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*Research article*

# Prevalence, Virulence Profiles, and Genetic Relatedness of *Escherichia coli* O45 from Raw meats, Southern Thailand

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**Abstract:** Non-O157 Enterohaemorrhagic *Escherichia coli* (EHEC) has become a major public health concern all around the globe. This study investigated the *E. coli* serogroup O45 from raw meats in southern Thailand using the immunomagnetic separation approach (IMS). Forty-nine *E. coli* O45 strains were obtained from 13 positive meat samples (13/105), showing the prevalence of O45 contamination as 12.4%. They were not members of EHEC. Exploration of other virulence genes exhibited that *fimH*, responsible for bacterial adherence, was found in all strains, while *astA* encoding for EAST-1 toxin was 63.2% of the strains. Moreover, *lpf* encoding long polar fimbriae was found as 30.6%. Phylogenetic group analysis revealed that the majority of *E. coli* O45 belonged to group D (88%), followed by group A (8%) and group B1 (4%). However, none belonged to group B2. The intactness of *stx*$_2$-phage integration sites showed that *sbcB* was occupied by prophages at the highest rate, followed by the Z2577 site. Antimicrobial susceptibility assay demonstrated relatively high bacterial resistance to cephalothin (78%), streptomycin (51%), cotrimoxazole (39%), tetracycline (31%), and chloramphenicol (23%). Furthermore, multi-drug resistant ability was uncovered in O45 strains at 49%. DNA profiling of *E. coli* O45 by BOX-PCR analyzed at 80% genetic similarity revealed 5 distinguishable clusters. More importantly, the strains from different samples and time intervals showed identical DNA fingerprints, suggesting that they may have originated from the same bacterial clone.

**Keywords:** *Escherichia coli*, O45, raw meat, Thailand, EHEC

## 1. Introduction

Diarrheal disease plays an important role as a public health problem, accounting for about 11% of child deaths worldwide, making diarrhea the second leading cause of mortality among children under 5 years of age [1]. Among the diarrheagenic *E. coli* (DEC) group, enterohaemorrhagic *E. coli* (EHEC) is the most important pathotype in human infections, showing the most devastating effect on the host. Only < 100 cells of EHEC are enough to cause illness [2].

EHEC, containing the cardinal virulence factors Shiga toxins (Stxs), is capable of causing food poisoning outbreaks in both the western and eastern hemispheres [3-5]. The symptoms of patients infected by EHEC vary from bloody diarrhea to renal failure and death [6]. Renal failure occurs when Stx is internalized into kidney cells through the cellular membrane-specific receptor

globotriaosylceramide (Gb3). After one adenine residue from 28S ribosomal RNA of 60S ribosomal subunit is removed, it inhibits protein synthesis [7].

Even though the EHEC serotype O157:H7 is the most crucial, EHEC in serotypes other than O157:H7 has recently attracted attention and is involved with severe human diseases [8]. There are 6 important EHEC serotypes (also called the big six) that have been demonstrated to carry *stx* genes, O26, O45, O103, O111, O121, and O145. These EHEC serotypes show a striking ability to cause sporadic infections and outbreaks in many countries worldwide. Although EHEC O45 infections were less found than serotype O157, the strain that carries *stx* is also thought to cause severe morbidity and mortality.

Natural reservoir hosts of EHEC are ruminants, especially cattle, that carry EHEC in their gut without pathological symptoms [9]. There are many routes of EHEC transfer from animals to humans, and the contamination through raw meats during slaughtering processes is one of the potential routes transferring EHEC to humans. Thus, raw meats, especially beef, are the important EHEC vehicles [10].

Although several EHEC serotypes are well-studied, a lack of information regarding the prevalence and characteristics of EHEC serotype O45 is documented in Thailand. Thus, this study aimed to investigate its prevalence, virulence profile, antimicrobial susceptibility, and the genetic similarity of EHEC O45 strains from meats marketed in southern Thailand. The data obtained in this study could provide information on possible infections and outbreaks of EHEC O45 in the southern area of Thailand and neighboring countries. This is essential from a public health standpoint.

## 2. Materials and Methods

### 2.1 Sample collection and immunomagnetic separation (IMS) of *E. coli* O45 from raw meats

To obtain *E. coli* O45 from raw meats, the IMS technique was carried out as previously described [11]. Raw meat samples, e.g., beef, chicken, and pork, were collected from 8 fresh markets throughout Hat-Yai City, Songkhla province, Thailand, and processed within 2 hours after collection. Briefly, 50 g of raw meat was homogenized with 450 ml of tryptic soy broth (TSB) for 1 minute. The liquid phase was obtained aseptically in a sterile bottle and incubated at 37ºC for 6 hours statically. Afterward, 1 ml of the enriched culture was transferred to a 1.5 ml sterile tube and mixed with 15 µl O45-specific immunomagnetic beads (Dynabeads, Thermo Scientific, USA) for 30 minutes with a gentle tube inverted every 5 minutes. The magnetic concentrator harvested the immunomagnetic beads-bacteria complex and washed it with 500 µl of phosphate buffer saline, pH 7.4 (PBS). Subsequently, it was re-suspended in 100 µl PBS, streaked on eosin methylene blue (EMB) agar, and incubated at 37ºC for 18 hours. For further analyses, ten to twenty green metallic sheen colonies were selected and kept at -80ºC (using 10% (v/v) glycerol as a cryoprotectant).

### 2.2 Genomic DNA preparation

Bacterial genomic DNA (gDNA) was extracted using boiling [12]. In brief, a single bacterial colony was grown in 3 ml of TSB at 37ºC for 3 hours with aeration (150 rpm orbital shaking). One ml of bacterial culture was boiled for 10 minutes, immediately immersed on ice for 5 minutes, then centrifuged at 11,000 *g* for 5 minutes. To prepare the PCR template, a ten-fold dilution of boiled supernatant was carried out using sterile deionized water (10-µl boiled supernatant: 90-µl sterile deionized water).

### 2.3 Identification of *E. coli* O45

For the identification of *E. coli* O45, PCR targeting *the wzy*O45 gene was performed. A 25-µl PCR reaction mixture was composed of 3.0 mM MgCl2, 0.1 mM of dNTPs, 0.4 µM of forward and reverse primers (Table 1), 1X Go*Taq* Flexi green buffer, 0.5 unit of GoTaq DNA polymerase (Promega, USA) and 2 µl of DNA template. Thirty-five cycles composing denaturation at 94ºC for 1 min, annealing at 50ºC for 1 min, and extension at 72ºC for 1 min were carried out in T100™ Thermal Cycler (Bio-Rad, Hercules, CA, USA). Amplicons were analyzed in 1.0% agarose gel electrophoresis, stained with ethidium bromide, and visualized under the WSE-5200 Printpraph 2M gel imaging system (ATTO Corp., Tokyo, Japan). The *uidA* gene specific for *E. coli* was also performed to confirm the being of *E. coli*.

