A comparison of immunomagnetic separation, direct culture and polymerase chain reaction for the detection of verocytotoxin-producing *Escherichia coli* O157 in human faeces

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Verocytotoxin-producing *Escherichia coli* O157 (O157 VTEC) has become well recognised as an important enteric pathogen. The number of organisms present in environmental and clinical samples may be low and efforts have been made to increase the sensitivity of O157 VTEC detection. Immunomagnetic separation (IMS) has been shown to improve O157 VTEC detection in bovine faeces and food samples. A milk-borne outbreak of O157 VTEC infection allowed us to compare the isolation rates from human faeces by IMS, direct faecal culture on sorbitol-MacConkey agar and a PCR test for verotoxin gene carriage. Of 142 faecal samples examined, 20 were positive on both direct culture and IMS and a further 13 on IMS alone. Therefore, IMS increased the detection rate of individual cases of O157 VTEC infection and also compared well with PCR. We recommend IMS for use in routine diagnostic laboratories where a more sensitive method than direct faecal culture is required for O157 VTEC isolation.

Introduction

Verocytotoxin (VT)-producing *Escherichia coli* O157 (O157 VTEC) has become widely recognised as an important enteric pathogen and a major cause of haemorrhagic colitis and the haemolytic uraemic syndrome [1–3]. Many laboratories culture O157 VTEC from clinical and environmental samples with sorbitol-MacConkey (SMAC) agar. O157 VTEC is recognised by the failure of most strains to ferment D-sorbitol within 24 h [4] followed by confirmation of O157 serogroup by latex agglutination [5, 6], identification as *E. coli* by biochemical testing and VT detection by either a Vero cell assay [7] or colony hybridisation with DNA probes specific for VT genes [8].

The numbers of O157 VTEC present in environmental samples, food and human faeces may be low and efforts have been made to increase the sensitivity of detection of this organism [9]. Improvements in sensitivity have been achieved by the modification of SMAC agar by the addition of cefixime and tellurite for higher selectivity [10] and of rhamnose, which leads to better discrimination from non-O157 *E.*

coli [11]. Selective liquid enrichment media for the isolation of O157 VTEC have also been developed, but with limited success to date [9].

The disadvantage of these cultural methods is that they do not differentiate between toxigenic and non-toxigenic strains of *E. coli* O157 and toxin has to be detected subsequently in the isolate. These methods also fail to detect toxigenic *E. coli* of serogroups other than O157. Techniques are available for VT detection in faeces, such as cell culture assay [12], although this may detect faecal cytotoxins other than VT because there is no VT antitoxin that will confirm the presence of VT by neutralisation [9]. The various VT ELISA tests are not sufficiently sensitive for this purpose [9]. VT gene probes have been used to detect VT gene DNA sequences in *E. coli* cultures [9] and PCR for VT genes is now used in primary faecal cultures [13].

Although there has been some success with the above methods, there remains the need for a simple, cheap and reliable procedure to increase the sensitivity of O157 VTEC detection in human faeces and in environmental samples such as food and water. Immunomagnetic separation (IMS) has been shown to improve *E. coli* O157 isolation rates from bovine faeces [14], milk [15] and artificially inoculated food [16–18] when combined with the use of SMAC agar [17, 18] and SMAC agar with cefixime [14–16].

A large milk-borne outbreak of O157 VTEC infection [19] provided the opportunity to compare the rate of detection of O157 VTEC in human faeces by IMS with the rates obtained by direct culture on SMAC agar and by PCR for VT genes in primary faecal cultures.

Materials and methods

A total of 142 faecal samples from 90 patients with suspected VTEC infection was collected. A single outbreak of phage type 2 VTEC infection [19] provided 126 samples from 79 patients and the other 16 samples were derived from 11 patients involved in smaller outbreaks of infection with phage types 2 and 28. The date of collection of the faeces was available for 122 samples. Nineteen patients provided more than one sample each (range 2–6 samples) totalling 57 samples. Nine of these patients had more than one sample taken in any one day. One hundred and twenty samples had been forwarded to us by other laboratories where they had initially been tested for VTEC by direct culture on SMAC agar and 29 had been found to be positive. The remaining 22 samples were tested for the first time in our laboratory.

All samples were stored at 4°C before analysis by direct culture on SMAC agar, simple enrichment and IMS as described below.

Direct culture

SMAC agar plates containing D-sorbitol (Oxoid) 1% were inoculated directly with a pea-sized sample of faeces with a standard cotton-wool swab and were incubated in air at 37°C for 18–24 h.

