

การหาปริมาณ *Escherichia coli* O26, O104, O111, และ O157 จากเนื้อวัวใน
ประเทศไทยและเนื้อวัวนำเข้าจากประเทศมาเลเซียด้วยวิธี Most Probable
Number-Polymerase Chain Reaction
Most Probable Number-Polymerase Chain Reaction-based
Quantification of *Escherichia coli* O26, O104, O111, and O157
from Thai and Imported Malaysian Beef

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บทคัดย่อ

มีรายงานถึงทำให้เกิดการติดเชื้ออย่างประปรายไปจนถึงการระบาดเป็นวงกว้าง ส่งผลให้เกิดความสูญเสียทางเศรษฐกิจและสังคมอย่างมหาศาลทั่วโลก ในการศึกษาครั้งนี้ได้ทำการตรวจสอบความชุกและหาปริมาณเชื้อ *E. coli* O26, O104, O111 และ O157 จากตัวอย่างเนื้อวัวขายปลีกทั้งเนื้อวัวไทยและเนื้อวัวที่นำเข้าจากประเทศมาเลเซีย โดยวิธี MPN-PCR จากการตรวจตัวอย่างเนื้อวัวจำนวน 102 ตัวอย่าง พบความชุกของเชื้อ *E. coli* O26, O104, O111, และ O157 เท่ากับ 57%, 6%, 0% และ 17% ตามลำดับ โดยพบว่าตัวอย่างเนื้อวัวปนเปื้อนด้วยเชื้อซีโรไทป์ O26 ในความชุกที่สูงที่สุด (P -value < 0.05) ในจำนวนตัวอย่างที่ให้ผลบวกนั้น ค่า MPN/g ที่น้อยที่สุดที่พบคือ < 3 MPN/g ในทุกซีโรไทป์ที่ตรวจ ในขณะที่ค่า MPN/g สูงสุดของเชื้อ *E. coli* O26, O104, O111 และ O157 เท่ากับ 75, 93, < 3, และ 27 ตามลำดับ นอกจากนี้มีการใช้เทคนิค Immuno-magnetic separation เพื่อยืนยันการตรวจไม่พบเชื้อ *E. coli* O111 ในตัวอย่างเนื้อวัวอีกด้วย ในการศึกษาี้แสดงให้เห็นว่าการปนเปื้อนเชื้อ *E. coli* O157 และ non-O157 ในตัวอย่างเนื้อวัวในความชุกที่สูง และปนเปื้อนในปริมาณมากก่อให้เกิดความเสี่ยงต่อสุขภาพของผู้บริโภคและสิ่งนี้มีความสำคัญด้านสาธารณสุข

คำสำคัญ: เนื้อวัว, Shiga toxin, *Escherichia coli*, การปนเปื้อน, O157, MPN-PCR

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ABSTRACT

The contamination of *Escherichia coli* O157 and non-O157 groups in foods has been reported to cause sporadic cases and outbreaks, resulting in significant economic and social losses worldwide. In this study, the prevalence of *E. coli* O26, O104, O111, and O157 in retail beef samples from either domestic Thai or imported Malaysian sources was investigated and their amounts were quantified using the MPN-PCR approach. Of the 102 samples, *E. coli* O26, O104, O111, and O157, were found in beef at rates of 57%, 6%, 0%, and 17%, respectively, with serotype O26 showing the highest contamination rate among the sample (P -value < 0.05). Among the positive samples investigated, the lowest MPN/g value were less than 3 for all serotypes, while the highest MPN/g values of *E. coli* O26, O104, O111, and O157, were 75, 93, < 3, and 27, respectively. Immuno-magnetic separation technique was also employed to confirm the lack of *E. coli* O111 in beef. The high level of *E. coli* O157 and non-O157 contamination in beef observed in this study are thought to pose health risks to consumers, making the finding crucial from a public health standpoint.

Key words: meat, Shiga toxin, *Escherichia coli*, contamination, O157, MPN-PCR

INTRODUCTION

Diarrheagenic *Escherichia coli* (DEC) play a role as causative agents of gastrointestinal illnesses around the world. A report on global child mortality demonstrates that diarrhea was responsible for 9.9% of 7.6 million deaths among children aged 1-59 months in 2010 and the situation in Southeast Asia follows a similar pattern (Liu *et al.*, 2012).

