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Molecular characterization of *Escherichia coli* isolated from different food sources

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Abstract

Abstract
Escherichia coli and *Escherichia coli* O157 were identified from "selom" *(Oenanthe stolonifera)*, "pegaga" *(Centella asiatica)*, beef, chicken, lamb, buffalo, "ulam Raja" *(Cosmos caudatus)* and "tenggek burung" *(Euodia redlevi)*. The bacteria were recovered using chromagenic agar. Isolated *Escherichia coli* and *Escherichia coli* 0157 were further characterized by plasmid profiling and enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR). The virulence genes of the isolates *(VT1, VT2, LT, ST, eaeA, inV)* that produces pathogenic *Escherichia coli* and *16S rRNA* gene were screened by a multiplex PCR assay. The plasmid profiling analysis showed that out of 176 isolates, only 103 isolates contained plasmids. ERIC-PCR analysis generated amplified products in the range of ~150 bp to > 1000 bp categorizing isolates into a total of 52 different profiles. Multiplex PCR showed that 20 (32.3%) of the isolates carried *eaeA* gene, 6 (9.7%) isolates possessed *inV* genes, only 1 (1.6%) have *VT2* genes and 1 (1.6%) as well carried *VT1* genes, 2 (3.2%) of the isolates harboured *LT* genes, and only 1 (1.6%) isolate possessed *ST* genes. There were no correlation between plasmid, ERIC-PCR and virulence genes profiles.

Introduction

Escherichia coli is among the common bacterial enteric pathogens capable of causing intestinal disease. Among the *Escherichia coli* causing intestinal disease, there are four well-described pathotypes: enterohaemorrhagic *Escherichia coli* (EHEC), enterotoxigenic *Escherichia coli* (ETEC), enteropathogenic *Escherichia coli* (EPEC), enteroaggregative *Escherichia coli* (EAEC) and enteroinvasive *Escherichia coli* (EIEC) (Nataro and Kaper, 1998). *Escherichia coli* O157 is a member of enterohemorrhagic *Escherichia coli* (EHEC) and has been identified as the cause of several outbreaks by causing diarrhea, hemorrhagic colitis, hemolytic uremic syndrome, thrombotic thrombocytopenic purpura (Hu *et al.*,1999), thus remain as a public health concern worldwide (Hodges and Kimball, 2005).

In Malaysia, few data is available on *Escherichia coli* and most studies concentrated on beef samples. Food poisonings have been occurred in Malaysia of which pathogenic *Escherichia coli* might be the causes despite no specific organism is correlated to the incidences of food poisonings being reported (Adzitey Frederick, 2011). "Selom" *(Oenanthe stolonifera)*, "pegaga" *(Centella asiatica)*, "ulam Raja" *(Cosmos caudatus)* and "tenggek burung" *(Euodia redlevi)* are commonly eaten as "ulam" among the Malay ethnic people. These "ulam" are usually consumed raw.

Plasmid has been used in the study of pathogens of animal (O'Brien *et al*., 1982; Nakamura *et al.*, 1986), fish (Aoki and Takahashi, 1987), fowl (Chaslus-Dancla *et al.*, 1987), and plants (Von Bodman and Shaw, 1987). It is speculated that plasmid profile analysis help to identify source of infection, discriminating isolates or assessing the effectiveness of control measures (Riley *et al*., 1983; Tenover *et al*., 1984; Nakamura *et al*., 1986). Molecular subtyping, or fingerprinting of *Escherichia coli* makes it possible to create a molecular profile. Enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR) is one of the molecular subtyping methods which is based on the analysis of the repetitive chromosomal sequences, called

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the Enterobacterial repetitive intergenic consensus (ERIC). ERIC-PCR enable the clonal characterization of different species of Enterobacteriaceae (Hulton *et al*., 1991; Versalovic *et al*., 1991) by generating a characteristic genomic fingerprinting. Differentiation between different bacterial strains can be carried out by using the genomic fingerprinting (Hulton *et al*., 1991; Dalla-Costa *et al*., 1998). The multiplex PCR method has been used to identify and differentiate pathogenic *Escherichia coli* strains in a number of studies. Assays have been developed in order to differentiate *Escherichia coli* virotypes by targeting virulence genes and other genes for infectious purposes (Lang *et al*., 1994; Tornieporth *et al*., 1995; Tsen and Jian, 1998; Reid *et al*., 1999).

To our knowledge, no study has been carried out to detect and characterize the presence of *Escherichia coli* in lamb, buffalo, pegaga *(Centella asiatica)*, ulam Raja *(Cosmos caudatus)*, selom (*Oenanthe stolonifera)* and tenggek burung *(Euodia redlevi)* in Malaysia. There are only a few studies about this topic, but they mainly focused on *Escherichia coli* O157:H7 from beef samples (*Son et al*., 1998, 2001; Sahilah, 1997; Sukhumungoon *et al*., 2011; Apun *et al*., 2006). Therefore, this study was initiated to detect and gather information about *Escherichia coli* recovered from various food sources in Malaysia ("selom" *(Oenanthe stolonifera)*, "pegaga" *(Centella asiatica*), "tenggek burung" *(Euodia redlevi)*, chicken, lamb, buffalo, "ulam Raja" *(Cosmos caudatus)* and beef. In this study, we isolated the bacteria by using chromagenic agar. The strains isolated were characterized by plasmid profiling, Enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR) and multiplex PCR for virulence genes detection.

