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Analysis of of *Escherichia coli* O157 isolated from imported beef and lamb in Malaysia and United Arab Emirates using RAPD-PCR

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Abstract: Enterohemorrhagic *Escherichia coli* (EHEC) of serotype O157 causing hemorrhagic colitis is on the increase in many parts of the world, hence specific surveillance of this pathogen is essential for identifying the sources and monitoring its spread. In this study, a total of 22 imported beef isolates of *E. coli* O157 isolated from wet market in Seri Serdang in Malaysia and 24 imported lamb isolates of *E. coli* O157 isolated from Center Slaughterhouse in Al-Ain in United Arab Emirates were examined. All the isolates were investigated by randomly amplified polymorphic DNA (RAPD) analysis with one primer: Gen1-50-9 (5´-AGAAGCGATG-3´). The primer generated polymorphisms in most of the isolates of *E. coli* O157 tested, producing bands ranging from 0.25 to 4.0 kilobases. The RAPD profiles revealed a high level of DNA sequence diversity within *E. coli* O157 isolates tested.

Key words: *Escherichia coli* O157, beef, lamb, RAPD-PCR

Introduction

Escherichia coli O157 is associated with a range of symptoms, including watery or bloody diarrhea, vomiting, hemorrhagic colitis, and hemolytic uremic syndrome, which is characterized by acute renal failure affecting mainly children and the immunocompromised (Griffin *et al.*, 1991). While the majority of foods linked to human outbreaks of *E. coli* O157 are not assessed quantitatively, some studies have indicated a low infective dose (Bell *et al.*, 1994 and Tilden *et al.*, 1996), highlighting the need for stringent control of contamination during food production.The ability to differentiate between strains of STEC O157 is complicated by the clonal nature of this group.

Natural populations of *E. coli* consist of a number of stable cell lines between which there is relatively little recombination of chromosomal genes. Traditional typing methods such as biochemical profiling (Ratnam *et al.*, 1988), plasmid profiling, verocytotoxin typing (Ostroff *et al.*, 1989), phage typing (Frost *et al.*, 1993), ribotyping (Martin *et al.*, 1996) and pulsed-field gel electrophoresis (PFGE) (Ooi, 2000) have had limited success in intraserogroup differentiation. As a consequence, there is a need to improve subtyping methods, with particular emphasis on the development of molecular methods to target the other minimal amount of variation observed between strains. Current methods are variable in their speed, technical complexity, cost and ability to discriminate reliably between STEC O157 (Grif *et al.*, 1998). To be able to trace reliably the source and mode of transmission of an outbreak, an epidemiological typing method must be able to discriminate reproducibly between different strains and identify identical strains. The potentially very severe sequelae arising from STEC O157 infection, including renal failure and death, mean that quick and effective epidemiological methods are required to trace the source and route of transmission of an outbreak.

Random amplification of polymorphic DNA by PCR (RAPD-PCR) analysis (Welsh *et al.*, 1990 and Williams *et al.*, 1990) is a molecular typing method that is finding increasing acceptance. A single, short (8-10 bp) primer of arbitrary sequence binds at complementary and partially mismatched sites to produce a useful strain-specific array of amplicons characteristic of the template DNA sequences of two strains and can add or remove binding sites or alter the distance between them, leading to changes in the amplicon profile. However, differences may exist undetected in the DNA sequence between two priming sites of an amplicon if they do not affect primer binding or amplicon length (Hilton *et al.*, 1998). Previous studies in which RAPD-PCR was used to subtype strains of STEC O157 have shown RAPD-PCR to be of considerable use in epidemiological studies and the technique compared favourably against selected typing methods (Grif *et al.*, 1998). The present study characterized representative isolates of STEC O157 isolated from Malaysia and United Arab Emirates by RAPD-PCR.

Materials and Methods

Bacterial Growth and Chromosomal DNA Isolation: The phenol extraction method was used for genomic DNA isolation. *Escherichia coli* O157:H7 isolates were grown in 5 ml Lauria Bertani (LB) broth (Sambrook *et al.*, 1989)

at 37°C with orbital shaking at 200 rpm. Bacterial cells were harvested by centrifugation from 1.5 ml of liquid culture and were washed in TE (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA) and resuspended in 700 il of the same buffer containing 1-mg/ml lysozyme. Subsequently, 50 il of 10% sodium dodecyl sulphate and 5 il of 20-mg/ml proteinase K were added to the mixed cell solution, and cells lysed for 20 minutes at 60°C. After incubation, the chromosomal DNA was extracted with an equal volume of TE saturated phenol-chloroform-isoamyl (25:24:1) alcohol (Promega, U.S.A.). The supernatant fluid was collected in an eppendrof tube and then about 200 il of 3M-potassium acetate and 400-il isopropanol were added to the suspension. The mixed solution was then incubated in ice for 10 minutes. The precipitated DNA was recovered by centrifugation at 12,000 rpm for 10 minutes. The supernatant was discarded and the pellet was washed with 0.5 ml of 70 % ethanol twice. The pellet was air-dried and dissolved in 50 il of sterile distilled water. The DNA was stored at -20°C until use. This was to avoid degradation of the genomic DNA.

