA profiling of EAEC O104 strains

Genomic DNA from representative EAEC O104 strains were digested with *Xba*I and separated by PFGE (Fig 3). One O104 strain was selected from each sample as a surrogate for PFGE analysis. In the case of more than one strain isolated in one sample, only one bacterial strain that represented the same genotype and phenotype was chosen to be a surrogate, for instance, NSP-8 was a surrogate of strains from sample number 45 (Table 2). In PFGE analysis, all 13 representative O104 strains showed 12 different patterns (numbers of DNA bands ranging from 20 to 31) with the exception of EAEC O104 strain NSP-12 from Thai beef that had identical PFGE pattern to NSP-31 from Malaysian beef, indicating that these two strains were derived from the same clone (Fig 3). In addition, PFGE patterns of EAEC O104 strain NSP-1 from Malaysia and strain NSP-2 from Thailand were very similar, with an extra DNA band located between 216 kb and 138 kb being present in NSP-2.

DISCUSSION

Cattle is an important reservoir host of several STEC serotypes, including

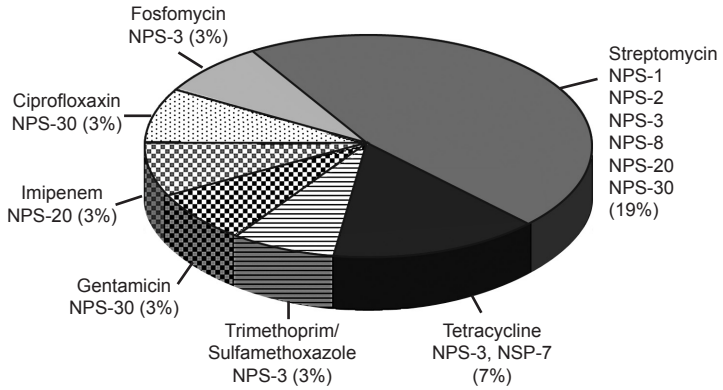

Fig 2–Antibiogram profiles of EAEC O104 strains from raw beef samples obtained from markets in Hat Yai City, Songkhla Province, Thailand during August 2015 - February 2016. Antimicrobial sensitivity was tested using a disk diffusion assay. Percent value is based on number of resistant strains among 31 strains (NSP-1 - 31).

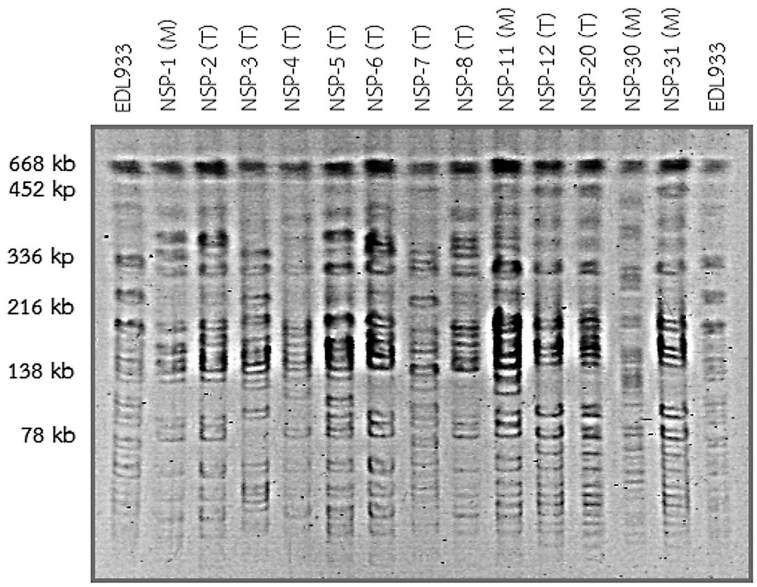

Fig 3–DNA profiling of EAEC O104 strains from raw beef samples obtained from markets in Hat Yai City, Songkhla Province, Thailand during August 2015 - February 2016. Genomic DNA from 13 representative EAEC O104 strains was digested with *Xba*I and separated by pulsed-field gel electrophoresis. M, imported beef from Malaysia; T, local Thai beef. EDL933, EHEC O157:H7 control.

a pivotal serotype O157 (Oporto *et al*, 2008). We surmised that cattle in southern areas of Thailand and those in neighboring Malaysia also carry *E. coli* O104 in their intestines and beef products could become contaminated during the slaughtering processes. The finding of *E. coli* O104 in 16% and 23% of Thai and imported Malaysian beef, respectively confirmed that EAEC O104 indeed existed in both countries. To the best of our knowledge, this is the first report in Thailand showing transborder transfer of EAEC O104 from beef samples from Malaysia to Thailand. Moreover, identical and very similar DNA profiles generated by *Xba*I/PFGE among EAEC O104 strains from local Thai and imported Malaysian beef samples indicate circulation of the same or closely related bacterial clones in the regions bordering the two countries.