Enrichment

An inoculum of faeces similar to that above was added to 10 ml of buffered peptone water (Oxoid) and incubated at 37°C for 6 h. This enrichment step constituted the first stage of the IMS procedure. To ensure that a subsequent positive result was due to the IMS and not to the enrichment, a standard loopful of the 6-h broth was streaked on SMAC agar and incubated as for direct culture.

Immunomagnetic separation

After incubation for 6 h, 1 ml of the broth culture was added to 20 µl of magnetic bead suspension (Dynabeads anti-*E. coli* O157; Dynal, Oslo) in a 1.5-ml sterile Eppendorf tube and the separation was performed as described previously [14]. The final bead pellet was resuspended in 100 µl of PBS-Tween 20, 50 µl of which was used to inoculate a third SMAC agar plate, which was incubated in air at 37°C for 18–24 h.

Identification of isolates

After incubation, all plates were inspected for pale colonies that did not ferment sorbitol. One colony from each pale colony type was serotested with an *E. coli* O157 latex agglutination kit (Oxoid). Latex positive cultures were confirmed as *E. coli* by use of API 20E strips (bioMérieux, France). Identification and verotoxin gene carriage were confirmed and *E. coli* O157 isolates were phage-typed at the Scottish *E. coli* O157 Reference Laboratory in Aberdeen.

VT gene PCR

Where possible, all faecal samples, irrespective of *E. coli* O157 culture results, were forwarded to the Aberdeen reference laboratory for detection of VT genes by PCR. The PCR was performed after IMS in all cases within 1–4 weeks. Faecal samples were returned to storage at 4°C while awaiting PCR. A total of 140 samples was tested with a PCR for the genes encoding verotoxins 1 and 2 as described previously [20]. The amplification products were not checked by hybridisation with specific DNA probes.

Results

IMS compared with direct culture

Of the 142 faecal samples examined, O157 VTEC was isolated from 33, representing 25 patients. Twenty samples (13 patients) were positive on both direct culture and IMS and 13 samples (12 patients) were positive on IMS only. Six new cases of O157 VTEC infection were detected by IMS. No sample was positive on direct culture but negative on IMS (Table 1).

Results of direct plating of enrichment broth compared with IMS

Of the 13 samples that were negative on direct culture and positive after IMS, only two were positive on direct plating after simple enrichment in peptone water. Twenty samples were positive on both direct culture and IMS, of which 19 were positive after enrichment (Table 2).

Table 1. Comparison of *E. coli* O157 detection by direct culture and IMS

<i>E. coli</i> O157 isolated by		Number of samples	Number of patients
direct culture	IMS		
+	–	0	0
+	+	20	13
–	+	13	12
–	–	109	65
Total		142	90

Table 2. Results after enrichment compared with results of direct culture and IMS for *E. coli* O157 detection

Direct culture	Enrichment result	IMS result	Number of samples
Negative	+	+	2
	—	+	11
	+	—	0
Positive	+	+	19
	—	+	1
	+	—	0

Results obtained on samples known previously to be positive before entry into the study

The faecal samples studied were of various ages before simultaneous performance of direct culture and IMS. Some of the samples that were known to have been positive originally on direct culture proved subsequently to be negative on both direct culture and IMS, whereas others were positive on IMS only. The date of collection of the faeces was available for 35 such samples and the mean age at the time of testing was calculated. Fourteen samples with a mean age of 12.78 days (range 3–31 days) remained positive on direct culture and IMS. Seven previously positive samples, now positive on IMS only, had a mean age of 29.14 days (range 20–38 days). Fourteen samples with a mean age of 33.64 days (range 26–66 days) were now negative on IMS as well as on direct culture (Table 3).

A *t* test was used to compare these means. The samples that were positive on IMS alone were significantly older than those which remained positive on direct culture ($p < 0.001$). There was no significant difference in the ages of the samples that were IMS positive alone and those that were completely negative ($0.1 < p < 0.5$).

Verotoxin gene PCR compared with IMS

One hundred and forty samples were examined by PCR. The remaining two samples in the study were of insufficient volume for further examination. The PCR was performed on DNA from primary faecal cultures. No growth was obtained from 43 samples although six of these had been positive on IMS.

