Molecular characterisation of verocytoxigenic *Escherichia coli* O157:H7 by random amplification of polymorphic DNA (RAPD) typing

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For the past two decades, polymerase chain reaction (PCR)-based genotyping methods have played an important role in bacterial typing schemes. One such PCR-based method, random amplification of polymorphic DNA (RAPD)-PCR, also known as arbitrarily primed-PCR (AP-PCR), has proved useful on account of its simplicity and utility for analysis of large-throughput samples.[1] This technique utilises a variable short-length arbitrary primer and does not require previous knowledge of the target DNA sequence data. The primer is amplified arbitrarily at low stringency, where the oligonucleotide binds at complementary and partially mismatched sites and generates bands that differ in length and nucleotide composition.

The RAPD-PCR methodology has been applied successfully for typing a wide variety of Gram-positive and Gram-negative bacteria. Various genus-specific and universal primers have been used, including the enterobacterial repetitive intergenic consensus (ERIC) sequence and M13 primers, and have successfully typed Gram-positive bacteria (e.g., methicillin-resistant *Staphylococcus aureus* [MRSA][2]) and Gram-negative organisms (e.g., *Pseudomonas aeruginosa*[3]). Therefore, the aim of this study is to examine use of the arbitrary primer M13 to genotype verocytotoxigenic *Escherichia coli* clinical isolates from Northern Ireland as a rapid means of strain differentiation and as an alternative to pulsed-field gel electrophoresis (PFGE).

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Isolates of *E. coli* O157:H7 (n=48) were used in the study, all of which were from clinical faecal specimens collected by the Northern Ireland Public Health Laboratory over a two-year period All *E. coli* O157 isolates were cultured on Columbia blood agar at 37°C overnight and passaged at least twice before DNA extraction. All DNA isolation procedures were carried out in a class II biological safety cabinet (MicroFlow, England) in a room physically separate from that used to set up nucleic acid amplification reaction mixes and also from the 'post-PCR' room, in accordance with the good molecular diagnostic procedure (GMDP) guidelines of Millar *et al.*[4], in order to minimise contamination and hence the possibility of false-positive results.

Genomic DNA from a single overnight colony of all *E. coli* isolates was extracted using the High Pure PCR Template Preparation Kit (Roche, England), in accordance with the manufacturer's instructions, and was quantified using the Ultrospec 2100 *pro* UV/visible spectrometer (Amersham Pharmacia Biotech, UK). All genomic DNA was within the concentration range 112–366 ng/μL and the purity coefficient of 2.01–2.13. Extracted DNA samples were visualised on 1% (w/v) agarose gel to access qualitatively the quality of the DNA.