### 2.4 *E. coli* pathotype classification and detection of virulence genes

Pathotype classification was investigated since *E. coli* in serogroup O45 tend to be a member of enterohaemorrhagic and Shiga toxin-producing *E. coli* (STEC). Indicator genes for 6 DEC categories were examined by PCR as following criteria, *stx+eae* for EHEC; *bfp+eae* for typical enteropathogenic *E. coli* (tEPEC) or *eae* alone for atypical enteropathogenic *E. coli* (aEPEC); *est/elt* for enterotoxigenic *E. coli*, ETEC; *aggR* for enteroaggregative *E. coli*, EAEC; *ipaH* for enteroinvasive *E. coli*, EIEC; *daaE* for diffusely adherent *E. coli*, DAEC (Table 1). Thermal cycling conditions were as follows: pre-heated at 95ºC for 3 minutes followed by 35 cycles of denaturation at 94ºC for 1 minute, annealing at 40ºC (for *est*), 50ºC (*elt*, *aggR*, *stx*$_2$), 55ºC (*stx*$_1$, *eae*, *daaE*), 60ºC (for *ipaH*), and extension at 72ºC for 1 minute except for *eae* for 70 seconds. The reactions were finalized at 72ºC for 5 minutes. Amplicons were analyzed as described above. Other *E. coli* virulence genes of *astA*, *agn43*, *cnf1*, *hlyA*, *fimH*, and *lpf* were investigated using PCR with appropriate primer pairs (Table 1). PCR components and conditions were the same as mentioned above except the annealing temperature as follows: 50ºC (for *astA*), 55ºC (*fimH*, *lpf*), 58ºC (*cnf1*, *hlyA*), and 67ºC (for *agn43*) for 1 minute.

### 2.5 Phylogenetic group classification of *E. coli* O45

The phylogenetic group might indicate the virulence capability of *E. coli* to some extent. Therefore, *E. coli* O45 was investigated for their phylogenetic groups in this study. Determination of the phylogenetic group was performed as described previously [23]. PCR targeting *chuA*, *yjaA*, and TspE4.C2 fragment was employed. The thermal cycling condition was pre-heated at 95ºC for 3 minutes, followed by 35 cycles of denaturation at 94ºC for 50 seconds, annealing at 54ºC for 50 seconds, and extension at 72ºC for 30 seconds. The reaction was finalized at 72ºC for 5 minutes. PCR products were analyzed as described above. Bacterial phylogenetic group classification was interpreted, followed by Clermont et al. [23].

### 2.6 Investigation of stx$_2$-phages occupancy in *E. coli* O45

Five *E. coli* genes, *sbcB*, *wrbA*, *yecE*, *yehV*, and Z2577, are essential sites for *stx*$_2$-phages to insert [32]. Thus, the intactness of all 5 genes was investigated by PCR using the components and condition described above except the different primers (Table 1) with the following annealing temperatures: 47ºC for *wrbA*, 50ºC for *sbcB* and *yehV*, 53ºC for Z2577, and 60ºC for *yecE*. The PCR products were analyzed by agarose gel electrophoresis as described above. If the *stx*$_2$ phage occupied a particular locus, PCR amplification was not allowed because of the large *stx*$_2$ phage genome.

### 2.7 Antimicrobial susceptibility assay

The disk diffusion method explored the antimicrobial susceptibility profile [33]. Briefly, a single colony was grown in 3 ml of Mueller-Hinton broth (MHB) at 37ºC for 3 hours with aeration. Then, the culture was centrifuged at 8,000 g for 30 seconds to obtain the cell pellet. The solution was adjusted to 0.5 McFarland turbidity standards (approximately 1.5 x $10^8$ cfu/ml) by densitometer (Biosan, Latvia) using 0.85% (w/v) sodium chloride solution (NSS). The adjusted bacteria were swabbed on the surface of Mueller-Hinton agar (MHA). Ten crucial antimicrobial agents, amikacin (30 μg), cephalothin (30 μg), chloramphenical (30 μg), fosfomycin (200 μg), gentamicin (10 μg), imipenem (10 μg), kanamycin (30 μg), streptomycin (10 μg), tetracycline (30 μg) and trimethoprim/sulfamethoxazole (25 μg) were applied. The plates were incubated at 37ºC for 18 hours. Vernier caliper measured clear zone and interpreted followed CLSI [33].

**Table 1.** Oligonucleotide primers used in this study

| Gene | Virulence factor | Primer name | Sequence (5′ to 3′) | Tm (°C) | Amplicon size (bp) | References |
|---|---|---|---|---|---|---|
| *wzy*O45 | O45 antigen | Forward- 5′O45<br>Reverse- 3′O45 | GGCTCATCATTTGGTGCTTTGTG<br>ATAAGGATTTTCAGCGCCCCTG | 55<br>54 | 404 | [13] |
| *stx*$_1$ | Shiga toxin 1 | Forward- EVT-1<br>Reverse- EVT-2 | CAACACTGGATGATCTCAG<br>CCCCCTCAACTGCTAATA | 49<br>48 | 350 | [11] |
| *stx*$_2$ | Shiga toxin 2 | Forward- EVS-1<br>Reverse- EVS-2 | ATCAGTCGTCACTCACTGGT<br>CCAGTTATCTGACATTCTG | 52<br>47 | 404 | [11] |
| *eae* | Intimin | Forward- AE-19<br>Reverse- AE-20 | CAGGTCGTCGTGTCTGCTAAA<br>TCAGCGTGGTTGGATCAACCT | 55<br>55 | 1,087 | [14] |
| *elt* | Heat-labile enterotoxin | Forward- TW20<br>Reverse- JW11 | GGCGACAGATTATACCGTGC<br>CGGTCTCTATATTCCCTGTT | 54<br>50 | 450 | [15] |
| *est* | Heat-stable enterotoxin | Forward- JW14<br>Reverse- JW7 | ATTTTTACTTTCTGTATTAGTCTT<br>CACCCGGTACAAGGCAGGATT | 45<br>56 | 190 | [15] |
| *ipaH* | Enteroinvasive mechanism | Forward- ipaIII<br>Reverse- ipaIV | GTTCCTTGACCGCCTTTCCGATACCGTC<br>GCCGGTCAGCCACCCTCTGAGAGTAC | 64<br>66 | 603, 619 | [16] |
| *daaE* | F1845 fimbriae | Forward-daaF-F<br>Reverse- daaF-R | GAACGTTGGTTAATGTGGGGTAA<br>TATTCACCGGTCGGTTATCAGT | 54<br>53 | 542 | [17] |
| *aggR* | Transcriptional activator of AAF/I | Forward-AggR-1<br>Reverse- AggR-2 | CAGAATACATCAGTACACTG<br>GAAGCTTACAGCCGATATAT | 48<br>48 | 433 | [18] |
| *bfpA* | Bundle forming pili | Forward- EP-1<br>Reverse- EP-2 | AATGGTGCTTGCGCTTGCTGC<br>GCCGCTTTATCCAACCTGGTA | 56<br>54 | 326 | [19] |