Material and Methods

Food samples collection

A total of 12 food samples comprising budu (fish sauce), belacan (shrimp sauce), cencaluk (fermented small shrimps), beef, chicken, lamb, buffalo meat, peanut, ulam raja *(Cosmos caudatus),* selom *(Oenanthe stolonifera)*, pegaga *(Centella asiatica)* and tenggek burung" *(Euodia redlevi)* were purchased around Selangor state, Malaysia between April and May 2011 for the isolation of *Escherichia coli.*

Isolation of Escherichia coli

Upon arrival at the laboratory, all the samples were analysed immediately. Portions (10 g) of each food sample was placed aseptically in a stomacher bag with 9 ml Trypticase Soy Broth (TSB; Merck, Darmstadt, Germany) and homogenized in a stomacher for 30 sec and incubated at 37°C overnight. A loopful of the broth culture was then plated onto CHROMagar ECC (CHROMagar Microbiology, Paris, France) and CHROMagar O157 (CHROMagar Microbiology, Paris, France). The plates were incubated at 37°C for 24 h. Mauve colonies were picked from the plates and were further colony-purified by streaking onto Trypticase Soy Agar (TSA; Merck, Darmstadt, Germany). The reference *Escherichia coli* EPEC, EAEC, EIEC, ETEC and EHEC included as positive controls in this study were provided by Prof. Nishibuchi Mitsuaki, Kyoto University.

Plasmid profiling

Plasmid extraction was carried out from an overnight culture at 37°C of each *Escherichia coli* strain in TSB. Plasmid DNA was extracted from culture cells following alkaline lysis method and ethanol precipitation. Once extracted, the plasmids were electrophoresed through 1.2% agarose gels. A 1 kb ladder (UBI, Canada) was used as a reference molecular weight marker. *Escherichia coli* V517, a strain carrying plasmid molecular weight standard was also included in the gel electrophoresis. After electrophoresis, the gels were stained in ethidium bromide solution for 10 sec, destained in running tap water for 10 min and then visualized.

DNA extraction

Genomic DNA of the isolates were extracted by using the phenol-chloroform method. DNA extraction from control *Escherichia coli* strains was conducted using the GENE ALLTM Cell SV mini (General Biosystem, Korea) according to the manufacturer's instructions. The quantity and quality of DNA were spectrophotometrically determined in a Biophotometer system (Eppendorf, Hamburg, Germany). All DNA preparations were stored at -20°C until used.

ERIC-PCR

ERIC-PCR was carried out by using the primer ERIC-1 (5'-ATGTAAGCTCCTGGGGATTCAC-3') and ERIC-2 (5'-AAGTAAGTGACTGGGGTGAGCG-3') as described by Versalovic *et al*. (1991). ERIC-PCR amplification reactions consisted of 25 µl volumes containing 2 µl genomic DNA, 2.5 µl 10×PCR buffer, 2 µl 10mM dNTPs, 0.25 µl 20mM MgCl2, 1 unit Taq polymerase (Intron Biotechnology) and 10 pmol of each primer. The PCR was performed using G-Storm thermal cycler (G-Storm, Somerton Biotechnology Centre, Somerset, United Kingdom). The cycling parameters were 4 min at 94°C for pre-

Table 1. Primer sets for multiplex PCR of pathogenic *Escherichia coli*

Type	Target gene	Primer sequence	Size	Reference
EHEC	*VT 1*	CTG GAT TTA ATG TCG CAT AGT G AGA ACG CCC ACT GAG ATC ATC	150	Lopes-Saucedo C *et al*., 2003
	VT 2	ATC CTA TTC CCG GGA GTT TAC G GCG TAT CGT ATA CAC AGG AGC	584	Vidal R *et al.*, 2004
ETEC	*LT*	GCA CAC GGA GCT CCT CAG TC TCC TTC ATC CTT TCA ATG GCT TT	218	Vidal R *et al.*, 2004
	ST	TCA CCT TTC CCT CAG GAT GC ATA TTA TTA ATA GCA CCC GG	179	Kimata K *et al*., 2005
EPEC	*eaeA*	CCC GAA TTC GGC ACA AGC ATA AGC CCC GGA TCC GTC TCG CCA GTA TTC G	881	Toma C *et al.*, 2003
EIEC	*inV*	TTT CCC TCT TGC CTG CAT ATG CGC CTC ACC ATA CCA TCC AGA AAG AAG	465	Wood PK *et al*., 1986
E. coli	*16S rRNA*	CCC CCT GGA CGA AGA CTG A *ACC GCT GGC AAC AAA GGA T*	401	Wang G *et al*., 2002 Brandal LT *et al., 2007*

denaturation, 35 cycles each of 45s at 94°C for denaturation, 1 min at 52°C for annealing, 3 min at 65°C for extension and a final extension at 65°C for 10 min. The PCR amplification products were resolved by electrophoresis in 2.0% agarose gel (SeaKem®, Cambrex Bio Science Rock Land, Inc Rockland, ME USA) which was stained with ethidium bromide and viewed under gel documentation system (Alpha Imager, Alpha Innotech, USA). 100 bp DNA ladder (Fermentas) was used as the standard DNA molecular weight marker.

Interpretation of ERIC-PCR data

Gel pictures were loaded into Bionumerics 6.6 Software (Applied Maths, Kortrijk, Belgium) and scored for banding patterns using densitometric curve-based characterization. Cluster analysis was performed and dendrogram was constructed. The Jaccard similarity coefficient and unweighted pair group method with arithmetic averages (UPGMA) was used for cluster analysis.

Multiplex PCR

Primers used in this multiplex assay were adopted from Kim *et a*l. (2010). Primers were prepared by First Base, Malaysia. In this multiplex PCR assay seven different primer pairs (*ST, LT, VT1, VT2, eaeA, inV* and *16S rRNA)* were used to determine the virulence factors of the *Escherichia coli* isolates. Sequences of the seven PCR primer pairs, their corresponding gene targets and size of expected amplification products are shown in Table 1.

Multiplex PCR was carried out in a total volume of 25 µl reaction mixture in a 0.2 ml thin-walled PCR tubes containing 2.5 µl of 10×PCR buffer (Tris-HCl, pH 9.0, PCR enhancers, (NH4)2SO4 and 20 mM $MgCl_2$), 0.5 µl Taq polymerase (Prime Taq DNA polymerase, GenetBio, Chungnam, South Korea), 2.5 µl dNTPs mixture (GenetBio, Chungnam, South Korea), 3 µl DNA templates, primers eaeA, inV, VT2 and 16S rRNA at 5 pmol/µl, VT1 and LT at 15 pmol/µl, ST at 20 pmol/µl). The remaining volume was adjusted by adding an appropriate amount of sterile water. DNA was amplified through 35 cycles of denaturation, annealing and polymerization in a thermocycler (Palm CyclerTM, Corbett Research). Initially, DNA denaturation at 95°C for 30 sec, annealing at 50°C for 40 sec and extension at 72°C for 1 min and a final extension at 72°C for 10 min.

