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References

1 Gürtler V, Mayall BC. Genomic approaches to typing, taxonomy and evolution of bacterial isolates. *Int J Syst Evol Microbiol* 2001; **51**: 3–16.
2 Kurlenda J, Grinholc M, Jasek K, Wegrzyn G. RAPD typing of methicillin-resistant *Staphylococcus aureus*: a 7-year experience in a Polish hospital. *Med Sci Monit* 2007; **13** (6): MT13–8.
3 Clarke L, Moore JE, Millar BC *et al*. Molecular epidemiology of *Pseudomonas aeruginosa* in adult patients with cystic fibrosis in Northern Ireland. *Br J Biomed Sci* 2008; **65**:18–21.
4 Millar BC, Xu J, Moore JE. Risk assessment models and contamination management: implications for broad-range ribosomal DNA PCR as a diagnostic tool in medical bacteriology. *J Clin Microbiol* 2002; **40**: 1575–80.
5 Schönian G, Meusel O, Tietz HJ *et al*. Identification of clinical strains of *Candida albicans* by DNA fingerprinting with the polymerase chain reaction. *Mycoses* 1993; **36**: 171–9.
6 Grif K, Karch H, Schneider C *et al*. Comparative study of five different techniques for epidemiological typing of *Escherichia coli* O157. *Diagn Microbiol Infect Dis* 1998; **32**: 165–76.
7 Power EG. RAPD typing in microbiology – a technical review. *J Hosp Infect* 1996; **34**: 247–65.

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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Table 1. Verocytotoxin-producing *E. coli* and related virulence gene targets used in this study, as defined in the corresponding references.

Primer	Target gene locus	Sequence (5' to 3')	Location within gene[1]	Product size (bp)	Reference
VT1-F VT1-R	Verocytotoxin 1 gene	ACA CTG GAT GAT CTC AGT GG CTG AAT CCC CCT CCA TTA TG	722–791 1373–1354	601	2
VT2-F VT2-R	Verocytotoxin 2 gene	CCA TGA CAA CGG ACA GCA GTT CCT GTC AAC TGA GCA CTT TG	624–644 1403–1384	780	2
eae-F eae-R	*eae* gene	TCG TCA CAG TTG CAG GCC TGG T CGA AGT CTT ATC AGC CGT AAA GT	803–824 1912–1890	1110	3
stx1-F stx1-R	Verocytotoxin 1 gene	ATA AAT CGC CAT TCG TTG ACT AC AGA ACG CCC ACT GAG ATC ATC	620–642 798–778	180	4
stx2-F stx2-R	Verocytotoxin 2 gene	GGC ACT GTC TGA AAC TGC TCC TCG CCA GTT ATC TGA CAT TCT G	841–861 1095–1074	255	4
eaeA-F eaeA-R	*eae* gene	GAC CCG GCA CAA GCA TAA GC CCA CCT GCA GCA ACA AGA GG	232–251 615–596	384	4
hlyA-F hlyA-R	Haemolysin gene	GCA TCA TCA AGC GTA CGT TCC AAT GAG CCA AGC TGG TTA AGC T	70–90 603–582	534	4
O157-F O157-R	*E. coli* O157 *rfb* gene	CGG ACA TCC ATG TGA TAT GG TTG CCT ATG TAC AGC TAA TCC	393–412 651–631	259	4
O111-F O111-R	*E. coli* O111 *rfb* gene	TAG AGA AAT TAT CAA GTT AGT TCC ATA GTT ATG AAC ATC TTG TTT AGC	24–47 429–406	406	4

in triplicate and viable count was expressed as $\log_{10}$ colony-forming units (cfu)/mL.

Following optimisation of the nine PCR-specific assays and the DNA extraction protocol, and quantifying the sensitivity of each assay in faecal suspensions, 94 routine faecal specimens were examined for the presence of the nine gene loci, using the optimised methods, in a proof-of-concept determination. The faecal samples were collected from GP samples (n=44) and from hospital in-patients (n=50) and were chosen randomly, regardless of the patient's illness.