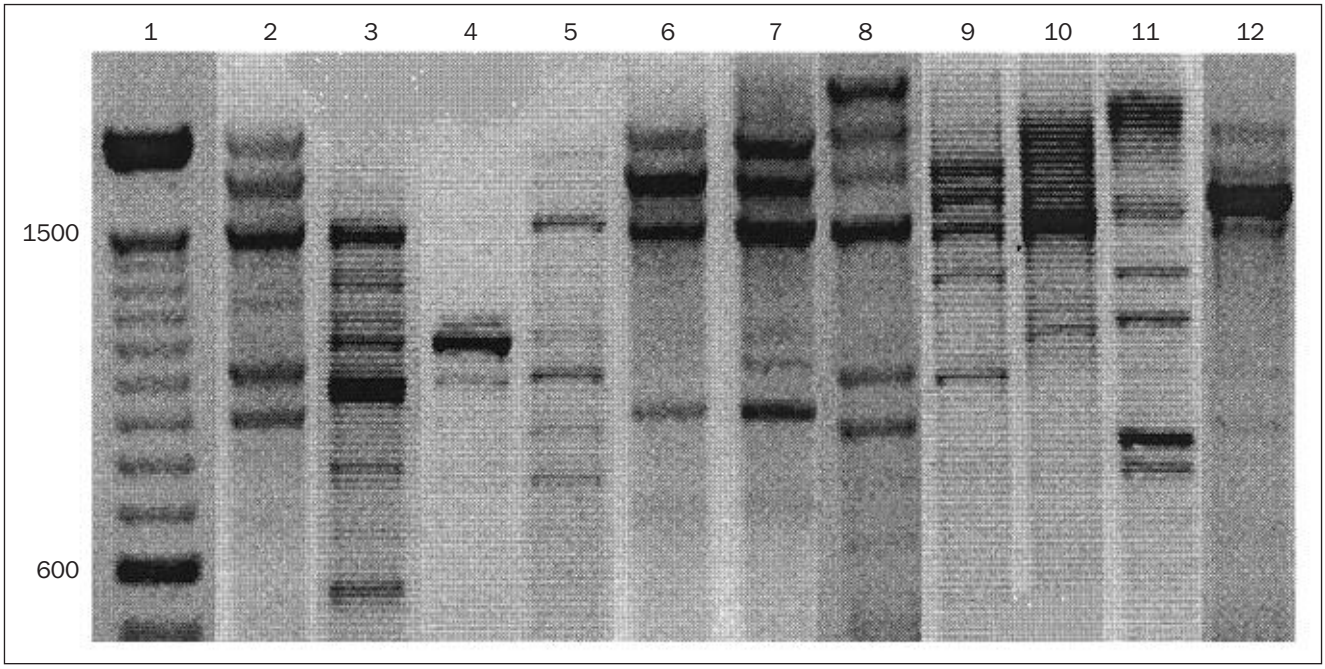

Fig. 1. A composite image displaying representative banding patterns of genotypes A–K obtained from 48 *E. coli* O157 isolates. Lane 1: molecular weight marker (Gibco); lane 2: genotype A; lane 3: genotype B; lane 4: genotype C; lane 5: genotype D; lane 6: genotype E; lane 7: genotype F; lane 8: genotype G; lane 9: genotype H; lane 10: genotype I; lane 11: genotype J; lane 12: genotype K.

Table 1. Comparison of laboratory attributes of pulse-field gel electrophoresis (PFGE) and random amplification of polymorphic DNA (RAPD) techniques.

Laboratory parameter	PFGE	RAPD
Nucleic acid amplification	No	Yes
Complete genome analysis	Yes	Yes
Prior DNA sequence knowledge required	No	No
Specialised *in situ* DNA extraction	Yes	No
Hands-on time	Moderate	Minimal
Running time	17 hours	3 hours
Technical expertise	Moderate	Moderate
Capital equipment	High	Moderate
Consumable costs	High	Moderate
Interpretation of results	Subjective but may be aided by gel capture software (e.g., bionumerics)	Subjective but may be aided by gel capture software (e.g., bionumerics)
Reproducibility	High	High, with appropriate controls

Amplification reactions were set up in accordance with GMDP guidelines.[4] Following opitimisation, reaction mixes (25 µL) were set up as follows: 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.75 mmol/L $MgCl_2$, 200 µmol (each) dATP, dCTP, dGTP and dTTP, 1.25 units *Thermus aquaticus* (*Taq*) DNA polymerase (Amplitaq, Perkin Elmer) and 20 pmol M13 arbitrary 15-mer primer (5′-GAG GGT GGC GGT TCT -3′).[5] Cycling parameters were an initial 5 min DNA denaturation cycle at 94°C followed by 40 cycles of 1 min at 94°C, 1 min at 50°C and 1 min at 72°C, then 72°C for 10 min and storage at 4°C. Amplicons were visualised on 1.5% agarose gel (ultrapure DNA grade, Bio-Rad) and the banding pattern was captured digitally using the Synapse Grabber software package (Synoptics, UK) system and saved as a bitmap file (*.bmp) for later analysis.

The genetic relationship between banding patterns was interpreted in accordance with the criteria as defined previously by Tenover *et al.*,[6] and individual M13 genotypes were assigned an arbitrary code based on ascending alphabetic order. Genotypes were assigned a new status when differing by a single band shift. Reproducibility of RAPD-PCR was examined with *E. coli* O157 isolates on at least two further occasions.

Use of RAPD-PCR with the M13 primer resulted in the generation of eleven genotypes (A–K): genotype A (58.3%), genotype B (10.4%), genotype C (8.2%), genotypes D, G and K (4.2% each) and genotypes E, F, H, I and J (2.1% each) (Fig. 1). The banding patterns consisted of between three bands (genotype C) to seven bands (genotype G), which varied in size from approximately 550 bp to >2000 bp (Fig. 1)

This study used RAPD to examine the genetic relatedness of human clinical *E. coli* O157 isolates, employing the M13 primer. The minisatellite probe M13 originated in the genome of bacteriophage M13 and its sequence, which is highly conserved, appears in bacterial, fungal, protozoal, animal and human genomes. For this reason and its relatively high occurrence rate in nature, it is important to eliminate all non-bacterial sources of DNA when undertaking a bacterial genotyping study, otherwise non-specific priming will occur that generates aberrant PCR amplicons, which will confuse the interpretation of results. Hence, bacterial cultures for genotypic analysis should be passaged at least twice in pure culture to remove any exogenous nucleic acid prior to M13 typing.

Use of this primer permitted the generation of banding patterns containing multiple amplicons, which aided in the differentiation of the isolates. This relative success in being able to generate several amplicons per isolate may have been due to use of a G+C-rich primer (67%) in conjunction with a target DNA template in *E. coli* that is 50–51% G+C. In addition, there was good reproducibility when examining isolates with M13, as similar banding patterns and hence clusters were obtained for the same isolates examined on different days. This reproducibility was due to the adoption of several control interventions during the study, including i) standardisation of the genomic DNA template, ii) use of single batches of reagents and a single thermal cycler, iii) optimisation and standardisation of the RAPD protocol, and iv) standardisation of the image-capture protocol.