RAPD Fingerprinting Primers: Ten-mer oligonucleotides (Gen 1-50-1 to Gen 1-50-10) of 50% G + C contents were screened in this study. Gene 1-50-9 (5´AGAAGCGATG´3) was selected for the study because it produce clear bands pattern in PCR products.

RAPD Amplifications

PCR-mixes were conducted in a final volume of 25 il containing of genomic DNA as a template (20-30 ng). 1X PCR buffer contained 200 mM Tris-HCl (pH 8.4), 500 mM KCl, 2.0 mM $MgCl_2$, 100 mM (each) deoxynucleotide triphosphate (dNTP), 0.5 iM primer, and 1 U of *Taq* polymerase (Promega). For all PCR experiments, negative controls containing sterile deionised water instead of DNA were always included. RAPD was carried out using a thermal cycler (Perkin Elmer 2400). The cycling parameters were 1 minute at 94°C (denaturation), 1 minute at 36°C (annealing) and 2 minutes at 72°C (extension) for a total of 45 cycles, with a final cycle extending amplification conditions to 72°C (elongation) for 5 minutes then after that cooled at 4°C.

Size Marker for PCR: The DNA ladder was used as size marker (1Kb DNA ladder, Promega, USA). It is ideal for determining the size of double stranded DNA from 250-10,000 base pairs. The ladder consists of 13 double-stranded, blunt-end fragments with sizes of 250, 500, 750, 1,000, 1,500, 2,000, 2,500, 3,000, 4,000, 5,000, 6,000, 8,000 and 10,000 base pairs.

Agarose Gel Electrophoresis: The PCR amplification products were resolved by electrophoresis in submerged horizontal agarose gels (1.0% w/v) in 1X TBE buffer pH 8.3. A lambda ladder (Promega) was used as size marker in all gels examined. The sizes of the DNA bands ranged from 0.25 to 10.0 kb. The required amount of agarose was dissolved in TBE buffer (pH 8.3) by boiling. The homogenous molten agarose solution was cooled to 50°C before casting. The resolving gels, 120 mm long, 150 mm wide and 5 mm deep was then placed horizontally in the buffer tank of the electrophoresis system. An aliquot of PCR amplification products (20 il) and a small (5-10 il) volume of tracking dye with 1 kb ladder were then loaded into sample wells. The tank was carefully filled with TBE buffer (pH 8.3) to completely submerge the gel by about 1 mm. Power of electrophoresis was supplied by a power pack (Model Biorad 300, USA.). Electrophoresis was carried out at room temperature from cathode (-) to anode (+) at constant voltage (70 V) until the tracking dye was 10-20 mm from the anode end of the gel.

Visualization of DNA Bands, Photography: Gel was stained in ethidium bromide (0.5 ìg/ml) solution for 30 minutes. The DNA-ethidium complex in the gels were visualized on a 320 nm UV transilluminatior (Model TM-36, UV products, Inc.). Photography was carried out with a Polaroid MP-4 land camera fitted with a red filter and Polaroid land 665 black and white films. The exposure time varied from 30 seconds to 40 seconds.

Interpretation of RAPD fingerprints: Scanned images were analyzed using the comparative analysis of electrophoresis patterns of Gel Compare (Kortrijk, Belgium). Bands were assigned on a presence-absence basis, regardless of intensity, using the cursor to mark the location. The computer software estimated band sizes for all data sets.