Genomic bacterial DNA, extracted directly from faecal samples, was screened for verocytotoxin 1 and 2, *eae, hlyA*, O111 and the O157 virulence factor, using a series of single PCR assays (Table 1). These were able to detect verocytotoxin II and the *eae* virulence factor from faecal samples (Table 2); however, no sample was positive for O157, O111 or enterohaemolysin (*hyl*) virulence factors. The presence of verocytotoxin 2 or *eae* alone would not confirm conclusively the presence of O157 isolates and this suggested that *E. coli* O157:H7 was not present in the screened faecal samples examined.

A reliable DNA purification method is very important for subsequent PCR analysis. The existence of inhibitors may decrease the sensitivity of the amplification, and therefore several methods have been used to extract DNA directly from faecal samples for subsequent PCR analysis. For example, the spin column method has been used to reduce the inhibitors in samples used to amplify part of the *omp*C region in salmonellas.[5] Stewart *et al*.[6] reported that a simple boiling method to extract DNA for PCR analysis had a greater sensitivity (approximately 3 cfu/g faeces) than that achieved using immunomagnetic separation (IMS).

Bilirubin and bile salts are major constituents of faecal material that inhibit PCR, even when present in low concentration.[1] Dilution of faecal samples can reduce the effect of inhibition, but it can also reduce the sensitivity of the assay. Subculturing faecal samples prior to extraction may increase the number of target organisms, reduce the level of inhibitors and enhance DNA purification,[4] but the advantage of using PCR to reduce test turnaround time may be lost.

Therefore, the development of direct DNA extraction from faeces for PCR template preparation is an important laboratory issue. In the present study, the method described by Lawson *et al*.[7] was used to extract and purify DNA directly from faeces. This method is based on that developed for bacterial extraction from serum and urine demonstrated by Boom *et al*.[8] These workers showed that nucleic acid will bind to diatoms, silica or glass particles in the presence of high concentrations of the chaotropic agent guanidium

Table 2. Frequency of virulence gene loci in a collection of random faecal specimens.

Virulence gene target	Number positive	Conventional result
VT1	0/94	NSG
VT2	1/94 (1.06%)	*Salmonella agona*
eae	5/94 (5.32%)	*E. coli* NSG *E. coli* *E. coli* NSG
Stx1	0/94	NSG
Stx2	1/94 (1.06%)	*E. coli* and *Campylobacter* sp.
*eae*A	3/94 (3.19%)	NSG NSG *E. coli*
*hly*A	0/94	NSG
0157	0/94	NSG
0111	0/94	NSG

NSG: no significant growth.

isothiocyanate (GuSCN). This is a powerful agent used in the purification and detection of DNA and RNA,[9] and has the ability to lyse cells and inactivate nucleases. Lawson *et al*.[7] also employed the method to remove PCR inhibitors, using polyvinylpyrrolidone (PVP).

Young *et al*.[10] described a PCR assay based on DNA extracted from soil that had a high organic content, following purification of the extract by electrophoresis through an agarose gel containing PVP. The mechanism by which PVP removes inhibitors is unclear, but Young *et al*. suggested that PVP absorbs phenolic compounds that bind covalently to DNA.

In the current study, polyoxyethylenesorbitan monoleate was used as an alternative to PVP. After DNA was extracted from the faecal material, the success of extraction was confirmed by PCR using p11p- and p13p-conserved primers for 16S rDNA, as previously described.[11] Inability to amplify a PCR product using these primers showed that PCR could not amplify any product from unextracted faecal suspension, even when the concentration of *E. coli* O157:H7 NCTC 12079 in the suspension was very high. Therefore, any PCR for 16S rDNA that failed to amplify was considered null and void and the extraction procedure was repeated, as inhibitors were still present in the extract. Using DNA extracted from spiked faecal samples, PCR produced strong positive amplicons from all the positive controls examined. This result suggests that DNA extraction employing this DNA extraction method may be useful for the direct detection of *E. coli* O157 from faecal samples.

The sensitivity of each PCR assay was assessed through a series of dilution assays. The sensitivity of the PCR using VTI and II, *eae* and O157 virulence factor was 4.4×10^5 cfu/g. The faecal extraction method used in this study was designed to extract DNA from bacteria and to purify the extracted DNA. In comparison to the results of work undertaken by Boom *et al*.,[8] a recent study showed that competition for binding to silica particles between DNA and a component present in faeces resulted in a low DNA extraction efficiency.[12] These workers suggested that choosing the correct ratio of silica particles to faeces would help to solve the problem. Other PCR assays using *eae*A and *hly*A virulence factors showed greater sensitivity (1 cfu/g) when DNA was extracted by the same method.