Among the 6 DEC pathotypes, enterohaemorrhagic *E. coli* (EHEC) exhibits the most devastating ability to human cells since it is capable of producing the principal virulence factor called Shiga toxin (Stx) (Nataro and Kaper, 1998). EHEC may contain Stx1 or Stx2 or both. Once Stx enters the human blood circulation, it binds to a receptor on renal cells called globotriaosylceramide (Gb3) (de Sablet *et al.*, 2008) and internalizes into the cell. Stx then causes the elimination of an adenine residue from 28S ribosomal RNA, resulting in inhibition of protein biosynthesis and cell death (Nataro and Kaper, 1998).

The most important EHEC serotype causing food poisoning outbreaks worldwide is EHEC O157:H7 (Themphachana *et al.*, 2014). The carriers for this O157 serotype are commonly meats, especially beef, since the natural reservoir host of this bacterial species is cattle. Massive outbreaks of this agent are reported from both eastern and

western hemispheres (Michino *et al.*, 1999; Riley *et al.*, 1983). Nevertheless, EHEC in the non-O157 group has lately been considered important and has gradually attracted attention from researchers all around the world. Among the EHEC non-O157 group, EHEC serotype O26 constitutes the most common serotype causing haemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) in Europe (European Food Safety Authority (EFSA), 2007; Bugarel *et al.*, 2011). Moreover, many outbreaks caused by *E. coli* O26 have been reported (Ethelberg *et al.*, 2009; Brown *et al.*, 2012). While EHEC O26 is the most common in Europe, EHEC O111, carrying at least one *stx* gene, is one of the most common cause of HC and HUS in United States (Brooks *et al.*, 2005). Furthermore, it also causes diseases in Europe (Karch *et al.*, 1999), Australia (Paton *et al.*, 1996), and Asia (Jeon *et al.*, 2006). In the past decade, Shiga toxin-producing *E. coli* O104 has exhibited a striking capability for causing sporadic infections and outbreaks worldwide, especially the latter that led to the largest outbreak in Germany in 2011. This outbreak resulted in 3,816 cases of gastroenteritis, 845 cases of HUS, and 54 deaths (Frank *et al.*, 2011). Thus, these data indicate that *E. coli* in the non-O157 group should be a greater concern.

Southern Thailand shares a partial border with Malaysia, and food products, especially beef, are imported from Malaysia to Thailand through this border due to high consumer demand. However, it is not clearly understood how much *E. coli* O157 and non-O157 are imported through the import process. To investigate this possibility, we aimed to estimate the prevalence and quantify the natural contamination of *E. coli* O26, O104, O111, and O157 in Thai and imported Malaysian beef sold in retail markets in Southern Thailand using MPN-PCR. This information is beneficial for the risk assessment of food-borne outbreaks caused by *E. coli* in both O157 and non-O157 groups in southern Thailand.

MATERIALS AND METHODS

1. Sample collection

To obtain data on the prevalence and quantity of *E. coli* O26, O104, O111, and O157 in beef, a total of 102 beef samples were collected from eight open markets throughout Hat-Yai city, Songkhla, Thailand. The beef samples were kept at room temperature and processed and analyzed within 2 hours after collection.

2. MPN-PCR-based quantification of *E. coli* O26, O104, O111, and O157

MPN-PCR was performed as described previously (Phetkhajorn *et al.*, 2014) with slight modifications. Briefly, a 25 g of beef was homogenized with 225 ml of tryptic soy broth (TSB) (Becton Dickinson, Sparks, MD). The liquid portion was used to perform the three-tube MPN method, 10-fold, 100-fold, and 1,000-fold dilutions. One ml of the aliquot from each dilution was transferred into triplicate MPN tubes, and then incubated at 37°C for 18 hours. One ml of culture from the turbid tube was then subjected to PCR template preparation using the boiling method. The boiled bacterial culture was immersed on ice for 10 minutes prior to be centrifuged at $11,000 \times g$ for 5 minutes. A ten-fold dilution of the boiled supernatant in sterile deionized water was prepared and used as the PCR

