Sains Malaysiana 39(1)(2010): 57–63

Detection of Shiga Toxin 1 and 2 *(stx*1 *and stx*2*)* Genes in *Escherichia coli* O157:H7 Isolated from Retail Beef in Malaysia by Multiplex Polymerase Chain Reaction (PCR)

(Pengesanan Gen Toksin Shiga 1 dan 2 (*stx*1 dan *stx*2) dalam *Escherichia coli* O157:H7 yang Dipencilkan daripada Daging Lembu Runcit di Malaysia dengan Menggunakan Tindak Balas Rantaian Polimerase Multipleks)

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ABSTRACT

Twenty (n=20) beef isolates of Escherichia coli *O157:H7 were examined for the detection of Shiga- toxin 1 and 2 (*stx1 *and* stx2*) genes by multiplex polymerase chain reaction (PCR) and characterized using Random Amplified Polymorphic DNA-Polymerase Chain Reaction (RAPD-PCR) fingerprinting. All isolates were obtained from the laboratory of Food Science and Biotechnology, University Putra Malaysia, Serdang, Selangor. In the detection of* stx*1 and* stx*2 genes, 14 of isolates (14/20) were positive to* stx*1 and* stx*2. 5 isolates (5/20) were positive to* stx*1 and 1 isolate (1/20) was negative by either of* stx1 *or* stx2 *genes. Using RAPD-PCR analysis, two oligonucleotides were chosen because they yielded clearly and reproducible band. There were OPAR8 (5'-TGGGGCTGTC-3') and OPAR20 (5'-ACGGCAAGGA-3'). Subsequently, all 20 isolates of* E.coli *O157:H7 were subtyped using OPAR8 and OPAR20. Primer OPAR8 produced 8 RAPD-PCR fingerprinting namely P1 to P11. Whereas, OPAR20 produced 16 RAPD-PCR fingerprinting of Q1-Q18. Combination of two primers was analyzed using Unweighted Pair Group Method with Arithmetic mean (UPGMA). Dendogram performed from cluster analysis showed that all the 20 isolates of* E.coli *O157:H7 differentiated into 20 individual isolates which may suggest the high level of local geographical genetic variation.*

Keywords: Escherichia coli *O157:H7; multiplex PCR; retail beef; Stx*1 *and stx*2 *genes*

ABSTRAK

Dua puluh (n=20) isolat Escherichia coli *O157:H7 yang diasingkan daripada daging lembu telah dikaji untuk mengesan gen toksin Shiga 1 dan 2 (*stx*1 dan* stx*2) dengan menggunakan multipleks rantaian tindak balas polimerase (PCR) dan pencirian dengan menggunakan cap jari Amplifikasi Polimorfik Asid Deoksiribonukleik-tindak balas rantaian polimerase (RAPD-PCR). Kesemua isolat telah diperolehi dari Makmal Sains Makanan dan Bioteknologi, Universiti Putra Malaysia Serdang, Selangor. Dalam pengesanan untuk gen* stx*1 dan* stx*2, 14 isolat (14/20) positif terhadap* stx*1 dan* stx*2. Manakala, 5 isolat (5/20) positif terhadap* stx*1 dan 1 isolat (1/20) negatif terhadap* stx*1 atau* stx*2 gen. Menggunakan analisis RAPD-PCR, 2 oligonukleotida telah dipilih kerana menghasilkan jalur yang jelas dan keputusan berulang. Mereka adalah OPAR8 (5'-TGGGGCTGTC-3') dan OPAR20 (5'-ACGGCAAGGA-3'). Oleh itu, kesemua 20 isolat E.coli O157:H7 telah disubtaipkan dengan menggunakan OPAR8 dan OPAR20. Primer OPAR8 menghasilkan 8 cap jari RAPD-PCR dinamakan P1 sehingga P11. Manakala OPAR20 menghasilkan 16 cap jari RAPD-PCR dinamakan Q1 hingga Q18. Gabungan dua primer dilakukan dengan menggunakan Kaedah Kumpulan Pasangan Tanpa penimbang dengan Keertian Aritmetik (UPGMA). Paparan dendrogram daripada analisis kelompok menunjukkan kesemua isolat* E.coli *O157:H7 boleh dibezakan kepada 20 isolat individu yang mencadangkan paras tinggi variasi genetik di geografi tempatan.*