**Table 1.** Oligonucleotide primers used in this study (continued)

| Gene | Virulence factor | Primer name | Sequence (5′ to 3′) | Tm (°C) | Amplicon size (bp) | References |
|---|---|---|---|---|---|---|
| *agn43* | Antigen 43 | Forward- 1-Kpn<br>Forward- 2-Bam | GAACCTGTCGGTACCGATGCCCTCCC<br>CGGGATCCGTTGCCACTGTACCGGGCTTGACGACC | 66<br>73 | ≈900 | [20] |
| *fimH* | Type 1 fimbriae | Forward- *fimH* –F<br>Reverse- *fimH* -R | TGCAGAACGGATAAGCCGTGG<br>GCAGTCACCTGCCCTCCGGTA | 56<br>60 | 508 | [21] |
| *astA* | EAST1 | Forward- EAST11a<br>Reverse- EAST11b | CCATCAACACAGTATATCCGA<br>GGTCGCGAGTGACGGCTTTGT | 51<br>58 | 111 | [22] |
| *chuA* | Heme transport | Forward- chuA1<br>Reverse- chuA2 | GACGAACCAACGGTCAGGAT<br>TGCCGCCAGTACCAAAGACA | 54<br>54 | 279 | [23] |
| *yjaA* | Unknown | Forward- yjaA1<br>Reverse- yjaA2 | TGAAGTGTCAGGAGACGCTG<br>ATGGAGAATGCGTTCCTCAAC | 54<br>52 | 211 | [23] |
| *TspE4.C2* | Unknown | Forward- TspE4.C2-1<br>Reverse- TspE4.C2-2 | GAGTAATGTCGGGGCATTCA<br>CGCGCCAACAAAGTATTACG | 52<br>52 | 152 | [23] |
| *uidA* | β-glucoronidase | Forward- uidA –F<br>Reverse- uidA -R | ATCACCGTGGTGACGCATGTCGC<br>CACCACGATGCCATGTTCATCTGC | 61<br>59 | 486 | [24] |
| *cnf1* | Cytotoxic necrotizing factor-1 | Forward- cnf1-F<br>Reverse- cnf1-R | GGCGACAAATGCAGTATTGCTTGG<br>GACGTTGGTTGCGGTAATTTTGGG | 57<br>57 | 552 | [22] |

**Table 1.** Oligonucleotide primers used in this study (continued)

| Gene | Virulence factor | Primer name | Sequence (5′ to 3′) | Tm (°C) | Amplicon size (bp) | References |
|---|---|---|---|---|---|---|
| *lpf* | Long polar fimbriae | Forward- lpf A1-F<br>Reverse- lpf A1-R | GGTCGTTTTTGCCTTAACCGC<br>AGGTTGAAATCGACCTGCGC | 54<br>54 | ≈500 | [25] |
| *wrbA* | Quinone oxidoreductase | Forward- wrbA1<br>Reverse- wrbA2 | ATGGCTAAAGTTCTGGTG<br>CTCCTGTTGAAGATTAGC | 46<br>46 | 600 | [26] |
| *yecE* | Unknown | Forward- EC10<br>Reverse- EC11 | GCCAGCGCCGAGCAGCACAATA<br>GGCAGGCAGTTGCAGCCAGTAT | 60<br>59 | 400 | [27] |
| *sbcB* | Exonuclease I | Forward- sbcB1<br>Reverse- sbcB2 | CATGATCTGTTGCCACTCG<br>AGGTCTGTCCGTTTCCACTC | 51<br>54 | 1,800 | [28] |
| *yehV* | Transcriptional regulator | Forward- Primer A<br>Reverse- Primer B | AAGTGGCGTTGCTTTGTGAT<br>AACAGATGTGTGGTGAGTGTCTG | 50<br>55 | 340 | [29] |
| Z2577 | Oxidoreductase | Forward- Z2577F<br>Reverse- Z2577R | AACCCCATTGATGCTCAGGCTC<br>TTCCCATTTTACACTTCCTCCG | 57<br>53 | 909 | [30] |
| hlyA | $\alpha$-hemolysin | Forward- hly1<br>Reverse- hly2 | AACAAGGATAAGCACTGTTCTGGCT<br>ACCATATAAGCGGTCATTCCCGTCA | 56<br>58 | 1,177 | [22] |
| Repetitive sequence | BOXA1R | CTACGGCAAGGCGACGCTGACG | 62 | variable | [31] | Repetitive sequence |

### 2.8 Genetic relationship of *E. coli* O45

DNA profiling of *E. coli* O45 was investigated by BOX-PCR [34]. Genomic DNA (gDNA) was obtained using a mini-prep spin column (Geneaid, Taipei, Taiwan). BOX-PCR was carried out in a 25-µl reaction mixture consisting of 0.2 µM of BOXA1R primer (Table 1), 0.2 mM dNTPs, 1X Go*Taq* Flexi green buffer, 3.0 mM of MgCl2, 1.25 units of GoTaq DNA polymerase, and 50 ng of DNA template. The thermal cycler condition was set with an initial denaturation (95ºC for 3 minutes) followed by 30 cycles of denaturation at 94ºC for 3 seconds and 92ºC for 30 seconds, annealing at 50ºC for 1 minute, and extension at 65ºC for 8 minutes. The amplification products were analyzed using 1.5% agarose gel electrophoresis for 1.5 hours at 90V and imaging as described above. Dendrograms for O45 were constructed using an unweighted pair-group method of arithmetic average (UPGMA) (Bioprofile software, France).

### 2.9 Statistical analysis

Data were analyzed using SPSS for Windows version 11.0 (SPSS, USA). One-way ANOVA was employed to analyze significant differences in *E. coli* O45 prevalence among meat types. Significance was set at p-value < 0.05.

## 3. Results and Discussion

### 3.1 Prevalence of *E. coli* O45 in raw meat samples

1,890 suspected isolates were obtained from 105 meat samples during *E. coli* O45 investigation by IMS. It exhibited that 49 of 1,890 from 13 samples (prevalence of 12.4%) were positive for *E. coli* O45 (Table 2). The rate of IMS-associated O45 detection in this study was in concordance with the work from Sirikaew et al. [32] and Wamaedesa et al. [35] that investigated the presence of *E. coli* O26 and O104 from raw meats by IMS and found the high prevalence of 12% and 17%, respectively. Therefore, these results suggest that *E. coli* in the serogroup O45 exists in southern Thailand in relatively high prevalence. The higher prevalence of *E. coli* O45 in chicken samples (29.6%) over pork (14.3%) and beef (2%) in this study is thought to be varied, depending upon individual study [32]. In this study, IMS was employed to assist in isolating *E. coli* O45 from meat samples. This IMS method is approximately 100-fold more effective than the conventional culture method for isolating target microorganisms [36]. Lozinak et al. [37] investigated the existence of Shiga toxin-producing *E. coli* (STEC) from several food samples, including raw meat in the United States, using real-time PCR and found *E. coli* O45 and O111 from raw chicken. The Ct value of *E. coli* O45 was much lower than *E. coli* O111, suggesting that *E. coli* O45 may be predominant in the culture. Nevertheless, at such contamination rates, the conventional culture method may not isolate *E. coli* O45 from the samples. Therefore, the IMS method using an antibody-coated magnetic bead is an essential process and must be applied for EHEC/STEC isolation.