Results

This study was conducted to investigate the diversity of *Escherichia coli* O157 in beef and lamb from Malaysia and United Arab Emirates, respectively. Ten oligonucleotide decamers were tested for their ability to generate RAPD markers from genomic DNAs of a subset of three isolates of *E. coli* O157 strains. Nine primers gave very poor

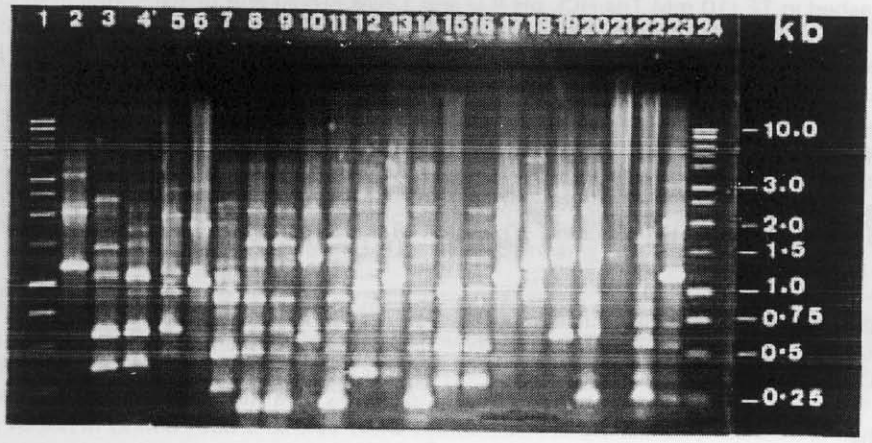

Fig. 1: RAPD-PCR fingerprints of *E. coli* O157 isolates no. 2-22 with primer Gen 1-50-9. Lane 1 and 24 contains DNA molecular weight markers in kilobase. Lanes 2, E1; 3 E2; 4, E3; 5, E5; 6, E7; 7, E8; 8, E9; 9, E10; 10, E12; 11, E13; 12, E14; 13, E16; 14, E18; 15, E19; 16, E20; 17, E21; 18, E22; 19, E23; 20, E24; 21, E25; 22, E26; 23, E27

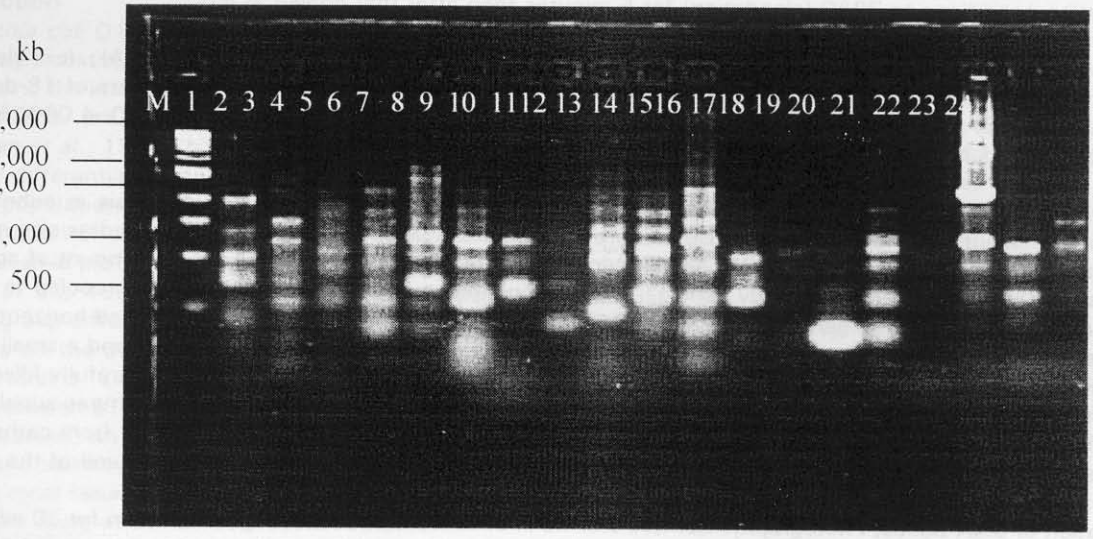

Fig. 2: Representative RAPD-PCr fingerprints of *E. coli* 0157 isolates no. 1-24 with primer Gen 1-50-9. Lane M contains DNA molecular weight markers in kilobase. Lanes 1, E1; 2, E2; 3, E3; 4, E4; 5, E5; 6, E6; 7, E7; 8, E8; 9, E9; 10, E10; 11, E11; 12, E12; 13, E13; 14, E14; 15, E15; 16, E16; 17, E17; 18, E18; 19, E19; 20, E20; 21, E21; 22, E22, 23, E23; 24, E24

amplification, and one of the primers generated a clear and reproducible patterns and were used to analyze all the isolates of imported beef and lamb isolated form Malaysia and United Arab Emirates. Fig. 1 showed the RAPD types of *E. coli* O157 obtained with primer Gen 1-50-09 (5´-AGAAGCGATG–3´) among the 22 isolates of beef from Malaysia. 18 RAPD types were apparent from primer Gen 1-50-09. The primer generated polymorphisms in all 22 isolates of *Escherichia coli* O157 tested, producing bands ranging from 0.25 to 4.0 kilobases. Representative results from the primer Gen1-50-9 to detect informative arrays of PCR products are shown in Fig. 2. Strain 18 was not typeable with primer Gen1-50-9 among the strains isolated from lamb from United Arab