Kata kunci: Daging lembu runcit; Escherichia coli *O157:H7; Gen Stx*1 *dan* Stx*2; PCR multipleks*

Introduction

Escherichia coli O157:H7 causes a spectrum of illness including diarrhea, haemorrhagic colitis, haemolytic uremic syndrome (HUS) and trombotic thrombocytopenic purpura (TTP) (Wells et al. 1983). The *E. coli* O157:H7 is categorized as enteheamorrhagic *E. coli* (EHEC) which caused worldwide outbreaks of haemorrhagic colitis and led in 10% of the cases to life-threatening haemorhagic-uremic syndrome (HUS) with a case-fatality rate ranging from 3% to 5%. HUS is characterized by acute renal failure, haemolytic anaemia and thrombocytopenia (WHO 2009).

It has been established that cattle is the major reservoir of *E. coli* O157:H7 with outbreaks of disease are associated with consumption of foods of bovine origins and related products (Griffin 1995). *E. coli* O157:H7 often produces two antigenically distinct types of *Stx*, *stx*1 and *stx*2 (Paton

& Paton 2002; Wang et al. 2002). The genes are originated form *Shigella dysenteriae* and also most common to Shigatoxigenic group of *E. coli* (STEC), serotype O157:H7, and other enterhaemorrhagic *E. coli* besides *S. dysenteriae* (Beutin 2006).

In Malaysia, there are no outbreak reports of foodborne disease cause by *E. coli* O157:H7, however this serotypes has been isolated from clinical samples as well as beef samples (Son et al. 1996, 1998). The incidence of *E. coli* O157:H7 in meat samples in local market indicated the exposure of these bacteria to public and can serve as a vehicle for the transmission of disease to man.

Typing of *E. coli* O157:H7 is important for epidemiology purposes such as to identify the sources of infection and monitroring the spread of bacteria. Thus, sensitive and efficient methods are needed for typing *E. coli* O157:H7. In this study we examined the Shiga- toxin 1 and 2 (*stx*1 and *stx*2) genes and genotypic characterized the 20 *E. coli* O157:H7 isolates using Random Amplified Polymorphic DNA-Polymerase Chain Reaction (RAPD-PCR) fingerprinting.

Material and Methods

Escherichia coli O157:H7 strains

Twenty (n=20) bacterial strains (EC1-EC20) were obtained from the laboratory of Food Science and Biotechnology, Universiti Putra Malaysia, Serdang, Selangor. All strains were isolated from retail beef and their geographical location was originated from Selangor area.

Preparation of whole-cell DNA for PCR and RAPD-PCR fingerprinting

A simple DNA extraction was done involving boiling, chilling and centrifugation (Jothikumar & Griffiths 2002). The cells were grown in 1.5 ml of Lauria-Bertani (LB)(Tryptone, 4.0 g/L, Yeast Extract, 5.0 g/L, Sodium chloride, 10.0 g/L) at 35°C for 20 hour were harvested and centrifuged at 12,000 rpm for 1 min. The supernatant was discarded. The pellet was then washed with 1.0 ml sterile distilled water and vortex. Then, it was boiled at 97°C for 10 min and immediately was frozen at -20°C for 10 minutes. The tube was centrifuged at 10,000 rpm for 3 minutes. The supernatant was used as a template.