### 3.2 Pathotype classification and virulence gene of *E. coli* O45

Even though *E. coli* O45 in this study did not belong to any DEC pathotypes, but they were possibly equipped with some other virulence genes. Thus, *E. coli* O45 strains were examined for their virulence genes, and the results revealed that *fimH* (encoding type 1 fimbrail tip responsible for bacterial adherence) was found in all O45 strains (100%). The *astA* gene encoding for enteroaggregative heat-stable enterotoxin 1 (EAST-1) was 63.2% (31 of 49 isolates). Additionally, *lpf* encoding long polar fimbriae was found at 30.6% (15 of 49 isolates) (Figure 1A). FimH is a virulent factor conferring autoaggregation, leading to the colonization of bacteria by the host [38]. At the same time, the long polar filaments play a crucial role in bacterial adhesion in *E. coli* O157:H7, including other pathogenic *E. coli* strains [39, 40]. These two virulence factors are very important for the early step of bacterial pathogenesis. Our results are in concordance with the study of Tiba et al. [41] and Van et al. [42] that revealed the high prevalence of *fimH* as 97.5% of 162 UPEC strains isolated from patients with cystitis and 92.1% of 38 multidrug-resistant strains isolated from raw meats and shellfishes sold in Vietnam. In addition, EAST-1, widely distributed among diarrheagenic E. coli, has been implicated in many outbreaks [43]. This suggests that although some *E. coli* strains are absent in indicative genes for DEC, they may carry other virulence genes responsible for pathogenesis. Hence, the presence of these genes in *E. coli* O45 in our study is noteworthy; they are virulent strains and potentially cause problems after consumption.

**Table 2.** Prevalence of *E. coli* O45 in raw meats, Hat-Yai, Thailand.

| Serotype | Source | Number of positive samples/ Total number of samples (%) | Number of positive isolates/ Total number of isolates (%) |
|---|---|---|---|
| O45 | Beef | 1/50$^{A}$ (2) | 2/933 (0.2) |
| | Chicken | 8/27$^{B}$ (29.6) | 41/495 (8.3) |
| | Pork | 4/28$^{AB}$ (14.3) | 6/462 (0.9) |
| | **Total** | **13/105 (12.4)** | **49/1,890 (2.6)** |

Uppercase letters (A, B, AB) indicate significant differences ($p$-value < 0.05) among 3 types of raw meat in *E. coli* O45.

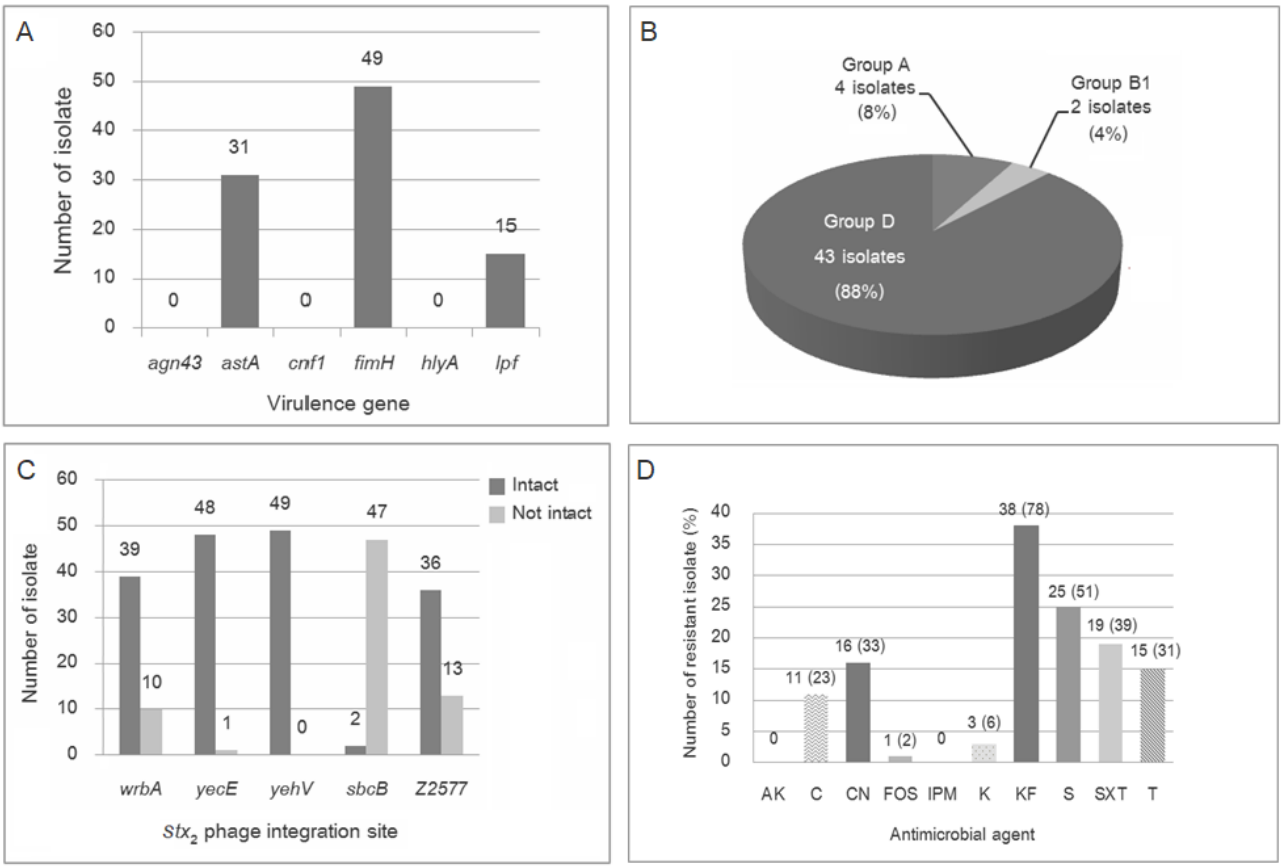


**Figure 1.** Investigation of virulence genes (A), phylogenetic group classification (B), determination of *stx*$_2$-phage integration intactness (C), and examination of antimicrobial susceptibility profiles (D) of 49 *E. coli* O45 from meats. AK, amikacin; C, chloramphenicol; CN, gentamicin; FOS, fosfomycin; IPM, imipenem; K, Kanamycin; KF, cephalothin; S, streptomycin; SXT, cotrimoxazole; T, tetracycline.