Amplified DNA fragments were analysed on 2.0% agarose gel. An aliquot of 15 µl of PCR reaction product was loaded onto the gel and run at 79 V for 45 min. 100 bp DNA ladder (Geneaid) was used as the standard DNA molecular weight marker. The gel was then stained with ethidium bromide and view under ultralviolet (UV) light.

Results

Isolation of Escherichia coli

A total of 176 *Escherichia coli* were successfully isolated from the different food sources: "selom" *(Oenanthe stolonifera)*, "pegaga" *(Centella asiatica)*, beef, chicken, lamb, buffalo, "ulam Raja" *(Cosmos caudatus)* and "tenggek burung"*(Euodia redlevi)*. The strains were isolated from beef (n=61), chicken (18), ulam raja (11), lamb (18), buffalo (28), pegaga (17), tenggek burung (5), belacan (1) and selom (17). Of these, 84 of the *Escherichia coli* isolates were *Escherichia coli* O157.

Plasmid profiling

Out of 176 *Escherichia coli* isolates, 103 (58.5%) were found to possess plasmids. The other 73 isolates were not typeable by plasmid profiling. The plasmid size obtained ranged from 0.75 kb to 10 kb. Some isolates harbor single sized plasmid while other had multiple plasmids with different sizes. On the basis of gel electrophoresis, the plasmid copies were found

Table 2. Plasmid Profile of *Escherichia coli* isolates

Sample code	Sample type	Plasmid Profile			Total no of bands
		HMW	MMW	LMW	
I1B3O	ulam raja	0	1	0	1
DB1O	beef	1	0		1
G2B4O	buffalo	0	0	1	1
G2B2O	buffalo	1	1	0	2
F1B2O	lamb				
G1B3O	buffalo				
D2B3O	beef				
F2B1O	lamb				
J2R2O	selom				
D1P5O	beef				
D2B4O	beef	1	0	0	1
D1P1O	beef	0	0	1	1
E1B1O	chicken	3	0	1	4
E1B2O	chicken	1	0	1	2
K2B1O	pegaga	0	0	1	1
G3B3O	buffalo	0	0	1	1
L2B1O	tenggekburung	1	0	0	1
G1B4O	buffalo	1	0	0	1
G1B2O	buffalo	1	3	1	5
JIB1O	selom				
D2B1O	beef	1	1	0	2
J2B3O	selom	1	0	0	1
K2B2O	pegaga	1	0	0	1
E1B4O	chicken				
K1B3O	pegaga	0	0	1	1
GB3O	buffalo	3	2	0	5
GB4O	buffalo	1	1	1	3
D4B5O	beef				
D3B4O	beef	0	1	0	1
D3B2O	beef	1	1	1	3
E2B4O	chicken	2	2	2	6
F1B3O	lamb				
D3P4O	beef	1	0	0	1
K1B2O	pegaga	0	1	0	1
D2B5O	beef	1	1	0	2
D1P2O	beef				
D1B5O	beef				
D2B2O	beef				
D4B2O	beef				
L2B3O	Tenggekburung	1	0	0	1
D5P1O	beef	4	3	0	7
D4B1O	beef	3	2	0	5
D5B5O	beef				
L2B4O	tenggek burung	1	1	0	2
D5B3O	beef	0	1	0	1
D1P3O	beef	2	0	0	2
IB3O	ulam raja	1	1	0	2
EB3O	chicken	0	2	0	2
GB2O	buffalo	0	2	0	2
G2B1O	buffalo	1	0	0	
I2B4O	ulam raja	1	0	0	
F2B3O	lamb	1	0	0	1
J2B2O	selom	1	0	0	1
EB2O	chicken	1	0	0	
F2B4O	lamb	1	0	1	2
G1R1O	buffalo	1	2	1	4
E2B2O	chicken				
K2B3O	pegaga	0	1	1	2
D3P2O	beef				
D2P2O	beef				
J1B2O	selom	2	1	0	3
GB1O	buffalo				
E2B2O	chicken				
D5B4O	beef				
D1B4O	beef	0	0	1	1
D4B3O	beef				
D5B2O	beef				
D4B4O	beef				
D5B1O	beef				
D3P1O	beef				

Sample code	Sample type	Plasmid Profile			Total no of bands
		HMW	MMW	LMW	
D1B1O	beef				
D2P4O	beef	0	0	1	1
E2B3O	chicken	4	1	1	6
K2B4O	pegaga	0	1	0	1
G1R2O	buffalo				
F2R1O	lamb				
D2P5O	beef	0	0	1	1
E2B1O	chicken	1	0	0	1
DB2O	beef	0	1	0	1
I1R1O	ulam raja				
D3B3O	beef	0	1	0	1
K1B1O	pegaga				
J1B3O	selom				
DB3O	beef				
D3B2E	beef				
B2P1E	belacan	0	1	0	1
D1P2E	beef				
E2R1E	chicken	1	0	0	1
DB1E	beef				
D1B5E	beef				
D3B5E	beef	0	0	1	1
DP2E	beef				
D2P1E	beef				
D1B2E	beef	2	1	1	4
DP1E	beef				
D1B4E	beef				
DB3E	beef				
E2R3E	chicken	1	2	1	4
D1P3E	beef				
D5B1E	beef				
FR4E	lamb				
I2R3E	ulam raja				
G2R4E	buffalo	1	0	1	2
I1R4E	ulam raja	1	0	0	1
K1R1E	pegaga	0	1	0	1
G1R2E	buffalo				
D4B4E	beef	1	0	0	1
G2R1E	buffalo				
F2R2E	lamb	1	1	2	4
K2R3E	pegaga				
GR4E	buffalo				
I2R1E	ulam raja	0	1	0	1
I1R2E	ulam raja	2	1	0	3
GR3E	buffalo				
F1R2E	lamb	1	0	0	1
D3P4E	beef	0	2	1	3
K2R2E	pegaga	1	0	0	1
D3P5E	beef				
I2R2E	ulam raja				
D3P2E	beef				
J2R2E	selom	1	0	0	1
F2R3E	lamb				
D2B4E	beef				
G1R3E	buffalo	1	0	1	2
F1R4E	lamb				
D3P1E	beef	0	0	1	1
D3B3E	beef				
GR1E	buffalo				
L2B2E	tenggek burung				
E1B1E	chicken	2	1	3	6
GB1E	buffalo				
D2B5E	beef				
E1R1E	chicken	0	1	1	2
D3P3E	beef				
D2P3E	beef				
L2B1E	tenggek burung	2	0	0	
2G3B1E	buffalo	1	0	0	1
E2B4E	chicken	1	0	1	2
F2R4E	lamb	0	2	1	3
E1B2E	chicken				