Multiplex PCR for detection of Shiga-toxin 1 and 2 (*stx*1 and *stx*2) genes

The multiplex detection gene and PCR condition were optimized using recommendations reported previously by Fode-Vaughan et al. (2003) using 20 strains and 2 pairs of primers were used which stx2F(5'-TTCTTCGGTATCCTATTCCC-3'), stx2R (5'-ATGCATCTCTGGTCATTGTA-3'), stx1F(5'-CAGTTAATGTGGTGGCGAAG-3'), and stx1R (5'-CTGTCACAGTAACAACCGT-3') designed by Olsvik and Strockbine (1993). The detection assay was performed in a 25 µl volume containing 5.0 µl of 5 × PCR buffer (100 mmol l^{-1} Tris–HCl, 35 mmol l^{-1} $MgCl_2$, 750 mmol l^{-1} KCl, pH 8.8), 1.0 µl of 10 mmol l^{-1} dNTPs (Promega, Madison, USA) 1.0 µl of 10 pmol μl^{-1} primer *stx*2F, *stx*2R, *stx*1F and *stx*1R, 0.2 µl of 1.0 units of *Taq* DNA polymerase (Promega, Madison, USA), 12.30 µl of sterile ultrapure deionized water and 2.0 µl of 100 ng DNA template. A negative-DNA control was performed by adding 1 µl of sterile ultrapure deionized water, a positive control was performed by adding 1 µl of the DNA sample. Amplification was performed in personal Eppendorf thermal-cycler (Eppendorf, Germany) with a temperature program consisting of the initial denaturation at 94°C for 5 minutes followed by 35 cycles of denaturation at 94°C for 2 minutes, annealing for 1 minute at 35°C and polymerization at 72°C for 2 minutes. Final elongation was at 72°C for 10 minutes. The amplification products were analyzed by electrophoresis in a 1.0% agarose in 0.5X TBE (0.1 M Tris, 0.1 M Boric acid, 0.1 mM EDTA) at 90 V for 40 minutes. Gels were stained with ethidium bromide. The amplified fragments were visualized with UV transilluminator (Syngene, USA). The 100 bp DNA ladder (Promega, USA) was used as a DNA size marker.

Random Amplified Polymorphic DNA-Polymerase Chain Reaction (RAPD-PCR) fingerprinting

The discriminatory ability and stability of RAPD-PCR fingerprinting were tested in a preliminary study against a panel of 4 different bacterial strains of *E. coli* O157:H7 with 10-mer random primers (Promega, USA). Primer OPAR8 and OPAR20 showed the greatest stability and discriminatory ability among the *E. coli* O157:H7 isolates, and was therefore used in this study. The RAPD-PCR fingerprinting assay was performed in a 25 µl volume containing 2.5 µl of 10× PCR buffer (100 mmol l^{-1} Tris–HCl, 35 mmol l^{-1} $MgCl_2$, 750 mmol l^{-1} KCl, pH 8.8), 0.5 µl of 10 mmol l^{-1} dNTPs (Promega, Madison, USA) 1.0 µl of 10 pmol μl^{-1} primer (Either OPAR8 or OPAR20), 0.3 µl of 1.5 units of *Taq* DNA polymerase (Promega, Madison, USA), 18.95 µl of sterile ultrapure deionized water and 1 µl of 100 ng DNA template. A negative-DNA control was performed by adding 1 µl of sterile ultrapure deionized water. Amplification was performed in personal Eppendorf thermal-cycler (Eppendorf, Germany) with a temperature program consisting of the initial denaturation at 94°C for 5 minutes followed by 45 cycles of denaturation at 94°C for 1 minute, annealing for 1 minute at 35°C and polymerization at 72°C for 2 minutes. Final elongation was at 72°C for 7 min. The amplification products were analyzed by electrophoresis in a 1.0% agarose in 0.5X TBE (0.1 M Tris, 0.1 M Boric acid, 0.1 mM EDTA) at 90 V for 40 minutes. Gels were stained with ethidium bromide. The amplified fragments were visualized with UV transilluminator (Syngene, USA). The 100 bp DNA ladder (Promega, USA) was used as a DNA size marker.

