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Immunomagnetic separation as a sensitive method for isolating *Escherichia coli* O157 from food samples

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SUMMARY

Minced beef samples inoculated with *Escherichia coli* O157 were cultured in buffered peptone water supplemented with vancomycin, cefsulodin and cefixime (BPW-VCC) and subcultured to cefixime tellurite sorbitol MacConkey (CT-SMAC) agar both directly and after immunomagnetic separation (IMS) of the organism with magnetic beads coated with an antibody against *E. coli* O157 (Dynabeads anti-*E. coli* O157, Dynal, Oslo). *E. coli* O157 was recovered from initial inocula of 200 organisms/g by direct subculture and 2 organisms/g by IMS. Twelve strains of *E. coli* O157 of different combinations of phage type, H antigen and toxin genotype were all recovered from initial inocula of two organisms/g by IMS. Non-specific binding of other organisms to the magnetic beads could be reduced by washing of the beads in PBS with Tween-20 0.002–0.005% *E. coli* O157 was not bound by magnetic coated with an unrelated antibody.

During investigation of a dairy herd that was possibly linked to a small outbreak of infection with *E. coli* O157, the organism was isolated from 2 of 279 foorestream milk samples from individual cattle; both isolates were made only by the IMS technique. IMS is rapid, technically simple, and a specific method for isolation of *E. coli* O157 and will be useful in epidemiological studies.

INTRODUCTION

Verocytotoxin-producing *Escherichia coli* (VTEC) are now recognized as a major cause of haemorrhagic colitis (HC) and the haemolytic–uraemic syndrome (HUS). In N. America, beef, beef products and untreated milk have been suggested as possible sources of VTEC infection for man [1, 2]. In the UK, verocytotoxin-producing (VT^+) *E. coli* O157, the most common serogroup associated with illness in man, have been isolated from cattle [3–5] but the organism has been isolated only rarely from food [4, 5] and its epidemiology therefore remains unclear. In view of the potential severity of infections caused by VT^+ *E. coli* O157 it is essential that the most sensitive methods available are used in studies designed to elucidate further the reservoirs of the organism and routes of transmission of the organism to man.

Most strains of VT^+ *E. coli* O157 do not ferment sorbitol whereas most other serogroups of *E. coli* do, and sorbitol MacConkey (SMAC) agar has proved useful

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for their isolation [6]. Improvements to sorbitol MacConkey medium [7, 8] have resulted in increased sensitivity of isolation of *E. coli* O157 from faecal samples, and enrichment culture in buffered peptone water with vancomycin, cefsulodin and cefixime has been effective for isolating the organism from carcass meat samples [4]. However, the detection of *E. coli* O157 present in low numbers in environmental samples is a problem due to the low sensitivity of enrichment and direct culture methods currently available.

The aims of this study were to evaluate the use of enrichment culture in modified buffered peptone water combined with a recently developed immunomagnetic separation technique for the isolation of *E. coli* O157 from food and environmental samples.

MATERIALS AND METHODS

Materials

Strains of *E. coli* O157 were isolated in a previous study [4] and were selected to include those of various phage types, H antigen types, and toxin genotypes (Table 1). Media used were buffered peptone water (CM509, Oxoid, Basingstoke) unmodified (BPW) or supplemented with vancomycin 8 mg/l, cefixime 0·05 mg/l and cefsulodin 10 mg/l (BPW-VCC [4]); nutrient broth (No. 2, Oxoid, Basingstoke); sorbitol MacConkey agar (CM813, Oxoid, Basingstoke) unmodified (SMAC) or supplemented with cefixime 0·05 mg/l and potassium tellurite 2·5 mg/l (CT-SMAC [8]); and plate count agar (LAB149, Lab M, Bury). Magnetic beads coated with an antibody against *E. coli* O157 (Dynabeads anti-*E. coli* O157) were supplied by Dynal AS, Oslo.