PCR for VT2 was negative on 79 samples, 15 of which had been positive on IMS, whereas 16 were

Table 3. Mean ages of previously positive samples with results of subsequent direct culture and IMS for *E. coli* O157 detection

Direct culture	IMS	Number of samples	Mean age of sample (days)
+	—	0	0
+	+	14	12.78
—	+	7	29.14
—	—	14	33.64

Table 4. Results of VT2 gene PCR compared with *E. coli* O157 detection by IMS

VT2 gene PCR result	IMS result	Number of samples
+	—	4
+	+	12
—	+	15
—	—	64
No growth	—	37
No growth	+	6

VT2 PCR positive, four of which had been negative on IMS (Table 4). Of the four samples that were VT2 PCR positive and IMS negative, two were from a single patient who had been consistently culture negative. The others were from two patients, previously culture positive at the laboratory of origin, and now negative on both direct culture and IMS in this study. Two samples were VT1 PCR positive. These were from one patient and were consistently negative for O157 VTEC on both direct culture and IMS. Repeated attempts were made to isolate PCR positive colonies from all four samples that had been consistently culture negative and PCR positive but no single PCR positive colonies could be identified.

Discussion

This study has demonstrated that IMS was more sensitive than direct faecal culture on SMAC agar for the isolation of O157 VTEC, increasing the number of positive samples from 20 to 33 (an increase of 65%). Overall, six new cases of infection were detected by IMS. This effect would appear to have been due to the IMS procedure itself and not a result of simple enrichment in buffered peptone water before IMS.

Many of the samples tested were forwarded to us by other laboratories investigating the outbreak [19] and some were known to have been positive on direct culture for O157 VTEC before a repeat direct culture at the same time as IMS. Some of these previously positive samples were found to be positive on IMS only or were completely negative. Those in the latter two categories were significantly older than those remaining positive on direct culture, indicating that as the number of viable organisms declined with storage of faeces, IMS was more able than direct culture to detect the small number present. It has been shown [21] that the O157 VTEC detection rate in faeces declines rapidly after the onset of diarrhoeal illness and IMS may have a role in extending the time during which the organism can be isolated from patients with suspected infection. This has important diagnostic implications in cases of haemolytic uraemic syndrome when the patient may present late in the diarrhoeal phase of the illness with very small numbers of O157 VTEC organisms in the faeces, the detection of which could be missed by direct culture on SMAC agar alone. In cases of haemolytic uraemic syndrome,

serological diagnosis of O157 VTEC infection may be useful in establishing the aetiology but may not be of adequate sensitivity. Results by this method are often delayed because this test is currently only available from reference laboratories.

IMS compared favourably with the VT gene PCR, and 15 samples were IMS positive and PCR negative. However, in most cases there was a considerable delay before PCR was performed. Since the PCR was performed on primary faecal cultures, the results may not be comparable on account of the possible loss of viable organisms. Simultaneous IMS and PCR would allow a truer comparison.

Four samples were VT2 PCR positive but IMS negative. Two of these had previously been positive on direct culture before negative results on simultaneous direct culture and IMS and this may indicate that PCR was more sensitive than IMS. The other two samples were from one patient, from whose faeces O157 VTEC had not been isolated at any stage. A further two samples, both from another patient, were VT1 PCR positive, although O157 VTEC had also not been isolated from these samples. Carriage of the VT1 gene was confirmed by DNA sequencing (unpublished observation). This could also be an indication that PCR was more sensitive than IMS but the occurrence of PCR positive, IMS negative samples might also be explained by the presence of non-O157 VTEC or verotoxigenic organisms other than *E. coli*.

In summary, IMS has been shown to be more sensitive than direct culture for the detection of O157 VTEC. Its sensitivity may be increased further by the use of SMAC agar containing cefixime and tellurite as recommended by the manufacturer of the immunomagnetic beads (Dynal, Oslo). As this study was part of the investigation of an outbreak of O157 VTEC infection, it was felt inappropriate to change from our standard use of non-selective SMAC agar. The VT gene PCR may be even more sensitive than IMS, although further work is needed to establish this point. However, PCR is likely to be valuable in detecting and evaluating the significance of organisms other than *E. coli* O157 that possess the VT1 and VT2 genes. PCR, at present, requires more expertise and equipment than does IMS and cannot at this stage be recommended as a routine diagnostic method. IMS is a simple, cheap procedure and is better suited to the diagnostic laboratory. Unlike PCR, IMS leads to the isolation of an organism on which toxin and phage typing can be performed and will, therefore, contribute to epidemiological studies of O157 VTEC infection.

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