DATA ANALYSIS

The banding patterns of individuals' strains were scored based on the presence or absence of the bands. The banding patterns scored were analyzed using the RAPDistance Package Software (version 1.04) program. The scoring was made in the form of binary code with the score '1' indicating presence of band and '0' the absence of band. The data obtained were recorded and entered in the software CorelDRAW Graphic Suite X3 where a dendogram was produced for further analysis. Clustering was based on the unweighted pair of group average method (UPGMA) and was performed with the RAPDistance software.

RESULTS

DETECTION OF SHIGA-TOXIN 1 AND 2 (*STX*1AND *STX*2) GENES

In this study, 2 pairs of primer were used for detection and confirmation of *E. coli* O157:H7 using which are *stx*2F, *stx*2R *stx*1F and *stx*1R as described by Fode-Vaughan et al. (2003). Two pair of primers were used for multiplex PCR analysis of twenty (n=20) isolates to detect *stx* gene. Most clinical signs of disease arise as a consequence of the production of Shiga toxin *stx*1, *stx*2 or combinations of these toxins (Griffin & Tauxe 1991). The DNA average sizes produced by the two primers are 180 bp and 255 bp, respectively (Paton & Paton 2002; Gehua et al. 2002). Among the twenty one samples, 14 isolates (14/20) were positive to *stx*1 and *stx*2, indicated by formation of 2 bands in a range of molecular weight of 180 bp – 255 bp. Whereas, 5 isolates (5/20)(EC2, EC7, EC12, EC14 and EC17) were positive to *stx*1, indicated by formation of only one band in a arrange of molecular weight of 180 bp while single isolate (1/20) (EC8) was lacked of both *stx*1 and *stx*2 which was indicated by no formation of band (Figure 1). Strain EC12 was used as a positive control (Lane 21). While, none band was observed on the negative control on the agarose gel in Lane 22 and excluded from the photograph.

RAPD-PCR FINGERPRINTING

A total of 10-mer of different oligonucleotide primers were used for RAPD-PCR analysis of a subset of 4 isolates to detect polymorphism within *Escherichia coli* O157:H7. The two primers produced a clear pattern and were used to analyse the whole set of 20 *E. coli* O157:H7strains.

Twenty (n=20) strains of *Escherichia coli* O157:H7 were used for RAPD-PCR analysis with OPAR8 and OPAR20. RAPD-PCR fingerprinting of *E. coli* O157:H7 obtained with primer OPAR8 represented by the Figure 2 and 3. The possible number of RAPD-PCR fingerprinting was estimated on the basis of changes in one or more clear bands or band sizes. Eleven (n=11) RAPD-PCR fingerprinting (P1-P11) were apparent from primer OPAR8. The number of RAPD bands produced for a given primer ranged from 1 to 7, with molecular sizes ranging from 0.3 to more than 1.5 kb with some of the bands appeared weak. However, no band produced with isolates EC1 and EC8 (Figure 3) with primer OPAR8.

The RAPD-PCR fingerprinting of *E. coli* O157:H7 strains obtained from primer OPAR20 is shown in Figure 4 and 5. Eighteen (n=18) RAPD-PCR fingerprinting (Q1-Q18) were obtained from primer OPAR20. The number of RAPD bands produced for a given primer ranged from 3 to 17, with molecular sizes ranging from 0.3 to more than 1.5 kb. Combination of both primers allowed the all *E. coli* O157:H7 differentiated into 20 genome types (Table 1).