template. Four O-serotype-specific gene targets, *wzx-wzy*_{O26}, *rfb*_{O104}, *rfb*_{O111}, and *rfb*_{O157}, were investigated by singleplex PCR (GoTaq Flexi system, Promega, USA) (Figure 1). The oligonucleotide primers used for amplifications and their annealing temperatures are shown in Table 1. PCR amplification was performed in a 25 µl reaction mixture comprising 1X GoTaq Flexi Green buffer, 3.0 mM MgCl₂, 0.1 mM dNTPs, 0.4 µM of each primer pair, 0.5 unit of GoTaq DNA polymerase, and 2 µl of DNA template. The thermocycling condition (conducted in a T100™ Thermal Cycler, Bio-Rad, Hercules, CA, USA) was as follows: 95°C for 3 minutes, followed by 35 cycles of 94°C for 1 minute, 50°C for 1 minute, and 72°C for 1 minute, with a final step at 72°C for 5 minutes. The amplicons were analyzed using 1.0 % agarose gel electrophoresis, stained with ethidium bromide, and images were captured using the WSE-5200 Printgraph 2M gel imaging system (ATTO Corp., Tokyo, Japan). *E. coli* O26 strain PSU192, O104 strain PSU237, O111 strain PE-27, and O157 strain PSU120 were used as positive strains for amplification of *wzx-wzy*_{O26}, *rfb*_{O104}, *rfb*_{O111}, and *rfb*_{O157} genes, respectively.

3. Isolation of *E. coli* O111 from beef by immunomagnetic separation (IMS) method

The investigation of *E. coli* O111 was further carried out in 86 beef samples using an IMS procedure as previously described (Sirikaew *et al.*, 2015). In brief, 50 g of raw beef were mixed with 450 ml of TSB (Becton Dickinson) in a sterile plastic bag, homogenized for 1 minute in a Stomacher CIR-400 machine (Seward, UK), and the supernatant was obtained by aseptically rinsing it into a sterile bottle. The supernatant was incubated at 37°C for 6 hours, and then 1 ml aliquot was mixed with anti O111 antibody-coated magnetic beads (Dynabeads, Invitrogen, USA) and incubated at room temperature with occasional gentle inversion of the tube for 30 minutes. The immunomagnetic beads

(IMB) were washed with 500 µl phosphate buffered saline, pH 7.4 (PBS), and isolated using a magnetic concentrator following the manufacturer's instructions. IMB was then streaked on eosin methylene blue (EMB) agar (Becton Dickinson). The plate was incubated at 37°C for 18 hours. Fifteen green metallic sheen colonies from each sample were selected for genome extraction and investigated for the presence of *E. coli* O111 by PCR. Amplicons were analyzed as mentioned above.

4. Statistical analysis

The data were analyzed using SPSS for Windows software, version 11.0 (SPSS, Chicago, IL, USA). One-way Analyses of Variance (ANOVA) was used to compare the significant differences in positive results among the four O serotypes. The level of significance was set at a *P*-value < 0.05.

RESULTS AND DISCUSSION

1. Prevalence of *E. coli* O26, O104, O111, and O157 in beef

To examine the prevalence of these four serotypes of *E. coli* in retail beef, the MPN-PCR approach was employed for the investigation. Out of 102 samples tested, *E. coli* O26, O104, O111, and O157 were found in beef with contamination rates of 58 samples (57%), 6 samples (6%), 0 sample (0%), and 17 samples (17%), respectively (Table 2). *E. coli* O26 showed the highest contamination rate in beef (*P*-value < 0.05). The relatively high contamination rates of *E. coli* O26, O104, and O157 in this present study are concerning, as these pathogens can cause severe illness with a low infectious dose; as few as 100 cells can lead to the disease (Nataro and Kaper, 1998). Of particular concern is serotype O157, which is frequently associated with food-borne outbreaks worldwide. In this present study, we demonstrate that *E. coli* O26, O104, and O157 indeed exist in beef samples, whether domestic or imported. This information is consistent with previous studies conducted in the same geographical area. Sirikaew *et al.* (2015) investigated the presence of *E.*

coli O26 using the IMS approach and found that 6 out of 55 beef samples (11%) contained the target bacteria. Although those O26 strains did not carry *stx* genes, 5 strains from beef harbored *escV*, a gene component in the type III secretion system, which is a dangerous virulent factor threatening humans. Moreover, Wameadesa *et al.* (2017) explored *E. coli* O104 from Thai and Malaysian beef. They successfully obtained 31 isolates of *E. coli* O104 from 12 beef samples (16% from Thai beef and 23% from imported Malaysian beef). Most of them carried virulence genes and were resistant to some antimicrobial agents. Additionally, some were extended-spectrum beta lactamase (ESBL)-producing *E. coli*, which can exacerbate infections in patients. More importantly, the possibility of trans-border transfer of *E. coli* O104 from Malaysian to Thailand was uncovered. This raises concerns about the potential transfer of other dangerous pathogens, posing health risk to people in southern Thailand.