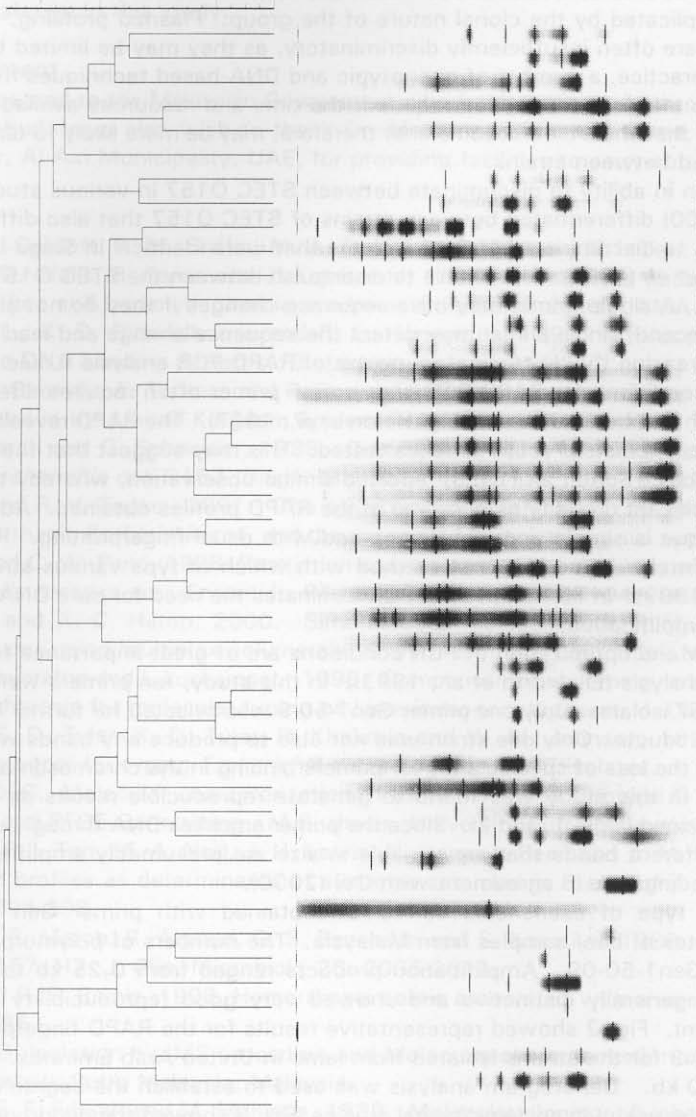

Fig. 13: Dendrogram showing genetic relationships between all strains of *E. coli* 0157 among isolates from Malaysia and United Arab Emirates beef and lamb samples respectively produced with primer Gen 1-50-9. Me, isolates from beef in Malaysia; E, isolates from lamb in United Arab Emirates

Emirates. The primer generated polymorphisms in the isolates of *E. coli* O157 tested, producing bands ranging from 0.25 to 4.0 kilobases. RAPD-PCR profiles were further analyzed using Gel Compar, to obtain a comprehensive result of the intraspecific relationships between all the strains analyzed from Malaysia and United Arab Emirates. Combination data from RAPD-PCR patterns obtained with primer Gen1-50-9, which yielded clear and reproducible patterns among all isolates examined, was made in a single dendrogram Fig. 3. The similarity in Fig. 3 was separated into separate clusters; isolates that are closely related are clustered together indicating close genetic distance.

Discussion

The ability to differentiate between strains of STEC O157, which are responsible for the majority of cases of enterohaemorrhagic disease, is complicated by the clonal nature of the group. Plasmid profiling, verocytotoxin typing, phage typing and ribotyping are often insufficiently discriminatory, as they may be limited to the number of types that can be described. In practice, a number of phenotypic and DNA-based techniques may be used in combination, depending on the amount of discrimination required, the time and resources available. Molecular methods such as RAPD-PCR examine the whole chromosome and, therefore, may be more likely to target the often-minimal amount of variation observed between strains.