E. coli O104 outbreak in northern Germany in 2011 resulted in considerable morbidity and mortality (Frank *et al*, 2011). This strain was a hybrid, carrying indicator genes from two DEC pathotypes, EAEC and STEC. In Denmark, *E. coli* O104 outbreak isolates in 2011 carries an additional gene *iutA* (Scheutz

et al, 2011), which codes for an aerobactin (iron sequester) that is present in about 80% of extra-intestinal pathogenic *E. coli* (Scheutz *et al*, 2011). The pathogenicity difference between *iutA*-carrying *E. coli* O104:H4 from Germany and Denmark is poorly characterized. However, the presence of *iutA* was shown to enhance the capability of *E. coli* O104:H4 to colonize mouse intestine (Torres *et al*, 2012). An investigation of the role of aerobactin in *E. coli* O104:H4 strain C3493 isolated from the stool sample of a patient with HUS during the European outbreak in 2011, compared to its *iutA* mutant designated strain CSS001 demonstrated that the *iutA* mutant is out-competed by its wide type strain in their competition to persist in the mouse cecum (Torres *et al*, 2012). In Asia, the first and the only case to date of HUS caused by *E. coli* O104:H4 was a 26 year-old woman in Korea in 2006 and after plasmapheresis and hemodialysis for 3 weeks, the patient recovered without any sequelae (Bae *et al*, 2006).

EAEC O104 strains in this study showed absence of *stx* but presence of EAEC-associated gene *aggA* coding for AAF/I major subunit and of *fimH* coding for Type 1 fimbriae tip, both of which play key roles in bacterial virulence. AAF/I protein mediates the adherence of bacteria to host epithelial cells and exhibits capability of biofilm formation and agglutination of human erythrocyte (Scheutz *et al*, 2011), while Type 1 fimbria confers autoaggregation, which leads to colonization of bacteria in human intestine (Schembri *et al*, 2001). The majority of O104 strains in this study lacked *iutA*, with only NSP-31 from Malaysian beef containing this gene. The absence of this gene may lead to a decrease in bacterial adherence capability to some extent. Nevertheless, the presence of other adherence genes, such as *aggA* and *fimH*, is thought to be sufficient for initiating pathogenicity. Worrisomely, the stx_2 phage integration sites in *E. coli* O104 in this study were still intact. This plausibly could lead to evolution of O104 strains to become more aggressive causing a potential outbreak in Thailand as had happened in northern Germany in 2011.

EAEC O104 in this study demonstrated an antibiogram pattern similar to other studies in the same geographical area, demonstrating that cephalothin, streptomycin, and tetracycline are ineffective (Sukhumungoon *et al*, 2011; Sirikaew *et al*, 2015). When compared to their corresponding EAEC pathotype isolated in 2013 and 2014, cephalothin and streptomycin were shown to be ineffective (Sukkua *et al*, 2015).

Yeast agglutination of EAEC O104 strains in this study is indicative of EAEC ability to bind to eukaryotic tissues and erythrocyte (Albert *et al*, 1993; Schembri *et al*, 2000) and this property is similar to that of EAEC O44 and O127a previously obtained from patients in our laboratory (Sukkua *et al*, 2016). Low level of clump formation of EAEC O104 may be due to a lower number of adherence genes compared to the typical EAEC that produces much more bacterial scum (Albert *et al*, 1993).

As regards extra-intestinal pathogenic *E. coli* (ExPEC), our results are in accordance with the work of Dezfulian *et al* (2003), who studied the phylogenetic group of ExPEC strains from animal food and found that virulent strains mainly belong to group B2 and D, while commensal strains belong to group A and B1. Although EAEC O104 samples in the present study mainly belonged to group B1 but the presence of other virulent genotypes and phenotypes supports their role in causing human infection.