2. MPN-PCR-based quantification of *E. coli* O26, O104, O111, and O157

Within the positive samples observed (58 samples of O26; 6 samples of O104; 17 samples of O157), the lowest MPN/g value were less than 3 for all serotypes, while the highest MPN/g values for *E. coli* O26, O104, O111, and O157 were 75, 93, < 3, and 27, respectively (Table 2). This range of *E. coli* contamination rates is considered relatively high and could pose health risks to the consumers. Focusing on each O serotype, it was revealed that the bacterial contamination rate (percentage of positive sample detected) in Malaysian beef was higher than in Thai beef. Conversely, the highest MPN/g values in Thai beef were higher than those in Malaysian beef across all groups (Table 2). These results suggested that although all 4 serotypes were present in lower amounts in Malaysian beef, they were more widespread. MPN-PCR is derived from the combination of conventional MPN approach and polymerase chain reaction (PCR) to assess the quantity of target

microorganism in the samples. This method is widely known to be accurate, sensitive, and specific. While conventional MPN requires 4-6 days to complete the processes, MPN-PCR takes only one day (Mäntynen *et al.*, 1997; Picozzi *et al.*, 2004). Phetkhajorn *et al.* (2014) employed MPN-PCR to investigate the presence of *est*- and *elt*-carrying enterotoxigenic *E. coli* (ETEC) in meat from southern Thailand and found that ETEC contamination in poultry was significantly higher than in other types of meat. Furthermore, Shiga toxin-producing *E. coli* (STEC) and enterohaemorrhagic *E. coli* (EHEC) were detected in beef using MPN-PCR (Sirikaew *et al.*, 2016). This study not only benefits the people in that area but also activates surveillance for enteropathogens throughout the country.

3. Investigation of *E. coli* O111 from beef by IMS method

Due to the absence of *E. coli* O111 contamination in both Thai and Malaysian beefs as detected by MPN-PCR, concrete confirmation of the lack of *E. coli* O111 in beef from southern Thailand was further investigated using IMS. A total of 1,141 green metallic sheen colonies on EMB agar from 86 beef samples (68 from local Thai beef and 18 from Malaysian beef) were examined. It was shown that none of them possessed the *rfb*_{O111} gene, suggesting that they did not belong to the O111 group. This result of the absence of O111 was consistent with the MPN-PCR results, which showed

an O111 value less than 3 MPN/g (Table 2). Immuno-magnetic beads specific to the somatic O antigen of the bacterial cell wall have been clearly demonstrated to increase the isolation efficiency of target bacteria by up to 100-fold (Chapman *et al.*, 1994). The bacterial isolation efficiency of IMS towards diarrheagenic *E. coli* was well studied. Wameadesa *et al.* (2017) employed IMS specific to *E. coli* O104 to investigate its presence in both Thai and imported beef, revealing the identification of numerous *E. coli* O104 strains from beef samples. In addition, enterohaemorrhagic *E. coli* (EHEC) O157:H7, *E. coli* O103, and atypical enteropathogenic *E. coli* (EPEC) O145 were also successfully isolated by IMS (Sukhumungoon *et al.*, 2011; Sae-lim *et al.*, 2018; Sae-lim *et al.*, 2017). These studies emphasize the effectiveness of bacterial isolation using the IMS approach. In this current study, IMS specific to O111 was applied to confirm the existence of O111 in beef samples, but the target microorganism was not detected. Nevertheless, our data are not consistent with the finding of Kayali *et al.* (2015), who also used IMS (pickpen-assisted IMS) to isolate Shiga toxin-producing *E. coli* (STEC) from beef and found *stx*₁-positive *E. coli* O111 in Malaysian beef. Thus, it was concluded that *E. coli* O111 was indeed absent during our investigation. Alternatively, the level of *E. coli* O111 contamination might have been below the detectable limit of IMS.