The *eae*A primer was designed to bind to enteropathogenic (EPEC) and enterohaemorrhagic (EHEC) strains. Paton and Paton[4] reported that *eae*A primers also reacted with *E. coli* O55. Many primer pairs have been used to amplify *E. coli eae* regions of EPEC, EHEC and VTEC strains. Gannon *et al*.[2] reported that several serogroups, especially *E. coli* O26, O55, O111, O128 and O145, have a tendency to cross-react. Furthermore, the central region of the *Yersinia pseudotuberculosis inv* gene locus showed 50% homology to that of the *E. coli* O157:H7 *eae* gene.[13] Paton and Paton[4] showed that the PCR primer *hly*A eliminated the possible cross-reaction with the gene encoding *E. coli* α-haemolysin, with which it shares approximately 70% DNA sequence homology.

The present study examined the benefits of direct PCR amplification from faecal samples. The choice of reliable, specific and sensitive primer sets for the PCR assays is essential, in order to obtain quality results that can facilitate detection, epidemiological studies and outbreak investigations. The combination of traditional methods (e.g., CT-SMAC medium), immunological detection and the PCR amplification of several target virulence gene loci may form the basis for an improved diagnostic service to provide optimised patient and outbreak management. □

References

1 Wilson IG. Inhibition and facilitation of nucleic acid amplification. *Appl Environ Microbiol* 1997; **63**: 3741–51.

2 Gannon VP, King RK, Kim JY, Thomas EJ. Rapid and sensitive method for detection of Shiga-like toxin-producing *Escherichia coli* in ground beef using the polymerase chain reaction. *Appl Environ Microbiol* 1992; **58**: 3809–15.

3 Beebakhee G, Louie M, De Azavedo J, Brunton J. Cloning and nucleotide sequence of the *eae* gene homologue from enterohemorrhagic *Escherichia coli* serotype O157:H7. *FEMS Microbiol Lett* 1992; **70**: 63–8.

4 Paton AW, Paton JC. Detection and characterization of Shiga toxigenic *Escherichia coli* by using multiplex PCR assays for *stx1, stx2, eaeA,* enterohemorrhagic *E. coli hlyA, rfb*O111, and *rfb*O157. *J Clin Microbiol* 1998; **36**: 598–602.

5 Amavisit P, Browning GF, Lightfoot D *et al*. Rapid PCR detection of *Salmonella* in horse faecal samples. *Vet Microbiol* 2001; **79**: 63–74.

6 Stewart DS, Tortorello ML, Gendel SM. Evaluation of DNA preparation techniques for detection of the *SLT*-1 gene of *Escherichia coli* O157: H7 in bovine faeces using the polymerase chain reaction. *Lett Appl Microbiol* 1998; **26**: 93–7.

7 Lawson AJ, Linton D, Stanley J, Owen RJ. Polymerase chain reaction detection and speciation of *Campylobacter upsaliensis* and *C. helveticus* in human faeces and comparison with culture techniques. *J Appl Microbiol* 1997; **83**: 375–80.

8 Boom R, Sol CJ, Salimans MM, Jansen CL, Wertheim-van Dillen PM, van der Noordaa J. Rapid and simple method for purification of nucleic acids. *J Clin Microbiol* 1990; **28**: 495–503.

9 Ciulla TA, Sklar RM, Hauser SL. A simple method for DNA purification from peripheral blood. *Anal Biochem* 1988; **174**: 485–8.

10 Young CC, Burghoff RL, Keim LG, Minak-Bernero V, Lute JR, Hinton SM. Polyvinylpyrrolidone-agarose gel electrophoresis purification of polymerase chain reaction-amplifiable DNA from soils. *Appl Environ Microbiol* 1993; **59**: 1972–4.

11 Millar B, Moore J, Mallon P *et al*. Molecular diagnosis of infective endocarditis–a new Duke's criterion. *Scand J Infect Dis* 2001; **33**: 673–80.

12 Boom R, Sol C, Weel J *et al*. Detection and quantitation of human cytomegalovirus DNA in faeces. *J Virol Methods* 2000; **84**: 1–14.