This is demonstrated by the variation in ability to discriminate between STEC O157 in various studies that used different primers. Hopkins *et al.* (2000) differentiated between strains of STEC O157 that also differed in phage type and Birch *et al.* (1996) are able to discriminate between isolates that were identical in Shiga-like toxin type and phage type. However, Schmidt *et al.* (1999) were unable to distinguish between the STEC O157 strains used in their study by RAPD-PCR analysis. A single primer may miss sequence changes if they do not alter or remove a primer-binding site. The use of a second, single primer may detect the sequence change and lead to a variation in the pattern of amplicons, thus increasing the discriminatory power of RAPD-PCR analysis (Louie *et al.*, 1996). However, optimization of reaction conditions for RAPD-PCR with a new primer often requires alterations in the reaction and cycling conditions, which can be time consuming (Hilton *et al.*, 1997). The RAPD revealed a high level of DNA sequence diversity within *Escherichia coli* O157 isolates tested. This may suggest that the strains might have originated from divergence sources. Son *et al.* (1998) reported similar observation, whereby the isolates of *Escherichia coli* O157:H7 gave a significant diversity as observed in the RAPD profiles obtained. Additionally, the procedure of the RAPD-PCR technique is simple and rapid compared with usual fingerprinting. RAPD analysis promises a discriminatory, easy to interpret and low cost method with which to type various strains of *E. coli* O157:H7 of different origins (Rozila 1999). In addition, RAPD-PCR eliminates the need for pure DNA; only a small amount of template is required for amplification.

The selection of an appropriate primer and optimization of PCR conditions are of great importance for maximizing the discriminatory power of RAPD analysis (Ellsworth *et al.*, 1993). In this study, ten primers were screened to detect polymorphic within *E. coli* O157 isolates. Only one primer Gen1-50-9 was selected for further RAPD analysis because it gave highly reproducible products. Only one strain was not able to produce any bands with the primer used. And this can be interpreted as the loss of specific sites for primers binding in the chromosomal DNAs of this isolate. The random primer utilized in this study was found to generate reproducible results as shown in the respective RAPD banding profiles obtained (Figs. 1, and 2). Since the primer amplifies DNA throughout the genome during the RAPD-PCR reactions, different bands that are variable in size are presumably amplified from target sequences at different loci; these findings are in agreement with Ooi (2000).

Figs. 1 and 2 showed the RAPD type of *Escherichia coli* O157 obtained with primer Gen 1-50-09 (5`-AGAAGCGATG-3`), among the isolates of beef samples from Malaysia. The numbers of polymorphic loci varied from 18 RAPD types from primer Gen1-50-09. Amplification products ranged from 0.25 kb to 4.0 kb. The patterns produced by primer were generally distinctive and showed very good reproducibility provided that amplification parameters are consistent. Fig. 2 showed representative results for the RAPD fingerprints of *E. coli* O157 obtained with primer Gen1-50-9 for the strains isolated from lamb in United Arab Emirates. Amplification products ranged from 0.25 kb to 4.0 kb. Dendrogram analysis was used to establish the degree of relatedness among strains, information that may be useful in epidemiological studies. Fig. 3 showed the dendrogram generated from RAPD-PCR genetic relationships, it was obtained from the combined data from the two different locations, beef from Malaysia and lamb from United Arab Emirates, using primer Gen1-50-9. The generated dendrogram was constructed based on the similarity and difference of the genetic distance information gained from scoring the presence and absence of a band in the RAPD-PCR banding profiles (Gel Compar software). Based on the dendrogram generated, there appears to be a genetic similarity among the strains of beef and sheep isolated from Malaysia and United Arab Emirates respectively. These results further suggest that there is the potential for a single strain of *E. coli* O157 to be distributed widely within a population between Malaysia and United Arab Emirates.

If genotypes of *E. coli* O157 that are pathogenic to human are present, detection of the presence in a population of specific gene sequences, such as by RAPD patterns that characterize pathogenic isolates is important in determining the risk posed by various source of transmission. It can be concluded that the techniques used in the present study could be useful in a national monitoring program in order to compare and identify potentially pathogenic strains of this important pathogen.

Under the conditions employed in this study, RAPD-PCR was appropriate for developing a typing scheme for *E. coli*,

which is in general agreement with the results of an earlier study on *E. coli* O157:H7 conducted in Malaysia (Son *et al.*, 1998). Temporal changes in species can be expected and further investigation may reveal even greater diversity than shown in this study.

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