### 3.3 Phylogenetic group classification of *E. coli* O45

The phylogenetic group could, in part, indicate the virulence capability of *E. coli*. The results demonstrated that the majority (43 of 49 strains) of *E. coli* O45 belonged to group D (88%) followed by group A (4 of 49 strains, 8%) and B1 (2 of 49 strains, 4%) but none belonged to group B2 (Figure 1B). Based on the phylogenetic group study from Dezfulian et al. [44], they demonstrated that the virulent strains of extraintestinal pathogenic *E. coli* (ExPEC) isolated from animal foods were found mainly to belong to groups B2 and D. In contrast, commensal strains belonged to group A and B1. This criterion was also applied to *E. coli* strains in this current study. Focusing on *E. coli* non-O157 in our previous studies, although some non-O157 groups such as *E. coli* O26 and EAEC O104 were found to be avirulent strains (most of the strains are members of phylogenetic group A and B1, respectively) [32, 35], *E. coli* O45 strains in this present study are thought to

be virulent. This result concurs with aEPEC O145 isolated from the same geographical area belonging to phylogenetic group D [45].

### 3.4 Investigation of stx2 phages occupancy in *E. coli* O45

The *stx*$_2$-phage carries the stx2 gene to the genome of *E. coli* through specific integration sites and makes it become EHEC/STEC. Once *stx*$_2$-phage occupies a specific site, no other phages can occupy such a site again. In this study, we investigated the intactness of 5 specific genes reported to be frequently occupied by *stx*$_2$-phage using PCR. The results revealed that in *E. coli* O45, the highest non-intact gene was *sbcB* (47 strains), followed by Z2577 (13 strains) and *wrbA* (10 strains), suggesting that these sites had been occupied by some prophages (Figure 1C). However, *yecE*, and *yehV* were still intact in most O45 strains. A high number of occupied *sbcB* genes in this study is in harmony with our previous work that showed *E. coli* O157:H7 strains from beef had *sbcB* gene occupied [46]. In the study, 41 bovine-origin *E. coli* O157 and non-O157 strains from Thailand from 1998 to 2012 were investigated for intactness of *stx*$_2$-phage integration sites and found that 40 strains (97.56%) revealed the *sbcB* gene occupancy. Integration of prophages is reported to play a key role in *E. coli* O157:H7 evolution and can increase its pathogenesis [47]. Prophage integration occurs through site-specific recombination or transposition. Housekeeping genes or regions close to tRNA genes are uncovered to be the sites for prophage integration [48]. It was reported that prophages preference integration sites in *E. coli* O157:H7 from Spain and sorbitol-fermenting *E. coli* O157: NM (non-motile) are *yehV* and *yecE*, respectively [49, 50]. However, in the current study, *yecE* and *yehV* are still intact in most O45 strains, suggesting the possible integration of *stx*$_2$-phage into these sites, reinforcing the O45 strains to be more powerful.

### 3.5 Antimicrobial susceptibility of *E. coli* O45

A widespread antimicrobial resistance is a big trouble for public health. In this current study, the results exhibited a high proportion of *E. coli* O45-resistant strains. Most of the strains were found to be resistant to cephalothin (78%), followed by streptomycin (51%), cotrimoxazole (39%), gentamicin (33%), tetracycline (31%), and chloramphenicol (23%), (Figure 1D). Additionally, when focused on the multi-drug resistant (MDR) strains, defined by the capability of resisting at least 3 antimicrobial classes, we found an MDR of 49%, which was considered relatively high. Antimicrobial-resistant capability can be emerged and transferred among bacterial species. The spread of this resistant capability is now becoming a problem worldwide owing to the frequent use of therapy, including prophylaxis and animal growth promotion [51]. The high level of antimicrobial-resistant phenotypes in *E. coli* O45, especially cephalothin, and tetracycline, in this current study is not surprising. Kim & Woo [52] demonstrated the antimicrobial resistance of *E. coli* collected from conventional and organic vegetables. They showed high cephalothin resistance at 67.8% and 71% in organic and conventional vegetables, respectively. In addition, streptomycin, cotrimoxazole, and tetracycline resistance were detected at high rates.

Due to the inexpensiveness of tetracycline, it has been extensively used for prophylaxis and illness therapy of humans and animals. Also, at sub-therapeutic levels, it is used as an animal growth promoter [53]. The study from Vietnam demonstrated that tetracycline was the most frequent antimicrobial resistance in raw meats [42]. More importantly, tetracycline-resistant genes are effectively transferred among bacterial species. Therefore, the ineffectiveness of this antimicrobial agent for treatment should be addressed in the case of infection by *E. coli* from meat consumption.

### 3.6 Genetic relationship of *E. coli* O45

The genetic relationship of *E. coli* O45 strains in this study was carried out using BOX-PCR as a source tracking tool (Figure 2). It was found that *E. coli* O45 could be distinguished into 5 distinct clusters based on 80% genetic similarity (Figure 3). Dendrogram analysis exhibited identical DNA fingerprints among strains from beef, pork, and chicken collected from different samples and time intervals, suggesting that they were closely related or may have originated from the same bacterial clone. Such a clone may circulate in the Thai environment throughout a period.

In bacterial typing, interspersed repetitive-element PCR (rep-PCR) is extensively employed since repetitive elements dispersed throughout the bacterial genome are highly conserved. Among repetitive elements, BOX elements help generate reliable and reproducible fingerprints. Of the BOX subunits comprising

*boxA* (57 bp), *boxB* (43 bp), and *boxC* (50 bp), only *boxA* is highly conserved among different bacterial species [31]. Therefore, in this study, a specific nucleotide primer BOXA1R targeting *boxA* was applied in BOX-PCR. A fluorescent-BOX-PCR has also been developed for subtyping *E. coli* and *Bacillus cereus* [54], exhibiting resolution power and discriminatory power higher than traditional BOX-PCR.

