Detection of Shiga Toxin 1 and 2 (stx1 and stx2) Genes in Escherichia coli O157:H7 Isolated from Retail Beef in Malaysia by Multiplex Polymerase Chain Reaction (PCR)

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 stx1 and stx2 genes, 14 of isolates (14/20) were positive to stx1 and stx2. 5 isolates (5/20) were positive to stx1 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 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; Stx1 and stx2 genes

INTRODUCTION

Escherichia coli O157:H7 causes a spectrum of illness including diarrhea, haemorrhagic colitis, haemolytic uraemic syndrome (HUS) and thrombotic 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 haemorrhagic-uraemic 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, stx1 and stx2 (Paton...
and most common to Shigella dysenteriae and also most common to Shigatoxigenic group of E. coli ( STEC), serotype O157:H7, and other enterohaemorrhagic 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 monitoring 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 (stx1 and stx2) 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 ultrapure deionized 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 12,000 rpm for 3 min. The supernatant was used as a template.

**MULTIPLEX PCR FOR DETECTION OF SHIGA-TOXIN 1 AND 2 (STX1 AND STX2) 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’-TTCTTCGCTATCCTATCCCC-3’), stx2R (5’-ATGCATCTCCGTGGTACATTGA-3’), stx1F (5’-CAGTTAATGGTGTCGGCAAG-3’), and stx1R (5’-CTGTACAGTAACAACCGT-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 MgCl₂, 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 l⁻¹ primer stx2F, stx2R, stx1F and stx1R, 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 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 MgCl₂, 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 l⁻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 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 (STX1 AND STX2) GENES

In this study, 2 pairs of primer were used for detection and confirmation of E. coli O157:H7 using which are stx2F, stx2R, stx1F and stx1R 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 stx1, stx2 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 stx1 and stx2, indicated by formation of 2 bands in a range of molecular weight of 180 bp – 255 bp, respectively (Paton & Paton 2002; Gehua et al. 2002).

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:H7 strains.

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)
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.

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.

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.
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 from 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 amplified 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

---

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

<table>
<thead>
<tr>
<th>Strains no.</th>
<th>RAPD-PCR fingerprinting observed with indicated primer</th>
<th>Genome types no.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OPAR8</td>
<td>OPAR20</td>
</tr>
<tr>
<td>EC1</td>
<td>ND</td>
<td>Q1</td>
</tr>
<tr>
<td>EC2</td>
<td>P1</td>
<td>Q2</td>
</tr>
<tr>
<td>EC3</td>
<td>P1</td>
<td>Q3</td>
</tr>
<tr>
<td>EC4</td>
<td>P2</td>
<td>Q4</td>
</tr>
<tr>
<td>EC5</td>
<td>P3</td>
<td>Q4</td>
</tr>
<tr>
<td>EC6</td>
<td>P3</td>
<td>Q5</td>
</tr>
<tr>
<td>EC7</td>
<td>P1</td>
<td>Q6</td>
</tr>
<tr>
<td>EC8</td>
<td>ND</td>
<td>Q7</td>
</tr>
<tr>
<td>EC9</td>
<td>P4</td>
<td>Q1</td>
</tr>
<tr>
<td>EC10</td>
<td>P2</td>
<td>Q8</td>
</tr>
<tr>
<td>EC11</td>
<td>P5</td>
<td>Q9</td>
</tr>
<tr>
<td>EC12</td>
<td>P6</td>
<td>Q10</td>
</tr>
<tr>
<td>EC13</td>
<td>P7</td>
<td>Q11</td>
</tr>
<tr>
<td>EC14</td>
<td>P8</td>
<td>Q12</td>
</tr>
<tr>
<td>EC15</td>
<td>P6</td>
<td>Q13</td>
</tr>
<tr>
<td>EC16</td>
<td>P9</td>
<td>Q14</td>
</tr>
<tr>
<td>EC17</td>
<td>P7</td>
<td>Q15</td>
</tr>
<tr>
<td>EC18</td>
<td>P10</td>
<td>Q16</td>
</tr>
<tr>
<td>EC19</td>
<td>P11</td>
<td>Q117</td>
</tr>
<tr>
<td>EC20</td>
<td>P8</td>
<td>Q18</td>
</tr>
</tbody>
</table>

ND-Not detected

*The designation of the RAPD-PCR fingerprinting for each primer were arbitrarily assigned.

*Genetic types were arbitrarily assigned on the basis of the combination of different RAPD-PCR fingerprinting.*
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 discriminating 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.

REFERENCES


Tenover and T.J. White, American Society for Microbiology, Washington, DC pp. 271-276.


A.M. Sahilah*
School of Chemical Sciences and Food Technology
Faculty of Science and Technology
Universiti Kebangsaan Malaysia
43600 UKM Bangi, Selangor D.E.
Malaysia

H. Nor’ Aishah & I. Noraida
Institute Biological Sciences
Faculty of Science
University of Malaya
50306 Kuala Lumpur
Malaysia.

A. Ahmad Azuhairi
Department of Community Health
Faculty of Medicine and Health Science
Universiti Putra Malaysia
43300 Serdang Selangor D.E.
Malaysia

*Corresponding author; email: sahilah@ukm.my

Received: 12 March 2009
Accepted: 27 April 2009