COMBINATION OF TWO PRIMER USING UNWEIGHTED PAIR GROUP METHOD WITH ARITHMETIC MEAN (UPGMA) ANALYSIS

Combination of two primers, OPAR8 and OPAR20 was analyzed using Unweighted Pair Group Method with Arithmetic mean (UPGMA) analysis. Figure 6 showed the combination of RAPD-PCR fingerprinting of *E. coli* O157:H7 obtained from primer OPAR8 and OPAR20, respectively. Dendogram performed twenty *E. coli* O157:H7 strains into 2 major clusters. Cluster A contained 2 sub cluster which are sub cluster I and sub cluster II. Sub cluster I

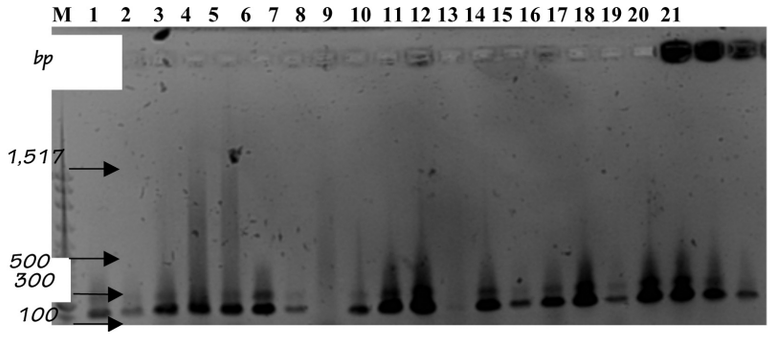
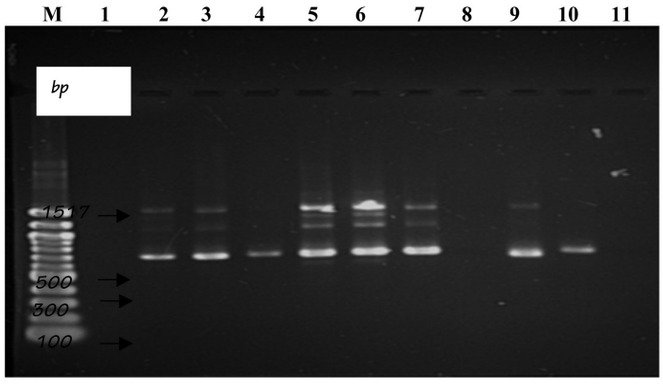
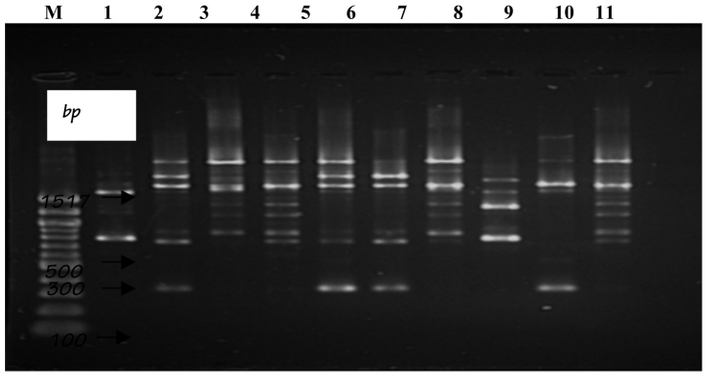


FIGURE 1. The electrophoresis patterns of multiplex polymerase chain reaction (PCR) detection of *Escherichia coli* O157:H7 isolates electrophoresed on 1.0 % agarose gel. M, Molecular weight sizes (base pairs, bp) are indicated by numbers on the left; lane 1-20: EC1, EC2, EC3, EC4, EC5, EC6, EC7, EC8, EC9, EC10, EC11, EC12, EC13, EC14, EC15, EC16, EC17, EC18, EC19, EC20. Lane 21: Positive control (EC 12)