In summary, although EAEC O104

strains circulating in Thai and Malaysian regions did not possess *stx* class genes but due to the intactness of stx_2 phage integration sites in their genome, there exists the possibility of stx_2 phage insertion into bacterial chromosome as has happened in the German outbreak of 2011, allowing them to become virulent. In addition, bacterial antimicrobial resistance and ESBL production may exacerbate pathogenicity in patients infected with O104 strain. Thus, more attention should be paid to the presence of EAEC O104 in raw meat not only in southern Thailand but in other regions of the country as well.

ACKNOWLEDGEMENTS

NW is a recipient of a fund from Food Safety and Health Research Unit, Prince of Songkla University. The authors thank Prof Dr Mitsuaki Nishibuchi for kindly providing EHEC O157:H7 strain EDL933 and Ms Kannika Sukkua for technical assistance in performing PFGE.

REFERENCES

Albert MJ, Qadri F, Haque A, Bhuiyan NA. Bacterial clump formation at the surface of liquid culture as a rapid test for identification of enteroaggregative *Escherichia coli*. *J Clin Microbiol* 1993; 31: 1397-9.

Bae WK, Lee YK, Cho MS. A case of hemolytic uremic syndrome caused by *Escherichia coli* O104:H4. *Yonsei Med J* 2006; 47: 437-9.

Bergeron CR, Prussing C, Boerlin P, *et al*. Chicken as reservoir for extraintestinal pathogenic *Escherichia coli* in humans, Canada. *Emerg Infect Dis* 2012; 18: 415-21.

Bielaszewska M, Mellmann A, Zhang W, *et al*. Characterisation of the *Escherichia coli* strain associated with an outbreak of haemolytic uraemic syndrome in Germany, 2011: a microbiological study. *Lancet Infect Dis* 2011; 11: 671-6.

Boyd DA, Tyler S, Christianson S, *et al*. Complete nucleotide sequence of a 92-kilobase plasmid habouring the CTX-M-15 extended-spectrum beta-lactamase involved in an outbreak in long-term-care facilities in Toronto, Canada. *Antimicrob Agents Chemother* 2004; 48: 3758-64.

Brüssow H, Canchana C, Hardt WD. Phages and the evolution of bacterial pathogens: from genomic rearrangements to lysogenic conversion. *Microbiol Mol Biol Rev* 2004; 68: 560-602.

Bugarel M, Beutin L, Martin A, Gill A, Fach P. Micro-array for the identification of Shiga toxin-producing *Escherichia coli* (STEC) seropathotypes associated with hemorrhagic colitis and hemolytic uremic syndrome in humans. *Int J Food Microbiol* 2010; 142: 318-29.

Cerna JF, Nataro JP, Estrada-Garcia T. Multiplex PCR for detection of three plasmid-borne genes of enteroaggregative *Escherichia coli* strains. *J Clin Microbiol* 2003; 41: 2138-40.

Clermont O, Bonacorsi S, Bingen E. Rapid and simple determination of the *Escherichia coli* phylogenetic group. *Appl Environ Microbiol* 2000; 66: 4555-8.

Clinical and Laboratory Standards Institute (CLSI). Performance standards for antimicrobial susceptibility testing; Twenty-fourth informational supplement M100-S24. Wayne: CLSI, 2014.

Danese PN, Pratt LA, Dove SL, Kolter R. The outer membrane protein, Antigen 43, mediates cell-to-cell interactions within *Escherichia coli* biofilms. *Mol Microbiol* 2000; 37: 424-32.

DeGreve H, Qizhi C, Deboeck F, Hernalsteens JP. The Shiga toxin VT2-encoding bacteriophage varphi297 integrates at a distinct position in the *Escherichia coli* genome. *Biochim Biophys Acta* 2002; 1579: 196-202.

Dezfulian H, Batisson I, Fairbrother JM, *et al*. Presence and characterization of extraintestinal pathogenic *Escherichia coli* virulence genes in F165-positive *E. coli* strains isolated from diseased calves and pigs. *J*

Clin Microbiol 2003; 41: 1375-85.

Frank C, Faber MS, Askar M, *et al*. Large and ongoing outbreak of haemolytic uraemic syndrome, Germany, May 2011. *Euro Surveill* 2011; 16: pii=19878.