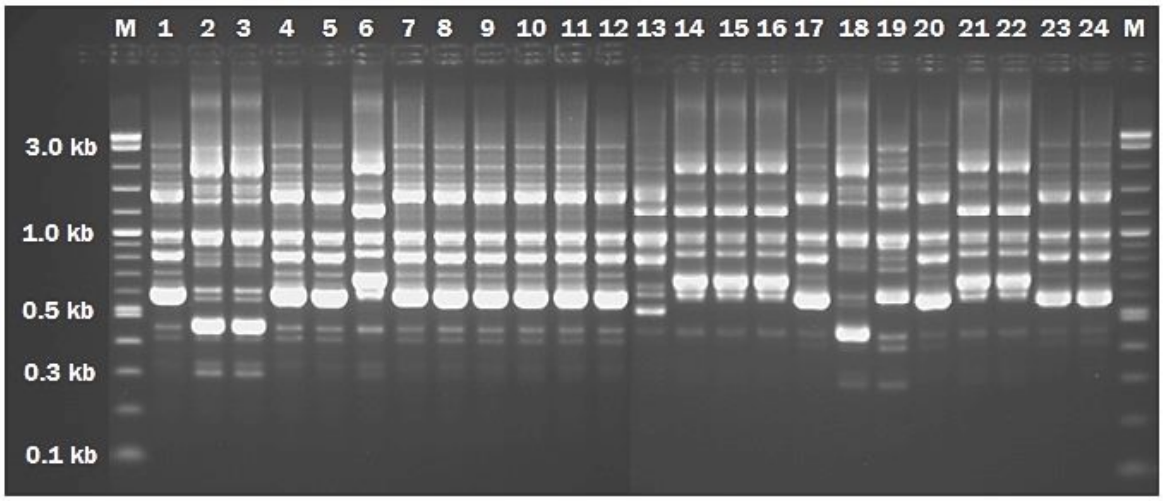


**Figure 2.** A DNA fingerprint was generated by BOX-PCR of 24 different surrogates of *E. coli* O45 isolated from raw meat samples collected in Hat-Yai, southern Thailand. PCR was performed using primers listed in Table 1 and analyzed by 1.5% agarose gel electrophoresis. Lane M, 2-log DNA markers; lane 1 to 24 are M48.5, C57.1, C57.2, C57.9, C58.18, P60.5, P63.5, C64.5, C64.6, C64.8, C71.6, C72.7, C72.19, P76.4, P76.20, C78.6, C78.10, C78.16, C80.1, C80.4, C80.15, C80.18, C87.1, P95.1, respectively. C, Chicken sample; P, Pork sample; B, Beef sample.

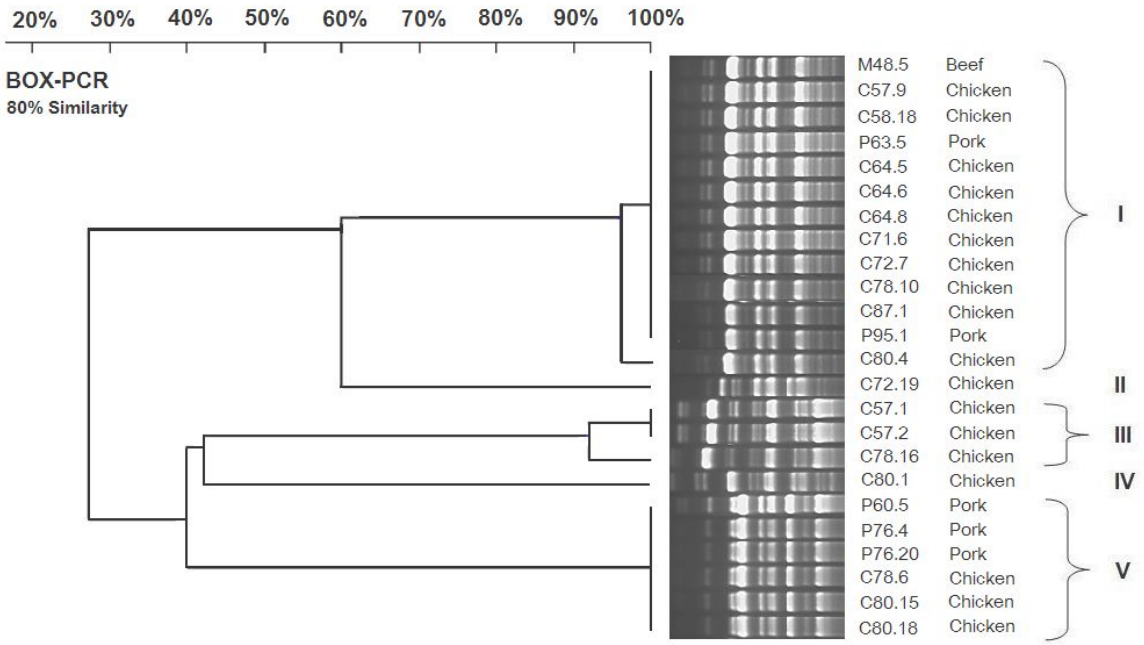


**Figure 3.** BOX-PCR-based dendrogram of 24 surrogates *E. coli* O45 strains from raw meat samples collected in Hat-Yai, Songkhla, southern Thailand. DNA profiles were generated by BOX-PCR using *boxA*. The dendrogram was constructed using an unweighted pair-group method of arithmetic average (UPGMA) (BioProfile software; Vilber Lourmat, Torey, France) and cut off at 80% similarity.

## 4. Conclusions

There is a contamination of *E. coli* O45 in meats at high levels. Even though O45 strains in this study are not in the EHEC/STEC group, some contain virulence factors and belong to phylogenetic group D, indicating the potential to cause illnesses. Moreover, they are resistant to numerous antimicrobial agents, and almost half of them show a multi-drug resistant phenotype and the possibility of gaining a *stx*$_2$-phage that makes them more dangerous in the future. These data are important from a public health standpoint.

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## References

[1] Centers for Disease Control and Prevention. Global diarrhea burden. Available: (Accessed 8 November 2023)

[2] Kaper, J.B.; Nataro, J.P.; Mobley, H.L.T. Pathogenic *Escherichia coli*. Nature *Reviews Microbiology*. **2004**, *2*, 123-140.

[3] Michino, H.; Araki, K.; Minami, S.; Takaya, S.; Sakai, N.; Miyazaki, M.; Ono, A.; Yanagawa, H. Massive outbreak of *Escherichia coli* O157: H7 infection in schoolchildren in Sakai City, Japan, associated with consumption of white radish sprouts. *American Journal of Epidemiology*. **1999**, *150*(8), 787-796.

[4] Dundas, S.; Todd, W.A.; Stewart, A.I.; Murdoch, P.S.; Chaudhuri, A.; Hutchinson, S.J. The central Scotland *Escherichia coli* O157: H7 outbreak: risk factors for the hemolytic uremic syndrome and death among hospitalized patients. *Clinical Infectious Diseases*. **2001**, *33*(7), 923-931.

[5] Rangel, J. M.; Sparling, P. H.; Crowe, C.; Griffin, P. M.; Swerdlow, D. L. Epidemiology of *Escherichia coli* O157: H7 outbreaks, United States, 1982–2002. *Emerging Infectious Diseases*. **2005**, *11*(4), 603-609.

[6] Nataro, J.P.; Kaper, J.B. Diarrheagenic *Escherichia coli*. *Clinical Microbiology Reviews*. **1998**, *11*(1), 142-201.

[7] Karmali, M.; Petric, M.; Steele, B.; Lim, C. Sporadic cases of a haemolytic-uraemic syndrome associated with fecal cytotoxin and cytotoxin-producing *Escherichia coli* in stools. *The Lancet*. **1983**, *321*(8325), 619-620.

[8] Paton, A.W.; Paton, J.C. *Enterobacter cloacae* producing a Shiga-like toxin II-related cytotoxin associated with a case of hemolytic-uremic syndrome. *Journal of Clinical Microbiology*. **1996**, *34*(2), 463-465.