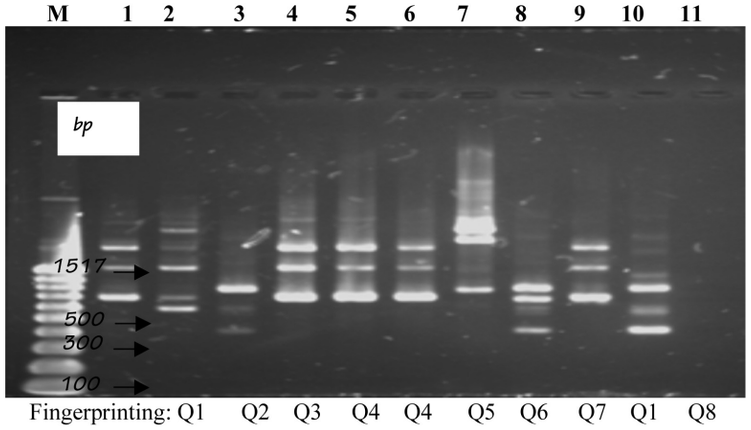
Fingerprinting: ND P1 P1 P2 P3 P3 P1 ND P4 P2

FIGURE 2. RAPD-PCR fingerprinting (P1-P4) of *Escherichia coli* O157:H7 isolates obtained with primer OPAR8 electrophoresed on 1.0% agarose gel. Lane M: 100 bp DNA ladder (molecular weight in base pair, bp); lane 1-10: EC1, EC2, EC3, EC4, EC5, EC6, EC7, EC8, EC9, EC10; Lane 11: Negative control



Fingerprintings: P5 P6 P7 P8 P6 P9 P7 P10 P11 P8

FIGURE 3. RAPD-PCR fingerprinting (P5-P11) of *Escherichia coli* O157:H7 isolates obtained with primer OPAR8 electrophoresed on 1.0% agarose gel. Lane M: 100 bp DNA ladder (molecular weight in base pair, bp); lane 1-10: EC11, EC12, EC13, EC14, EC15, EC16, EC17, EC18, EC19, EC20; Lane 11: Negative control.



Fingerprinting: Q1 Q2 Q3 Q4 Q4 Q5 Q6 Q7 Q1 Q8

FIGURE 4. RAPD-PCR fingerprinting (Q1-Q8) of *Escherichia coli* O157:H7 isolates obtained with primer OPAR20 electrophoresed on 1.0% agarose gel. Lane M: 100 bp DNA ladder (molecular weight in base pair, bp); lane 1-10: EC1, EC2, EC3, EC4, EC5, EC6, EC7, EC8, EC9, EC10; Lane 11: Negative control

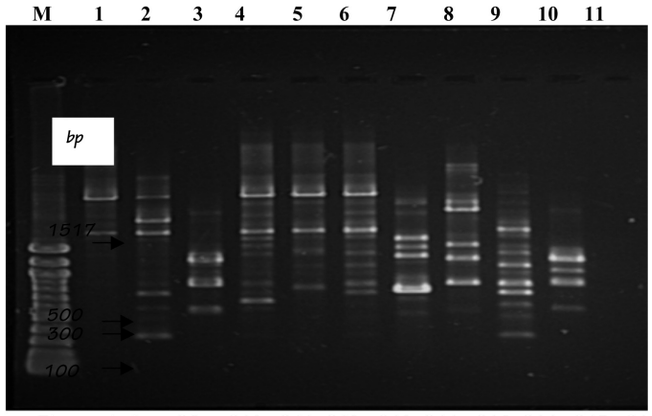


FIGURE 5. RAPD-PCR fingerprinting (Q9-Q18) of *Escherichia coli* O157:H7 isolates obtained with primer OPAR20 electrophoresed on 1.0% agarose gel. Lane M: 100 bp DNA ladder (molecular weight in base pair, bp); lane 1-10: EC11, EC12, EC13, EC14, EC15, EC16, EC17, EC18, EC19, EC20; Lane 11: Negative control

TABLE 1. Genotypic diversity of *Escherichia coli* O157:H7 using random amplified polymorpic DNA-PCR (RAPD-PCR)