D2P5E	beef				
FR2E	lamb				
G2R3E	buffalo	1	0	0	1
E2B1E	chicken				
D2B3E	beef				
F1R1E	lamb	1	5	2	8
D3B1E	beef				
I1R1	ulam raja	2	2	0	4
GR2E	buffalo	0	1	0	1
G2R2E	buffalo	0	0	2	2
K2R1E	pegaga				
J1R1E	selom	1	1	1	3
J1R2E	selom	1	0	0	1
J1R3E	selom	1	1	0	2
J1R4E	selom	0	1	1	2
J2R1E	selom	1	0	1	2
J2R3E	selom	1	0	0	1
JR1E	selom	1	0	0	1
R2E	selom	1	0	0	1
JR3E	selom	1	0	0	1
JR4E	selom	1	0	0	1
K1R2E	pegaga	1	0	0	1
K1R3E	pegaga	1	0	0	1
K1R4E	pegaga	0	1	0	1
K2R4E	pegaga				
KR1E	pegaga	1	0	0	1
KR2E	pegaga	1	1	0	2
FR1E	lamb	0	1	1	2
FR3E	lamb	0	0	1	1
F1R3E	lamb	0	1	0	1
F2R1E	lamb				
I2R4E	ulam raja	1	0	0	1
G1R1E	buffalo	0	0	1	1
G1R4E	buffalo	0	1	0	1
GB2E	buffalo	1	0	0	1
E2B3E	chicken	0	1	0	1

High molecular weight: 4.0-10.0 kb; Medium molecular weight: 1.5-4.0 kb; Low molecular weight: 0.5-1.5 kb.

to vary between 1 and 8. Table 2 showed the plasmid profiles of *Escherichia coli* isolates.

ERIC-PCR

All 176 isolates were subjected to ERIC-PCR amplification but only 60 out of 176 isolates were typeable by ERIC-PCR. Primers targeted to ERIC sequence elements yielded complex strain-specific fingerprint patterns with multiple bands of distinct intensities (Figure 1). Some had amplicon bands in common, but strain-to-strain variation could be detected by the presence or absence of some other bands. ERIC-PCR accurately differentiates the isolates by means of the number and positions of the amplified DNA fragments, which are visible in the gels (Figure 1). ERIC-PCR generated number of amplified products ranging from ~150 bp to > 1000 bp. The isolates produced different strains by ERIC-PCR ranging from 1 to 10 bands. Not a single band was consistently present in all isolates showing 100% polymorphism.

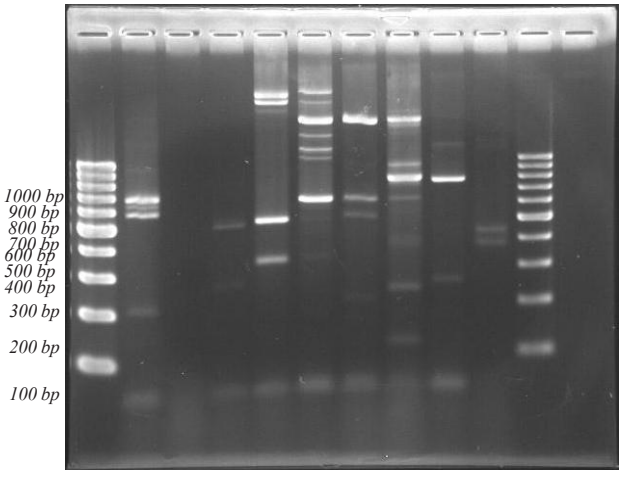

Figure 1. Agarose gel pictures for ERIC-PCR. M denoted the molecular weight marker using 100 bp ladder while the numbers on the top represented the sample numbers.

Dendrogram (Figure 2) was generated from the ERIC-PCR. At 48% cutoff value, a total of 52 different profiles were recognized on the basis of distribution of ERIC elements in the genome of the *Escherichia coli* isolates. Based on clustering, isolates could be grouped into 8 mini clusters having two strains, whereas others formed their own unique pattern.

Multiplex PCR

Isolates which harboured plasmids and were typeable by ERIC-PCR, were screened by a multiplex PCR assay for the presence of virulence genes. This include *Escherichia coli* isolates from beef (n=19), chicken (n=10), buffalo meat (n=9), pegaga (n=4), tenggek burung (n=4), ulam raja (n=5), lamb (n=6), selom (n=5).

The target genes specific to EHEC (*VT1* and *VT2*), ETEC (*LT* and *ST*), EPEC (*eaeA),* EIEC (*inV*) and *16S rRNA* produced amplicons at 150 bp, 584 bp, 218 bp, 179 bp, 881bp, 465 bp and 401 bp respectively on the control *Escherichia coli* strains (data not shown).