[9] Arthur, T.M.; Barkocy-Gallagher; G.A., Rivera-Betancourt; M.; Koohmaraie, M. Prevalence and characterization of non-O157 Shiga toxin-producing *Escherichia coli* on carcasses in commercial beef cattle processing plants. *Applied and Environmental Microbiol*ogy. **2002**, *68*(10), 4847-4852.

[10] Barkocy-Gallagher, G.A.; Arthur, T.M.; Rivera-Betancourt, M.; Nou, X.; Shackelford, S.D.; Wheeler, T.L.; Koohmaraie, M. Seasonal prevalence of Shigatoxin–producing *Escherichia coli*, including O157: H7 and non-O157 serotypes, and *Salmonella* in commercial beef processing plants. *Journal of Food Protection*. **2003**, *66*(11), 1978-1986.

[11] Sukhumungoon, P.; Nakaguchi, Y.; Ingviya, N.; Pradutkanchana, J.; Iwade, Y.; Seto, K.; Son, R.; Nishibuchi, M.; Vuddhakul, V. Investigation of *stx*$_2$+ *eae*+ *Escherichia coli* O157: H7 in beef imported from Malaysia to Thailand. *International Food Research Journal*. **2011**, *18*(1), 381-386.

[12] Pannuch, M.; Sirikaew, S.; Nakaguchi, Y.; Nishibuchi, M.; Sukhumungoon, P. Quantification of enteropathogenic *Escherichia coli* from retailed meats. *International Food Research Journal*. **2014**, *21*(2), 547-551.

[13] Ju, W.; Shen, J.; Li, Y.; Toro, M.A.; Zhao, S.; Ayers, S.; Najjar, M.B.; Meng, J. Non-O157 Shiga toxin-producing *Escherichia coli* in retail ground beef and pork in the Washington DC area. *Food Microbiology*. **2012**, *32*(2), 371-377.

[14] Gannon, V.; Rashed, M.; King, R.K.; Thomas, E. Detection and characterization of the *eae* gene of Shiga-like toxin-producing *Escherichia coli* using polymerase chain reaction. *Journal of Clinical Microbiology*. **1993**, *31*(5), 1268-1274.

[15] Stacy-Phipps, S.; Mecca, J.J.; Weiss, J.B. Multiplex PCR assay and simple preparation method for stool specimens detect enterotoxigenic *Escherichia coli* DNA during infection. *Journal of Clinical Microbiology*. **1995**, *33*(5), 1054-1059.

[16] Sethabutr, O.; Venkatesan, M.; Murphy, G.S.; Eampokalap, B.; Hoge, C.W.; Echeverria, P. Detection of Shigellae and enteroinvasive *Escherichia coli* by amplification of the invasion plasmid antigen H DNA sequence in patients with dysentery. *Journal of Infectious Diseases*. **1993**, *167*(2), 458-461.

[17] Vidal, M.; Kruger, E.; Durán, C.; Lagos, R.; Levine, M.; Prado, V.; Toro, C.; Vidal, R. Single multiplex PCR assay to identify simultaneously the six categories of diarrheagenic *Escherichia coli* associated with enteric infections. *Journal of Clinical Microbiology*. **2005**, *43*(10), 5362-5365.

[18] Tsukamoto, T. PCR methods for detecting enteropathogenic *Escherichia coli* (localized adherence) and enteroaggregative *Escherichia coli*. *Kansenshogaku zasshi*. **1996**, *70*(6), 569-573.

[19] Gunzburg, S.T.; Tornieporth, N.G; Riley, L.W. Identification of enteropathogenic *Escherichia coli* by PCR-based detection of the bundle-forming pilus gene. *Journal of Clinical Microbiology*. **1995**, *33*(5), 1375-1377.

[20] Danese, P.N.; Pratt, L.A.; Dove, S.L; Kolter, R. The outer membrane protein, Antigen 43, mediates cell-to-cell interactions within *Escherichia coli* biofilms. *Molecular Microbiology*. **2000**, *37*(2), 424-432.

[21] Johnson, J.R.; Stell, A.L. Extended virulence genotypes of *Escherichia coli* strains from patients with urosepsis in relation to phylogeny and host compromise. *The Journal of Infectious Diseases*. **2000**, *181*(1), 261-272.

[22] Yamamoto, S.; Terai, A.; Yuri, K.; Kurazono, H.; Takeda, Y.; Yoshida, O. Detection of urovirulence factors in *Escherichia coli* by multiplex polymerase chain reaction. *FEMS Immunology and Medical Microbiology*. **1995**, *12*(2), 85-90.

[23] Clermont, O.; Bonacorsi, S; Bingen, E. Rapid and simple determination of the *Escherichia coli* phylogenetic group. *Applied and environmental microbiology*. **2000**, *66*(10), 4555-4558.

[24] Heininger, A.; Binder, M.; Schmidt, S.; Unertl, K.; Botzenhart, K.; Döring, G. PCR and blood culture for detection of *Escherichia coli* bacteremia in rats. *Journal of clinical microbiology*. **1999**, *37*(8), 2479-2482.

[25] Torres, A.G.; Kanack, K.J.; Tutt, C.B.; Popov, V.; Kaper, J.B. 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 Microbiology Letters*. **2004**, *238*(2), 333-344.

[26] Tóth, 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. *Applied and Environmental Microbiology*. **2003**, *69*(12), 7242-7247.

[27] De Greve, H.; Qizhi, C.; Deboeck, F.; Hernalsteens, J.P. The Shiga-toxin VT2-encoding bacteriophage ϕ297 integrates at a distinct position in the *Escherichia coli* genome. *Biochimica et Biophysica Acta (BBA)-Gene Structure and Expression*. **2002**, *1579*, 196-202.

[28] Ohnishi, M.; Terajima, J.; Kurokawa, K.; Nakayama, K.; Murata, T.; Tamura, K.; Ogura, Y.; Watanabe, H.; Hayashi, T. Genomic diversity of enterohemorrhagic *Escherichia coli* O157 revealed by whole genome PCR scanning. *Proceedings of the National Academy of Sciences*. **2002**, *99*(26), 17043-17048.

[29] Shaikh, N.; Tarr, P.I. *Escherichia coli* O157: H7 Shiga toxin-encoding bacteriophages: integrations, excisions, truncations, and evolutionary implications. *Journal of Bacteriology*. **2003**, *185*(12), 3596-3605.

[30] Koch, C.; Hertwig, S.; Appel, B. Nucleotide sequence of the integration site of the temperate bacteriophage 6220, which carries the Shiga toxin gene *stx*$_1$ox3. *Journal of Bacteriology*. **2003**, *185*(21), 6463-6466.

[31] Versalovic, J.; Schneider, M.; De Bruijn, F.; Lupski, J.R. Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. *Methods in Molecular and Cellular Biology* 1994, *5*(1), 25-40.

[32] 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 Journal of Tropical Medicine and Public Health.* **2015**, *46*(2), 241-253.