Strains no.	RAPD-PCR fingerprinting observed with indicated primer[a]		Genome types no.[b]
	OPAR8	OPAR20	
EC1	ND	Q1	1
EC2	P1	Q2	2
EC3	P1	Q3	3
EC4	P2	Q4	4
EC5	P3	Q4	5
EC6	P3	Q5	6
EC7	P1	Q6	7
EC8	ND	Q7	8
EC9	P4	Q1	9
EC10	P2	Q8	10
EC11	P5	Q9	11
EC12	P6	Q10	12
EC13	P7	Q11	13
EC14	P8	Q12	14
EC15	P6	Q13	15
EC16	P9	Q14	16
EC17	P7	Q15	17
EC18	P10	Q16	18
EC19	P11	Q117	19
EC20	P8	Q18	20

ND-Not detected

[a]- The designation of the RAPD-PCR fingerprinting for each primer were arbitrarily assigned.

[b]- Genetic types were arbitrarily assigned on the basis of the combination of different RAPD-PCR fingerprinting.

has 6 strains of *E. coli* O157:H7 which are EC18, EC14, EC5, EC9, EC11 and EC8. Sub cluster II contained 7 strains which are EC12, EC15, EC16, EC19, EC7, EC4 and EC10. Cluster B divided into one sub cluster which is sub cluster III. It contained 7 strains which were EC1, EC2, EC6, EC20, EC17, EC13 and EC3.

DISCUSSION AND CONCLUSION

In this study, the detection of *stx*1 and *stx*2 genes have been shown among 20 beef isolates of *Escherichia coli* O157:H7. Fourteen (n=14) strains were positive to *stx*1 and *stx*2, 5 strains were positive to *stx*1 and a single strain was negative by either of *stx*1 or *stx*2 genes while a single isolate was negative either *stx*1 or *stx*2. All enterohemorrhagic *Escherichia coli* (EHEC) strains cause serious disease in humans and possess at least one Shiga-like toxin (*stx*1 or *stx*2) gene (Griffin 1995; Paton & Paton 2002; Wang et al. 2002). The detection of Shiga-like toxins is very useful for the identification of EHEC and Non-EHEC strains were negative for both *stx*1 and *stx*2. *The stx*1 *and stx* 2 *primers gave negative results f*rom other bacteria tested, including *Listeria monocytogenes*, *Listeria grayii*, *Listeria ivanovii*, *Salmonella enterica* serovar Typhimurium var. Copenhagen PT 10 SA, *S. enterica* serovar Enteritidis, *Shigella sonnei*, *Yersinia enterocolitica*, and *Proteus vulgaris* (Jothikumar & Griffiths 2002). In this work, EC8 showed negative result to *stx*1 and *stx*2 primers which clearly indicated the EC8 did not belong to the EHEC *E. coli*. The primers used are a powerful primer to amplify *stx*1 and *stx*2 sequences in pathogenic EHEC *E. coli* and able to distinguish among nonpathogenic *E. coli* isolates.

All *E. coli* isolates were examined for random amplied polymorphic DNA-PCR (RAPD-PCR). Two 10-mer arbitrary primers (OPAR8 and OPAR20) were used to generate RAPD-PCR fingerprints to the whole set of 20 *E. coli* O157:H7. The selections of those primers were based on good yield bands observed on the agarose gel. Several