Although the reproducibility of RAPD typing has been reported by various groups, there have also been reports of problems. Grif *et al.*[7] reported that RAPD suffered from a lack of standardisation using different methods in different laboratories, which may affect the quality of results. It can also suffer from the same factors that affect ordinary PCR, including magnesium concentration, primer and reagent batch-to-batch variation and the quality of the thermal cycler. It has also been suggested that for RAPD to be a definitive typing technique then the reproducible generation and interpretation of RAPD fingerprints needs to be developed. To achieve this, the use of an automated system for DNA preparation, manufactured RAPD master mixes, the same thermal cycler, and standard procedures for visualisation of fingerprints may aid reproducibility. The above notwithstanding, RAPD does offer an effective nucleic acid (DNA) amplification-based molecular alternative to PFGE genotyping (Table 1).

In conclusion, this small study demonstrates that use of M13 RAPD permits the rapid genotyping and discrimination of clinical *E. coli* O157:H7 isolates in a reproducible manner. With the adoption of several controls to aid reproducibility, this technique may be useful as an alternative to PFGE, particularly in the epidemiological investigation of outbreaks where speed may be a significant factor. □

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Direct molecular (PCR) detection of verocytotoxigenic and related virulence determinants (*eae*, *hyl*, *stx*) in *E. coli* 0157:H7 from fresh faecal material

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Verocytotoxin-producing *Escherichia coli* (VTEC) is closely associated with foodborne diseases such as haemorrhagic colitis (HC) and haemolytic uraemic syndrome (HUS). Infection with VTEC organisms may cause a wide range of symptoms, although some infected individuals may remain asymptomatic or not sufficiently ill to attend their family doctor or hospital. When symptoms are severe (i.e., requiring medical attention) generally they include severe diarrhoea.

While polymerase chain reaction (PCR) assays have been applied as an alternative approach to the detection of microorganisms by routine examination, direct PCR detection of enteric bacteria in faecal samples is made problematic by the presence of potent PCR inhibitors. Faeces comprises a mixture of substances that include bilirubin, bile salts, urobilinogens and polysaccharides, which, even when present at low concentrations, are known to inhibit PCR methods.[1]

There is evidence in the literature to indicate that symptomatic patients may not always grow a positive faecal culture on SMAC medium, possibly because only relatively few organisms are present. Therefore, the main aim of the present study is to examine laboratory methods for the direct molecular detection of verocytotoxin gene loci directly from faeces and to establish optimised molecular protocols for the detection of VTEC and related virulence genes in a small surveillance study. Such an approach could hasten implementation of appropriate treatment, control and prevention.

Faecal or spiked faecal-saline suspensions (100 µL) were mixed with 40 µL diatomaceous earth (DE, Sigma) and 900 µL lysis buffer, and incubated at room temperature for 10 min. Lysis buffer contained 120 g guanidine thiocyanate (Sigma) in 100 mL 0.1 mol/L Tris HCl (pH 6.4, Sigma), 22 mL 0.2 mol/L EDTA solution (pH 8.3, Prolabo) and 2.6 g Triton X-100 (Sigma). After incubation, the preparation was centrifuged at 11,600 x*g* for 15 sec and the pellet was washed (x2) with washing buffer (120 g guanidine thiocyanate [Sigma] in 100 mL 0.1 mol/L Tris HCl [pH 6.4]), then with 70% (v/v) ethanol (x2) and finally with acetone (x1). The washed pellet was dried on a heating block at 54°C for 10 min, resuspended in 100 µL TE buffer (10 mmol/L Tris HCl, 1 mmol/L EDTA [pH 8.0]), incubated at 54°C for 10 min then centrifuged at 11,600 x*g* for 2 min. The supernatant was removed to a fresh Eppendorf tube and stored at –20°C until required.

A 150 µL volume of polyoxyethylenesorbitan monoleate-TE (10% [w/v] polyoxyethylenesorbitan monoleate [Sigma] in TE buffer) was added to 50 µL nucleic acid suspension and incubated at room temperature for 10 min. In order to remove PCR inhibitors, 100 µL 2 mol/L ammonium acetate and 600 µL isopropanol were added and held at –20°C for 30 min to precipitate the DNA. The mixture was centrifuged at 11,600 x*g* for 10 min and the supernatant was discarded carefully using a Pasteur pipette. Purified DNA was resuspended in 50 µL TE buffer and then stored at –20°C until required.

The PCR methodology was performed on extracted genomic DNA from faeces (and a PCR control) employing nine VTEC and related virulence gene targets, using 16S ribosomal DNA (rDNA) universal or broad-range primers (Table 1). All PCR assays used were optimised for primer concentration, magnesium concentration, annealing temperature and PCR cycle number. The sensitivity of each PCR primer pair was determined by use of a spiked faecal suspension. Briefly, a fresh faecal suspension was prepared from a sample previously demonstrated by culture and molecular methods to be negative for the molecular gene loci examined. For each gene locus examined, 100 µL (w/v) of a serial dilution of *E. coli* NCTC 12079 in 0.1% peptone saline was diluted to 10^{-9} and then 900 µL faecal suspension was used. Bacterial genomic DNA was extracted, followed by amplification with the nine gene loci employing the optimised conditions defined previously. Viable cell count was performed in the peptone saline component by spreading 100 µL on nutrient agar (Oxoid CM0003) and incubating for 24 h at 37°C. Three dilutions were used

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