54 (87.1%) out of 62 *Escherichia coli* isolates yielded strong PCR amplification of the 401 bp 16S rRNA gene which was being employed as the internal standard for pathogenic *Escherichia coli* identification. PCR showed that 20 (32.3%) of the isolates carried *eaeA* gene, 6 (9.7%) isolates possessed *inV* genes, only 1 (1.6%) have *VT2* genes and 1 (1.6%) as well carried *VT1* genes, 2 (3.2%) of the isolates harboured *LT* genes, and only 1 (1.6%) isolate possessed *ST* genes.

Of the 20 isolates carrying *eaeA* gene, 8 were detected from beef, one from tenggek burung, 2 isolates were recovered from buffalo, 3 from ulam raja, 2 isolates from chicken, 2 isolates as well from lamb and one isolate from pegaga and the other one from selom. The amplification of the *inV* gene gave positive PCR products for 6 isolates: two were

Table 3. The distribution of the toxin genes among the isolates. "+", present; "-", absent. Sources: D, beef; E, chicken; F, lamb; G, buffalo; I, ulam raja; J, selom; K, pegaga; L, tenggek burung

Sample code	*eaeA*	*inV*	*VT2*	*VT1*	*LT*	*ST*	*16sRNA*
DB1O	-	+	-	-	-	-	+
G2B4O	-	+	-	-	-	-	+
G2B2O	-	+	-	-	-	-	+
D2B4O	+	-	-	-	-	-	+
D1P1O	+	-	-	-	-	-	+
E1B1O	-	-	-	-	-	-	+
E1B2O	-	-	-	-	-	-	-
K2B1O	-	-	-	-	-	-	+
G3B3O	+	-	-	-	-	-	+
L2B1O	-	-	-	-	-	-	+
GB3O	-	-	-	-	-	-	+
D3B4O	-	+	-	-	-	-	+
D3B2O	-	-	-	-	-	-	+
E2B4O	-	-	-	-	-	-	+
D3P4O	-	-	-	-	-	-	+
K1B2O	-	-	-	-	-	-	-
D2B5O	-	-	-	-	-	-	+
L2B3O	-	-	-	-	-	-	+
D5P1O	-	-	-	-	-	-	+
D4B1O	+	-	-	-	-	-	+
L2B4O	+	-	-	-	-	-	+
D5B3O	+	-	-	-	-	-	+
D1P3O	-	-	-	-	-	-	+
IB3O +	-	-	-	-	-	+	
GB2O	+	+	-	-	-	-	+
F2B3O	-	-	-	-	-	-	-
J2B2O	-	-	-	-	-	-	+
F2B4O	-	-	-	-	-	-	+
K2B3O	+	-	-	-	-	-	+
J1B2O	-	-	-	-	-	-	+
D1B4O	+	-	-	-	-	-	+
D2P4O	-	-	-	-	-	-	-
E2B3O	+	-	-	-	-	-	+
K2B4O	-	-	-	-	-	-	+
D2P5O	+	-	-	-	-	-	+
DB2O	-	-	-	-	-	-	+
D3B3O	-	-	-	-	-	-	+
E2R1E	+	+	-	+	+	+	+
D3B5E	+	-	-	-	+	-	+
F2R2E	+	-	-	-	-	-	+
I2R1E	-	-	-	-	-	-	+
I1R2E	+	-	+	-	-	-	+
D3P4E	+	-	-	-	-	-	+
J2R2E	-	-	-	-	-	-	-
E1B1E	-	-	-	-	-	-	+
E1R1E	-	-	-	-	-	-	+
L2B1E	-	-	-	-	-	-	+
G3B1E	-	-	-	-	-	-	+
E2B4E	-	-	-	-	-	-	+
F2R4E	-	-	-	-	-	-	+
G2R3E	-	-	-	-	-	-	+
F1R1E	-	-	-	-	-	-	+
I1R1 -	-	-	-	-	-	+	
GR2E	-	-	-	-	-	-	+
G2R2E	-	-	-	-	-	-	+
JR1E -	-	-	-	-	-	-	
JR2E +	-	-	-	-	-	+	
FR3E +	-	-	-	-	-	+	
I2R4E	+	-	-	-	-	-	+
E2B3E	-	-	-	-	-	-	-
EB3O	-	-	-	-	-	-	+
D3P1E	-	-	-	-	-	-	-

detected from beef, 3 from buffalo, and one isolate was from chicken. In addition, the two isolates carrying *VT1* genes were recovered from beef and chicken respectively. The only one isolate carrying *VT2* gene was detected from ulam raja. Besides, there is one isolate detected from chicken carrying the six virulence genes except *VT2* gene. This isolate contained the necessary virulence genes required to cause human disease, and must be considered as potential pathogens that could be involved in future outbreaks. None of the *Escherichia coli* isolates harbored a complete array of the tested virulence

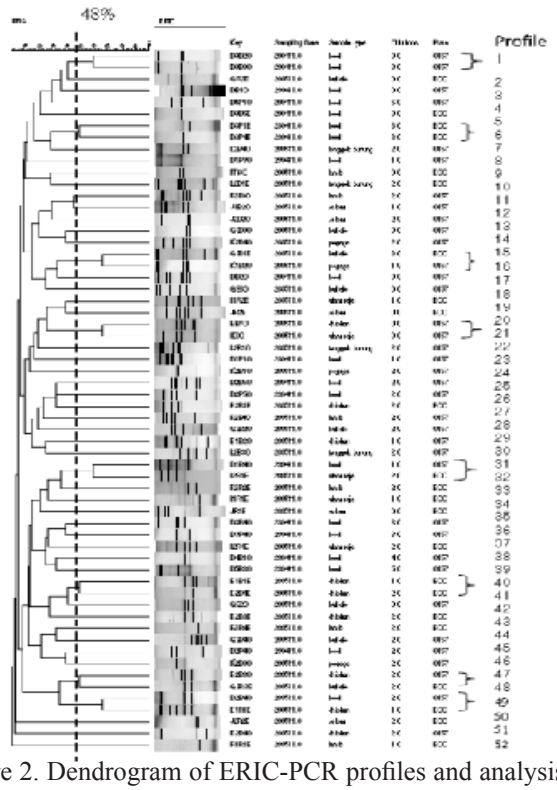

Figure 2. Dendrogram of ERIC-PCR profiles and analysis of genetic relatedness among *Escherichia coli* isolates

factors. The occurrence of these virulence genes were summarized in Table 3.