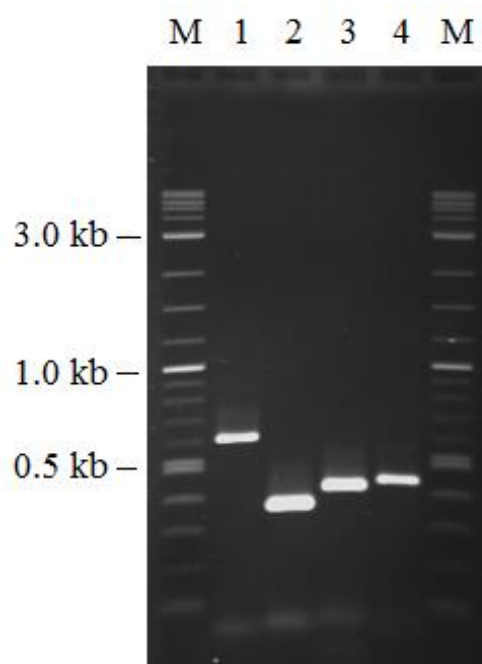


Figure 1 PCR amplification of four target genes in positive strains of *E. coli*. Lane 1: *E. coli* O26 strain PSU192 (*wzx-wzy*_{O26}, 596 bp), Lane 2: *E. coli* O104 strain PSU237 (*rfb*_{O104}, 351 bp), Lane 3: *E. coli* O111 strain PE-27 (*rfb*_{O111}, 406 bp), Lane 4: and *E. coli* O157 strain PSU120 (*rfb*_{O157}, 400 bp). PCR reactions are performed as described in Materials and Methods and analyzed by 1% agarose gel electrophoresis. Lane M contained a 2-log DNA ladder (New England Biolabs, USA).

Table 1 Oligonucleotide primers used in the study.

Target gene	Primer name	Sequence (5' to 3')	Amplicon size (bp)	Reference
<i>wzx-wzy</i> _{O26}	wzx-wzyO26f wzx-wzyO26r	AAATTAGAAGCGCGTTCATC CCCAGCAAGCCAATTATCACT	596	Durso <i>et al.</i> (2005)
<i>rfb</i> _{O104}	O104rfbO-f O104rfbO-r	TGAACTGATTTTTAGGATGG AGAACCTCACTCAAATTATG	351	Bielaszewska <i>et al.</i> (2011)
<i>rfb</i> _{O111}	rfbO111f rfbO111r	AGAGAAATTATCAAGTTAGTTCC ATAGTTATGAACATCTTGTTTAGC	406	Durso <i>et al.</i> (2007)
<i>rfb</i> _{O157}	O157-F O157-R	CGTGATGATGTTGAGTTG AGATTGGTTGGCATTACTG	400	Maurer <i>et al.</i> (1999)

Table 2 MPN/g value of *E. coli* O26, O104, O111, and O157 from beef in Hat-Yai city.

Serotype	No. of positive sample/ Total sample	Percentage of positive sample	MPN/g		
			Min ^δ	Med	Max
O26	^A 58/102	56.9	< 3	3 to 35	75
Thai beef	33/68	48.5	< 3	3 to 35	75
Malaysian beef	25/34	73.5	< 3	3 to 27	35
O104	^{BC} 6/102	5.9	< 3	3 to 53	93
Thai beef	3/68	4.4	< 3	3	93
Malaysian beef	3/34	8.8	< 3	3	53
O111	^D 0/102	0	< 3	< 3	< 3
Thai beef	0/68	0	< 3	< 3	< 3
Malaysian beef	0/34	0	< 3	< 3	< 3

Table 2 Continuous

Serotype	No. of positive sample/ Total sample	Percentage of positive sample	MPN/g		
			Min ^δ	Med	Max
O157	^{BC} 17/102	16.7	< 3	3 to 15	27
Thai beef	9/68	13.2	< 3	3 to 15	27
Malaysian beef	8/34	23.5	< 3	3 to 3.6	11

^δMin, minimum MPN value; Med, medium MPN values between minimum and maximum among positive samples; Max, maximum MPN value of the positive samples; Uppercase letters indicate significant differences in the number of positive samples among the O serotypes.

CONCLUSION

In summary, the *E. coli* in both non-O157 and O157 groups were found to have a high prevalence contamination level in beef. These serotypes can be life-threatening, leading to sporadic outbreaks worldwide. Thus, the presence of *E. coli* O26, O104, and O157 in both domestic Thai and imported beef raises serious concerns. Therefore, periodic surveillance should be conducted for the sake of public health in Thailand.

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