Gannon VPJ, Rashed M, King RK, Thomas EJG. Detection and characterization of the *eae* gene of Shiga-like toxin-producing *Escherichia coli* using polymerase chain reaction. *J Clin Microbiol* 1993; 31: 1268-74.

Gunzburg ST, Tornieporth NG, Riley LW. Identification of enteropathogenic *Escherichia coli* by PCR-based detection of the bundle-forming pilus gene. *J Clin Microbiol* 1995; 33: 1375-7.

Heninger A, Binder M, Schmidt S, Unartl K, Botzenhart K, Doring G. PCR and blood culture for detection of *Escherichia coli* bacteremia in rats. *J Clin Microbiol* 1999; 37: 2479-82.

Johnson JR, Brown JJ. Colonization with and acquisition of uropathogenic *Escherichia coli* strains as revealed by polymerase chain reaction-based detection. *J Infect Dis* 1998; 177: 1120-4.

Johnson JR, Stell AL. Extended virulence genotypes of *Escherichia coli* strains from patients with urosepsis in relation to phylogeny and host compromise. *J Infect Dis* 2000; 181: 261-72.

Karch H, Denamur E, Dobrindt U, *et al*. The enemy within us: lessons from the 2011 European *Escherichia coli* O104:H4 outbreak. *EMBO Mol Med* 2012; 4: 841-8.

Karmali MA, Steele BT, Petric M, Lim C. Sporadic cases of haemolytic-uraemic syndrome associated with faecal cytotoxin and cytotoxin-producing *Escherichia coli* in stools. *Lancet* 1983; 1: 619-20.

Koch C, Hertwig S, Appel B. Nucleotide sequence of the integration site of the temperate bacteriophage 6220, which carries the Shiga toxin gene *stx* (1ox3). *J Bacteriol* 2003; 185: 6463-6.

Kunsmann L, Rüter C, Bauwens A, *et al*. Virulence from vesicles: novel mechanisms of host cell injury by *Escherichia coli* O104:H4 outbreak strain. *Sci Rep* 2015; 5: 13252.

Monstein HJ, Östholm-Balkhed A, Nilsson MV, Nilsson M, Dornbusch K, Nilsson LE. Multiplex PCR amplification assay for the detection of ^{bla}SHV, ^{bla}TEM and ^{bla}CTX-M genes in enterobacteriaceae. *APMIS* 2007; 115: 1400-8.

Müller D, Hagedorn P, Brast S, *et al*. Rapid identification and differentiation of clinical isolates of enteropathogenic *Escherichia coli* (EPEC), atypical EPEC, and Shiga toxin-producing *Escherichia coli* by a one-step multiplex PCR method. *J Clin Microbiol* 2006; 44: 2626-9.

Ohnishi M, Terajima J, Kurokawa K, *et al*. Genomic diversity of enterohemorrhagic *Escherichia coli* O157 revealed by whole genome PCR scanning. *Proc Natl Acad Sci USA* 2002; 99: 17043-8.

Oporto B, Esteban JI, Aduriz G, Juste RA, Hurtado A. *Escherichia coli* O157:H7 and non-O157 Shiga toxin-producing *E. coli* in healthy cattle, sheep and swine herds in Northern Spain. *Zoonoses Public Health* 2008; 55: 73-81.

Paterson DL, Hujer KM, Hujer AM, *et al*. Extended-spectrum- β lactamases in *Klebsiella pneumoniae* bloodstream isolates from seven countries: dominance and widespread prevalence of SHV-and CTX-M-type β lactamases. *Antimicrob Agents Chemother* 2003; 47: 3553-60.

Riley LW, Remis RS, Helgerson SD, *et al*. Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. *N Engl J Med* 1983; 308: 681-5.

Schembri MA, Sokurenko EV, Klemm P. Functional flexibility of the FimH adhesion: insights from a random mutant library. *Infect Immun* 2000; 68: 2638-46.

Schembri MA, Christiansen G, Klemm P. FimH-mediated autoaggregation of *Escherichia coli*. *Mol Microbiol* 2001; 41: 1419-30.

Scheutz F, Neilsen EM, Frimodt-MØller J, *et al*. Characteristics of the enteroaggregative Shiga toxin/verotoxin-producing *Esch-*

erichia coli O104:H4 strains causing the outbreak of haemolytic uraemic syndrome in Germany, May to June 211. *Euro Surveill* 2011; 16: pii=19889.