Discussion

In this study, plasmid profiling was performed to obtain a molecular strain typing of the isolates. However, some of the isolates were not typeable by plasmid profiling. The plasmid content of most bacterial strains is usually a stable feature, although there are cases in which plasmids are lost during subculture. A study by Levine *et al*. (1985) showed that the EAF plasmid was lost in a high proportion of colonies recovered from the stools of volunteers.

From the results, we observed that isolates arising from the same food sample type did not always have the same plasmid profile. In fact, different profiles of isolates from the same food sample were found. Besides, same plasmid profile also occurred in isolates arising from different food sample type. The best results are usually obtained by combining plasmid profiles with other typing data. Plasmid profiling in this study does not demonstrate high discriminatory power in term of clustering based on the sources of isolates.

ERIC-PCR is a recognized method of studying bacterial diversity (Versalovic *et al*., 1991; Wolska and Szweda, 2008). The technique is simple, fast, less labour intensive, does not require expensive setup, can be performed in any place with moderate facilities, and eliminate the need for pure DNA and only a small amount of template is required for the amplification reaction. The *Escherichia coli* isolates were subjected to ERIC-PCR to further verify the genetic relationship among isolates. It was of interest to determine whether these isolates are genetically diverse or clonal.

The *Escherichia coli* isolates produced many different ERIC patterns (52 different ERIC profiles among the 60 isolates analysed). The differences in band sizes and recognition of 52 distinct profiles among 60 isolates analyzed, reflected apparent polymorphism among isolates based on amplification of ERIC sequences. Additionally, identification of the 52 distinct ERIC profiles also showed the variable copy numbers and location of ERIC sequences which are known to vary greatly. This indicates high diversity among the *Escherichia coli* isolates. Ling *et al*. (2000) characterized a total of 30 strains of *Escherichia coli* O157:H7 isolated from beef and chicken burger by ERIC-PCR. In that study they found that the ERIC polymorphism patterns obtained showed a significant discriminatory fingerprint among the 30 *Escherichia coli* O157:H7 strains. Nearly every isolate had a unique fingerprint and that there were no bands that were highly conserved among the isolates. Their study suggested that there is considerable genetic heterogeneity among the *Escherichia coli* O157:H7 strains by ERIC-PCR. Study carried out by Son *et al*. (1998) also showed that *Escherichia coli* O157:H7 from beef samples in Malaysia had diverse profiles after analyzed by arbitrarily primed polymerase chain reaction. The numbers of polymorphic DNA fragments obtained from the ERIC-PCR were used for cluster analysis of the *Escherichia coli* isolates. In this study, we found that there is no specific trend of clustering of the *Escherichia coli* isolates with regard to ERIC-PCR on the food sample types.

From the dendrogram analysis, we observed that *Escherichia coli* isolates were arbitrarily grouped within the dendrogram regardless of the food sample types. Besides, *Escherichia coli* O157 were found distributed heterogeneously among the food sample types tested. ERIC profile number 3 and 17 showed that a food sample can carry two different unrelated *Escherichia coli* strains. In fact, different profiles of isolates from the same food sample were observed. Therefore, it is advisable to analyze multiple isolates from each food sample since a sample may harbor strains with different genetic profiles as evidence by the results of this study. Moreover, the same profile also occurred in isolates arising from different samples, as shown in ERIC profile 32, indicating the widespread diffusion of some biotypes.

In addition to the detection of *Escherichia coli* from beef, chicken, lamb, buffalo, pegaga, ulam raja, and selom, the virulence genes of the isolates were determined by multiplex PCR to know if these isolates possess the same virulence factor profile that *Escherichia coli* strains isolated from human infections have. We choose the method of Kim *et al*. (2010) because in this multiplex assay, all seven primer pairs successfully targeted seven genes from the four major virotypes (i.e., VTEC/EHEC, EPEC, EIEC and ETEC) when mixed in a single reaction tube.

After screened by the multiplex assay, we found that some isolates from the meat type food samples (beef, chicken, lamb and buffalo) were contaminated with *Escherichia coli* carrying toxin genes. The meat products might be contaminated during slicing, chopping and hand mixing. Training for food handlers on safe food handling and proper cooking are therefore important to reduce or eliminate the risk from pathogenic bacteria originating from raw foods.

Isolates from pegaga, selom, tenggek burung and ulam raja were found to be contaminated by *Escherichia coli* carrying toxin genes as well. Contamination of the vegetable food sample types may occur when farmers grow them in fields, processing and distribution, in addition to polluted rinsing water, human handling, animals, unhygienic equipment or transportation vehicles, cross-contamination and high storage temperatures (Beuchat, 2002; Johannessen *et al*., 2002).

Besides, we also found that isolates from the same food sample types can carry different combination of virulence genes. This revealed that a food sample type could harbor at the same time different *Escherichia coli* strains, regarding their virulence patterns. This correlates with the findings of other researcher. Previous investigation on *Escherichia coli* isolates obtained from stool (Woodward *et al*., 1992; Stacy-Phipps *et al*., 1995; Paton and Paton, 1998; Tsen *et al*., 1998), natural water (Lang *et al*., 1994) and food samples (Tsen *et al*., 1996) also demonstrated the presence of multiple virulence genes in many clinical and environmental isolates of *Escherichia coli*. Results from those studies and the present analysis together strongly indicate that many diverse strains of *Escherichia coli* that carry different combinations of virulence genes are present in the environment; which highlights the need for more effective monitoring methods that can rapidly detect, identify and type these pathogens for risk assessment purposes.