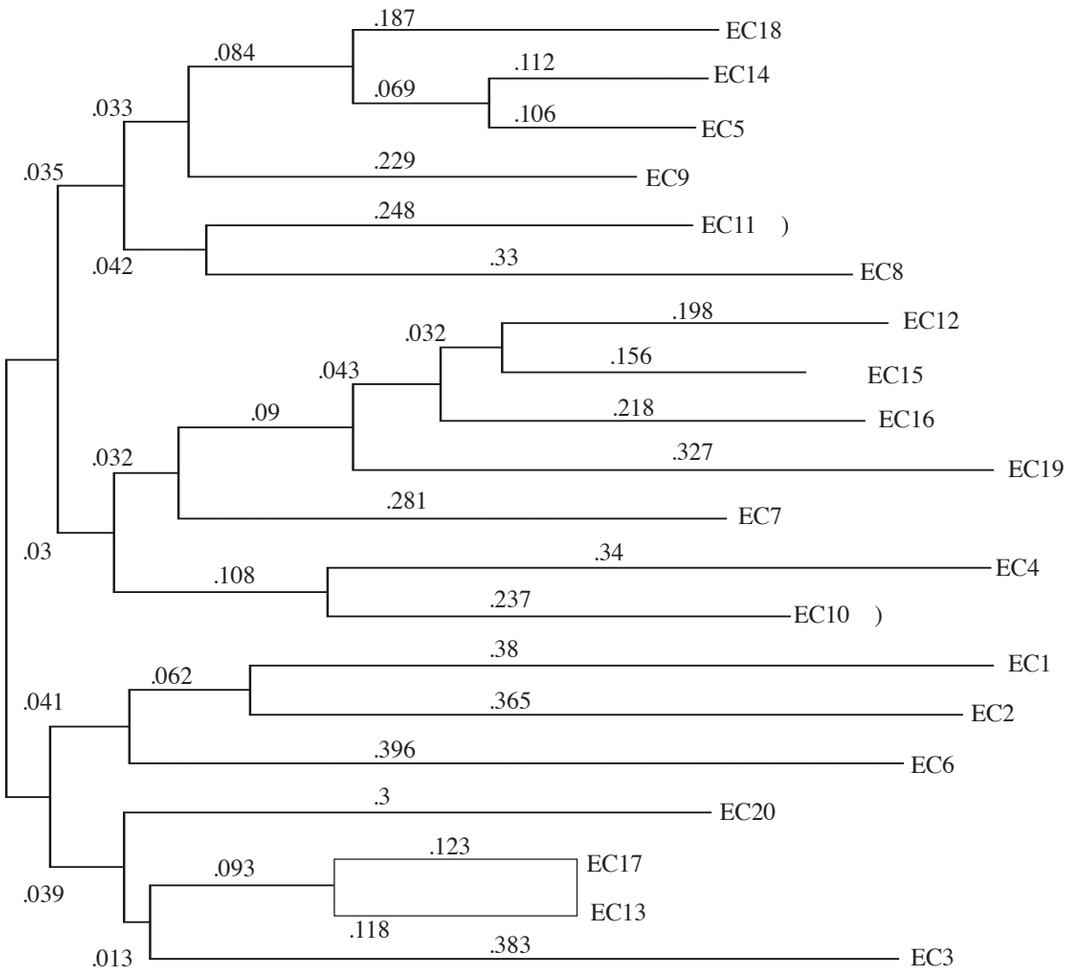


FIGURE 6. Dendogram generated from the random amplified polymorphic DNA-PCR (RAPD-PCR) fingerprinting among 20 beef isolates of the *Escherichia coli* O157:H7 with primer OPAR8 and OPAR20

isolates failed to produce any bands with the two primers used. This can be interpreted as the loss of specific sites for primers binding in the chromosomal DNAs of these isolates since these DNAs gave appropriate bands when they examined using the primers in reciprocal (Table 1).

The OPAR20 primer was more powerful in discriminatory of all *E. coli* O157:H7 tested which generated 18 fingerprints compared to OPAR8 which was only produced 11 fingerprints. The RAPD-PCR analysis using OPAR8 and OPAR20 in combination allowed all strains of *E. coli* O157:H7 differentiated into 20 genome types. Consistent with Unweighted Pair Group Method with Arithmetic mean (UPGMA) analysis, dendogram performed from cluster analysis showed that all the 20 isolates of *E.coli* O157:H7 differentiated into 20 individual isolates which may suggest the high level of local geographical genetic variation.

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Received: 12 March 2009
Accepted: 27 April 2009