[33] Clinical and Laboratory Standards Institute (CLSI). Performance standards for antimicrobial susceptibility testing; Twenty-third informational supplement M100-S25**.** Wayne, PA, USA 2015.

[34] Sukhumungoon, P.; Tantadapan, R.; Rattanachuay, P. Repetitive sequence based-PCR profiling of *Escherichia coli* O157 strains from beef in southern Thailand. *Southeast Asian Journal of Tropical Medicine and Public Health***. 2016**, *47*(1), 55-65.

[35] Wameadesa, N.; Sae-lim, A.; Hayeebilan, F.; Rattanachuay, P.; Sukhumungoon, P. Enteroaggregative *Escherichia coli* O104 from Thai and imported Malaysian raw beef. *The Southeast Asian Journal of Tropical Medicine and Public Health*. **2017**, *48*(2), 338-350.

[36] Chapman, P.; Wright, D; Siddons, C. A comparison of immunomagnetic separation and direct culture for the isolation of verocytotoxin-producing *Escherichia coli* O157 from bovine faeces. *Journal of Medical Microbiology*. **1994**, *40*(6), 424-427.

[37] Lozinak, K.A.; Jani, N.; Gangiredla, J.; Patel, I.; Elkins, C.A.; Hu, Z.; Kassim, P.A.; Myers, R.A.; Laksanalamai, P. Investigation of potential Shiga toxin-producing *Escherichia coli* (STEC) associated with a local foodborne outbreak using multidisciplinary approaches. *Food Science and Human Wellness.* **2016**, *5*, 163-168.

[38] Schembri, M.A.; Christiansen, G.; Klemm, P.; FimH mediated autoaggregation of *Escherichia coli*. *Molecular Microbiology*. **2001**,. *41*, 1419-1430.

[39] Fitzhenry, R.; Dahan, S.; Torres, A.G.; Chong, Y.; Heuschkel, R.; Murch, S.H.; Thomson, M.; Kaper, J.B.; Frankel, G.; Phillips, A.D. Long polar fimbriae and tissue tropism in *Escherichia coli* O157:H7. *Microbes and Infection.* **2006**, *8*, 1741-1749.

[40] Jordan, D.M.; Cornick, N.; Torres, A.G.; DeanNystrom, E.A.; Kaper, J.B.; Moon, H.W. Long polar fimbriae contribute to colonization by *Escherichia coli* O157:H7 in *vivo*. *Infection and Immunity*. **2004**, *72*, 6168-6171.

[41] Tiba, M.R.; Yano, T.; Leite, D.D.S. Genotypic characterization of virulence factors in *Escherichia coli* strains from patients with cystitis. *Revista do Instituto de Medicina Tropical de Sao Paulo*. **2008**, *50*(5), 255-260.

[42] Van, T.T.H.; Chin, J.; Chapman, T.; Tran, L.T; Coloe, P.J. Safety of raw meat and shellfish in Vietnam: an analysis of *Escherichia coli* isolations for antibiotic resistance and virulence genes. *International Journal of Food Microbiology.* **2008**, *124*(3), 217-223.

[43] Nishikawa, Y.; Ogasawara, J.; Helander, A.; Haruki, K. An outbreak of gastroenteritis in Japan due to *Escherichia coli* O166. *Emerging Infectious Diseases.* **1999**, *5*, 300.

[44] Dezfulian, H.; Batisson, I.; Fairbrother, J.M.; Lau, P.C.K.; Nassar, A.; Szatmari, G; Harel, J. Presence and characterization of extraintestinal pathogenic *Escherichia coli* virulence genes in F165-positive *E. coli* strains isolated from diseased calves and pigs. *Journal of Clinical Microbiology.* **2003**, *41*, 1375-85.

[45] Sae-lim, A.; Jearanai, P.; Rattanachuay, P.; Sukhumungoon, P. Prevalence, virulence profiles, and genetic relationship of atypical enteropathogenic *Escherichia coli* O145 from beef, southern Thailand. *Southeast Asian Journal of Tropical Medicine and Public Health*. **2017**, *48*(6), 1248-1259.

[46] Sukhumungoon, P.; Nakaguchi, Y. Shiga toxin 2-converting bacteriophages occupy *sbcB* gene as a primary integration site in bovine-origined *Escherichia coli* O157: H7 and non-O157 from Thailand. *Life Science Journal*. **2013**, *10*, 2334-2340.

[47] Ooka, T.; Terajima, J.; Kusumoto, M.; Iguchi, A.; Kurokawa, K.; Ogura, Y.; Asadulghani, M.; Nakayama, K.; Murase, K.; Ohnishi, M. Development of a multiplex PCR-based rapid typing method for enterohemorrhagic *Escherichia coli* O157 strains. *Journal of Clinical Microbiology.* **2009**, *47*(9), 2888-2894.

[48] Schmidt, H.; Zhang, W.L.; Hemmrich, U.; Jelacic, S.; Brunder, W.; Tarr, P.; Dobrindt, U.; Hacker, J.; Karch, H. Identification and characterization of a novel genomic island integrated at *selC* in locus of enterocyte effacement-negative, Shiga toxin-producing *Escherichia coli*. *Infection and Immunity*. **2001**, *69*(11), 6863-6873.

[49] Mellmann, A.; Lu, S.; Karch, H.; Xu, J.G.; Harmsen, D.; Schmidt, M. A.; Bielaszewska, M. Recycling of Shiga toxin 2 genes in sorbitol-fermenting enterohemorrhagic *Escherichia coli* O157: NM. *Applied and Environmental Microbiology*. **2008**, *74*(1), 67-72.

[50] Serra-Moreno, R.**;** Jofre, J.**;** Muniesa, M. Insertion site occupancy by *stx*$_2$ bacteriophages depends on the locus availability of the host strain chromosome. *Journal of Bacteriology*. **2007**, *189*(18), 6645-6654.

[51] Rasheed, M.U.**;** Thajuddin, N.**;** Ahamed, P.**;** Teklemariam, Z.**;** Jamil, K. Antimicrobial drug resistance in strains of *Escherichia coli* isolated from food sources. *Revista do Instituto de Medicina Tropical de São Paulo*. **2014**, *56*(4), 341-346.

[52] Kim, S.**;** Woo, G.J. Prevalence and characterization of antimicrobial-resistant *Escherichia coli* isolated from conventional and organic vegetables. *Foodborne Pathogens and Disease.* **2014**. *11*, 815-821.

[53] Chopra, I.; Roberts, M. Tetracycline antibiotics: Mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiology and Molecular Biology Reviews*. **2001**. *65*, 232-260.

[54] Brusetti, L.; Malkhazova, I.; Gtari, M.; Tamagnini, I.; Borin, S.; Merabishvili, M.; Chanishvili, N.; Mora, D.; Cappitelli, F.; Daffonchio, D. Fluorescent-BOX-PCR for resolving bacterial genetic diversity, endemism and biogeography. *BMC Microbiology*. **2008**, *8*(1), 220.