Sethabutr O, Venkatesan M, Murphy GS, Eampokalap B, Hoge CW, Echeverria P. Detection of Shigellae and enteroinvasive *Escherichia coli* by amplification of the invasion plasmid antigen H DNA sequence in patients with dysentery. *J Infect Dis* 1993; 167: 458-61.

Serra-Moreno R, Jofre J, Muniesa M. Insertion site occupancy by stx_2 bacteriophages depends on the locus availability of the host strain chromosome. *J Bacteriol* 2007; 189: 6645-54.

Shaikh N, Tarr PI. *Escherichia coli* O157:H7 Shiga toxin- encoding bacteriophages: integrations, excisions, truncations, and evolutionary implications. *J Bacteriol* 2003; 185: 3596-605.

Sirikaew S, Rattanachuay P, Nakaguchi Y, Sukhumungoon P. Immuno-magnetic isolation, characterization and genetic relationship of *Escherichia coli* O26 from raw meats, Hat Yai City, Songkhla, Thailand. *Southeast Asian J Trop Med Public Health* 2015; 46: 241-53.

Stacy-Phipps S, Mecca JJ, Weiss JB. Multiplex PCR assay and simple preparation method for stool specimens detect enterotoxigenic *Escherichia coli* DNA during course of infection. *J Clin Microbiol* 1995; 33: 1054-59.

Sukhumungoon P, Nakaguchi Y, Ingviya N, *et al*. Investigation of stx_2^+ eae^+ *Escherichia coli* O157:H7 in beef imported from Malaysia to Thailand. *Int Food Res J* 2011; 18: 381-6.

Sukkua K, Patungkaro W, Sukhumungoon P. Detection and molecular characterization of enteroaggregative *Escherichia coli* from diarrheal patients in tertiary hospitals, southern Thailand. *Southeast Asian J Trop Med Public Health* 2015; 46: 901-10.

Sukkua K, Rattanachuay P, Sukhumungoon P. *Ex vivo* adherence to murine ileal, biofilm formation ability and presence of adherence-associated of human and animal diarrheagenic *Escherichia coli*. *Southeast Asian J Trop Med Public Health* 2016; 47: 55-65.

Themphachana M, Nakaguchi Y, Nishibuchi M, *et al*. First report in Thailand of a *stx*-negative *Escherichia coli* O157 from a patient with diarrhea, Thailand. *Southeast Asian J Trop Med Public Health* 2014; 45: 881-9.

Torres AG, Kanack KJ, Tutt C, Popov V, Kaper JB. Characterization of the second long polar (LP) fimbriae of *Escherichia coli* O157:H7 and distribution of LP fimbriae in other pathogenic *E. coli* strains. *FEMS Microbiol Lett* 2004; 238: 333-44.

Torres AG, Cieza RJ, Rojas-Lopez M, *et al*. *In vivo* bioluminescence imaging of *Escherichia coli* O104:H4 and role of aerobactin during colonization of a mouse model of infection. *BMC Microbiol* 2012; 12: 112.

Toth I, Schmidt H, Dow M, Malik A, Oswald E, Nagy B. Transduction of porcine enteropathogenic *Escherichia coli* with a derivative of a Shiga toxin 2-encoding bacteriophage in a porcine ligated ileal loop system. *Appl Environ Microbiol* 2003; 69: 7242-7.

Tsukamoto T. [PCR methods for detection of enteropathogenic *Escherichia coli* (localized adherence) and enteroaggregative *Escherichia coli*]. *Kansenshogazu Zasshi* 1996; 70: 569-73.

Vidal M, Kruger E, Duran C, *et al*. Single multiplex PCR assay to identify simultaneously the six categories of diarrheagenic *Escherichia coli* associated with enteric infections. *J Clin Microbiol* 2005; 43: 5362-5.

Vila J, Vargas M, Henderson IR, Gascon J, Nataro JP. Enteroaggregative *Escherichia coli* virulence factors in traveler's diarrhea strains. *J Infect Dis* 2000; 182: 1780-3.

Yamamoto T, Echeverria P. Detection of the enteroaggregative *Escherichia coli* heat-stable enterotoxin 1 gene sequences in enterotoxigenic *E. coli* strains pathogenic for humans. *Infect Immun* 1996; 64: 1441-5.