In this study, after amplification with the protocol described, 14 isolates were found to carry *eaeA* gene alone. The proportion of colonies with *eaeA* is low. These values are in good agreement with the study of Pierard *et al*. (1997) whereby STEC isolated from raw meat had low occurrence of *eaeA* genes. Although the *eaeA* gene is an established virulence factor in human enteropathogenic *Escherichia coli* (Donnenberg *et al*., 1993), the implications for food safety of *eaeA* positive *Escherichia coli* being present in food is not clear. However the presence of the *eaeA* gene alone could suggest the dangerousness of the *Escherichia coli* strain.

The percentage of the isolates carrying *VT2, VT1, LT* and *ST* toxin genes were very low as well in the present study. This is not a bias in the protocol, or bound to the inhibitor effects of food samples on the multiplex PCR, this rather attests to the low occurrence of *Escherichia coli* carrying these genes. Heuvelink *et al.* (1996) also observed that there was a lack of expression of *stx* (synonymous with *VT* and *SLT* (Calderwood *et al*., 1996)) genes in STEC isolated from retail raw meats.

When comparing result of plasmid profiling with toxin gene profiles, there is no significant correlation between toxin gene and with the number of plasmid harbored and sizes of the plasmid. In addition, plasmid profile did not correlate with ERIC-PCR profile. The ERIC-PCR profile and toxin gene profile does not show any direct correlation as well.

In conclusion, the *Escherichia coli* isolated from the various food sources (beef, lamb, chicken, buffalo, pegaga, tenggek burung, selom, and ulam raja) showed different plasmid, ERIC and toxin gene profile. The isolates were highly diverse. The present survey may only be representative of the risk of *Escherichia coli* contamination at the precise period of investigation. Therefore, increased and consistent monitoring for the presence of *Escherichia coli* in various food sources is needed in addition to monitor the level of virulence genes in order to ascertain the potential public health risk of these emerging strains.

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References

Adzitey Frederick. 2011. *Escherichia coli*, it Prevalence and Antibiotic Resistant in Malaysia: A Mini Review. Microbiology Journal 1 (2): 47-53.

Aoki, T., and A. Takahashi. 1987. Class D tetracycline resistance determinants of R plasmids from the fish

pathogens *Aeromonas hydrophila, Edwardsiella tarda,* and *Pasteurella piscicida*. Antimicrobial Agents and Chemotherapy 31:1278-1280.

Apun, K., Chang, P.P., Sim, E.U.H. and Micky, V. 2006. Clonal diversity of *Escherichia coli* isolates from marketed beef in East Malaysia. World Journal of Microbiology and Biotechnology 22: 661-667.

Beuchat, L. R. 2002. Ecological factors influencing survival and growth of human pathogens on raw fruits and vegetables. Microbes and Infection 4: 413-423.

Calderwood, S. B., Acheson, D.W.K., Keusch, G.T., Barrett, T.J., Griffin, P.M., Strockbine, N.A., Swaminathan, B., Kaper, J.B., Levine, M.M., Kaplan, B.S., Karch, H., O'Brien, A.D., Obrig, T.G., Takeda, Y., Tarr, P.I. and Wachsmuth, I.K. 1996. Proposed new nomenclature for SLT (VT) family. American Society for Microbiology News 62: 118–119.

Chaslus-Dancla, E., Gerbaud, G., Lagorce, M., Lafont, J. and Courvalin, P. 1987. Persistence of an antibiotic resistance plasmid in intestinal *Escherichia coli* of chickens in the absence of selective pressure. Antimicrobial Agents and Chemotherapy 31:784-788.

Dalla-Costa, L.M., Irino, K., Rodrigues, J., Rivera, I.N.G. and Trabulsi, L.R. 1998. Characterization of diarrhoeagenic *Escherichia coli* clones by ribotyping and ERIC-PCR. International Journal of Medical Microbiology 47: 227-234.

Donnenberg, M.S., Tzipori, S., McKee, M.L., O'Brien, A.D., Alroy, J. and Kaper, J.B. 1993. The role of the eae gene of enterohemorrhagic *Escherichia coli* in intimate attachment in vitro and in a porcine model. Journal of Clinical Investigation 92: 1418–1424.

Heuvelink, A. E., Wernars, K., and De Boer, E. 1996. Occurrence of *Escherichia coli* O157 and other verocytotoxin producing E. coli in retail raw meats in the Netherlands. Journal of Food Protection 59: 1267–1272.

Hodges, J.R. and Kimball, A.M. 2005. The global diet trade and novel infections. Globalization and Health 1: 1-7.

Hulton C S J., Higgins C. and Sharp P.M. 1991. ERIC sequences a novel family of repetitive elements in the genome of *Escherichia coli*, *Salmonella typhimurium* and other enterobacteria. Molecular Microbiology 5: 825-834.

Hu, Y., Zhang, Q. and Meitzler, J.C. 1999. Rapid and sensitive detection of *Escherichia coli* 0157:H7 in bovine faeces by a multiplex PCR. Journal of Applied Microbiology 87: 867-876.

Johannessen, G. S., Loncarevic, S. and Kruse, H. 2002. Bacteriological analysis of fresh produce in Norway. International Journal of Food Microbiology 77: 199-204.

Kim, K.H., Joon, C., Cheung, C.Y., Jong, M. L., Cho, S. Y., Kang C. S. and Kim, D.H. 2010. Development of Multiplex PCR Assays to Identify *Escherichia coli* Pathogenic Genes in Food. Journal of Food Science and Biotechnology 19(5): 1205-1210.

Lang, A. L., Tsai, Y. L., Mayer, C. L., Patton, K. C. and Palmer, C. J. 1994. Multiplex PCR for detection of the heat-labile toxin gene and shiga-like toxin I and II genes in *Escherichia coli* isolated from natural waters. Applied and Environmental Microbiology 60: 3145-3149.

Levine, M. M., Nataro, J. P. and Karch, H., Baldini, M.M., Kaper, J.B., Black, R.E., Clements, M.I. and O'Brien, A.D. 1985. The diarrheal response of humans to some classic serotypes of enteropathogenic *Escherichia coli* is dependent on a plasmid encoding an enteroadhesiveness factor. Journal of Infectious Diseases 152: 550-559.

Ling, O., Rasul, G., Ismail Abdul Karim, M., Purwati, E. and Lihan, S. 2000. Enterobacterial repetitive intragenic consensus (ERIC) genotyping of *Escherichia coli* O157:H7. Pakistan Journal of Biological Sciences 3: 35-37.

Nakamura, M,. Sato, S., Ohya, T., Suzuki, S. and Ikeda, S. 1986. Plasmid profile analysis in epidemiological studies of animal *Salmonella typhimurium.* Journal of Clinical Microbiology 23: 360-365.

Nataro, J.P. and Kaper, J.B. 1998. Diarrheagenic *Escherichia coli*. Clinical Microbiology Reviews 11: 142-201.

O'Brien, T. F., Hopkins, J.D., Gilleece, E.S., Medeiros, A.A., Kent, R.L., Blackburn, B.O., Holmes, M.B., Reardon,J.P., Vergeront, J.M., Schell, W.L., Christenson, E., Blssett, M.L. and Morse, E.V. 1982. Molecular epidemiology of antibiotic resistance in *Salmonella* from animals and human beings in the United States. New England Journal of Medicine 307:1-6.

Paton, A.W. and Paton, J.C. 1998. Detection and characterization of Shiga toxigenic *Escherichia coli* by using multiplex PCR assays for *stx1, stx2, eaeA*, enterohemorrhagic *E. coli hlyA, rfbO111*, and *rfbO157.* Journal of Clinical Microbiology 36: 598–602.

Pierard, D., Van Damme, L., Moriau, L., Stevens, D. and Lauwers, S. 1997. Virulence factors of verocytotoxin-producing *Escherichia coli* isolated from raw meats. Applied and Environmental Microbiology 63: 4585–4587.

Son, S., Ling, O.W., Rusul, G., Karim, M.I. and Nishibuchi, M. 2001. Detection of *Escherichia coli* O157:H7 by multiplex PCR and their characterization by plasmid profiling, antimicrobial resistance, RAPD and PFGE analyses. Journal of Microbiological Methods 46: 131–139.

Reid, S.D., Selander, R.K. and Whittam, T.S. 1999. Sequence diversity of flagellin (fliC) alleles in pathogenic *Escherichia coli.* Journal of Bacteriology 181: 153–160.

Riley, L.W., Remis, R.S., Helgerson, S.D., McGee, H.B., Wells, J.G., Davis, B.R., Hebert, R.J., Olcott, E.S., Johnson, L.M., Hargrett, N.T., Blake, P.A. and Cohen, M.L. 1983. Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. New England Journal of Medicine 308: 681-685.

Sahilah, A.M. 1997. Plasmid profiling and antibiotic resistance of *Escherichia coli* and *Escherichia coli* O157 strains. University Putra Malaysia, MSc thesis.

Son, R., Sahilah, A.M., Rusul, G., Zainuri, A., Tadaaki, M., Norio, A., Yung, B.K., Jun, O. and Nishibuchi, M. 1998. Detection of *Escherichia coli* O157:H7 in beef marketed in Malaysia. Applied and Environmental Microbiology 64: 1153-1156.

Stacy-Phipps, S., Mecca, J.J., Weiss, J.B., 1995. Multiplex PCR assay and simple preparation method for stool specimens detect enterotoxigenic *Escherichia coli* DNA during course of infection. Journal of Clinical Microbiology 33: 1054–1059.

Sukhumungoon, P., Nakaguchi, Y., Ingviya, N., Pradutkanchana, J. and Iwade, Y., Seto, K., Son, R., Nishibuchi, M. and Vuddhakul, V. 2011. Investigation of stx2+, eae+ *Escherichia coli* O157:H7 in beef imported from Malaysia to Thailand. International Food Research Journal 18: 381-386.

Tenover, P.C., Williams, S., Gordo, K.P., Harris, N., Nolam, J. and Plords, J. 1984. Utility of plasmid finger-printing for epidemiological studies of Campylobacter jejuni infections. Journal of Infectious Disease 149: 279-289.

Tornieporth, N.G., John, J., Salgado, K., De Jesus, P., Latham, E., Melo, M.C.N., Gunzberg, S.T. and Riley, L.W. 1995. Differentiation of pathogenic *Escherichia coli* strains in Brazilian children by PCR. Journal of Clinical Microbiology 33: 1371–1374.

Tsen, H. Y., Chi, W. R. and Lin, C. K. 1996. Use of novel polymerase chain reaction primers for the specific detection of heat labile toxin I, heat stable toxin I and II enterototoxigenic *Escherichia coli* in milk. Journal of Food Protection 59: 795-802.

Tsen, H.-Y. Jian, L.-Z. 1998. Development and use of a multiplex PCR system for the rapid screening of heat labile toxin I, heat stable toxin II and shiga-like toxin I and II genes of *Escherichia coli* in water. Journal of Applied Microbiology 84: 585–592.

Tsen, H.Y., Lin, C.K. and Chi, W.R. 1998. Development and use of 16S rRNA targeted PCR primers for the identification of *Escherichia coli* cells in water. Journal of Applied Microbiology 85: 554-560.

Versalovic, J., Koeuth, T. and Lupski, J.R. 1991. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Research 19: 6823-6831.

von Bodman, S. B. and Shaw, P.D. 1987. Conservation of plasmids among plant-pathogenic *Pseudomonas syringa*e isolates of diverse origins. Plasmid 17: 240-247.

Wolska, K. and Szweda, P. 2008. A Comparative evaluation of PCR ribotyping and ERIC PCR for determining the diversity of clinical *Pseudomonas aeruginosa* isolates. Polish Journal of Microbiology 57: 157-163.

Woodward, M. J., Carroll, P. J. and Wray, C. 1992. Detection of entero-and verocytotoxin genes in *Escherichia coli* from diarrhoeal disease in animals using the polymerase chain reaction. Veterinary Microbiology 31: 251-261.