Optimizing immunomagnetic separation of E. coli *O157*

Effect of bead volume and tube-pretreatment

E. coli O157 strain P1446 was grown in nutrient broth for 4 h at 37 °C and stored at 4 °C for 24 h while c.f.u./ml were estimated by a conventional serial dilution method with surface spreading of dilutions on to plate count agar. The culture was diluted in nutrient broth to give about 200 c.f.u./ml and 1 ml of this dilution was added to each of four untreated 1·5 ml polypropylene microcentrifuge tubes (Greiner Labortechnik, Dursley) and four tubes which had been siliconized by treatment with dichlorodimethylsilane (Sigma) by a standard method [9]. Three untreated and three siliconized tubes were seeded with 5, 10 and 20 μl respectively of magnetic beads coated with an antibody against *E. coli* O157 and the fourth tubes were used without addition of beads. The beads were suspended evenly in the broth culture by vortex mixing and were placed in a rotating mixer so that they were mixed by inversion every 2–3 s for 30 min. Tubes were placed in a magnetic separator rack (MPC-10, Dynal, Oslo), the magnets placed in position and left for 5 min. The culture supernate was removed by aspiration with a Pasteur pipette, the magnetic slide was removed from the rack, the beads washed by resuspension in 1 ml of phosphate buffered saline (PBS) pH 7·2 with Tween-20 0·002% v/v (PBST) and the magnetic slide replaced for 2 min. The beads were washed in PBST in this way twice more, the magnetic slide replaced for 2 min, the supernate removed and the beads resuspended in approximately 25 μl of PBS. The

Table 1. *Strains of* E. coli *O157 used in the study*

Strain number	H antigen	Phage type	Toxin genotype VT_1	Toxin genotype VT_2
P1394	—	2	−	+
P1396	7	2	−	+
P1431	—	4	−	+
P1446	7	4	+	+
P1426	—	8	+	+
P1506	7	8	−	+
P1401	7	14	+	+
P1523	7	14	−	+
P1524	7	14	+	−
P1430	—	49	−	+
P1519	7	49	−	+
P1400	—	RDNC*	−	−

* RDNC, strain reacted with the phage set but did not conform to a recognized phage type.

whole of the bead suspension was surface spread, using a sterile glass spreader, onto CT-SMAC plates and incubated for 18 h at 37 °C. Sorbitol non-fermenting colonies were confirmed as *E. coli* O157 by agglutination with a latex test kit (DR620, Oxoid, Basingstoke) [10] and were counted and the percentage recovery calculated. Experiments were performed in duplicate. Untreated microtubes were used for all further tests.

Reduction of non-specific binding of organisms to beads

E. coli O157 strain P1446 and a sorbitol fermenting *E. coli* (NCTC 10418) were grown in nutrient broth and c.f.u./ml estimated as above. Cultures were diluted in nutrient broth to give a mixed suspension containing approximately 10^4 c.f.u./ml of each organism. 1 ml of this suspension was added to each of six 1·5 ml microcentrifuge tubes pre-seeded with 20 μl of magnetic beads and IMS performed as above but using PBS with Tween-20 at 0·0002, 0·002, 0·02, 0·05, 0·1 and 0·2 % v/v to wash the beads in tubes 1–6 respectively; the beads were washed four times and after each wash 100 μl of the supernate was spread as described above onto the surface of SMAC medium. Plates were incubated for 18 h at 37 °C. Sorbitol non-fermenting colonies were identified as *E. coli* O157 as above and the numbers of these and sorbitol fermenting colonies was recorded for each plate. Experiments were performed in duplicate.

Isolation of E. coli *O157 from minced beef*

Inoculation of beef with E. coli *O157 and enrichment culture*

Eight samples of raw minced beef, each taken from a different retail outlet, were pooled in a sterile container and mixed well with two sterile spatulae. Portions of 10 g were weighed into sterile glass jars and stored at −20 °C until required. The total viable count (TVC) of organisms in the beef samples were estimated by a conventional serial dilution method with surface spreading of dilutions on to plate count agar which was incubated aerobically at 37 °C for 24 h. *E. coli* O157 strain

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P1446 was grown in nutrient broth and c.f.u./ml estimated as above. Dilutions of the culture were prepared in nutrient broth and 1 ml volumes added to 10 g portions of minced beef (previously thawed and at 4 °C) to give samples containing approximately 2000, 200, 20 and 2 *E. coli* O157 per 10 g; an uninoculated 10 g portion of beef was used as a control. Each 10 g portion of beef was added to 90 ml of BPW-VCC, mixed well to disperse the meat and incubated for 6 h at 37 °C.

Subculture and IMS

Enrichment cultures were then used to compare the two isolation methods: (1) direct subculture: 25 μl of each culture was surface spread as above on to CT-SMAC medium and incubated for 18 h at 37 °C and (2) IMS: 1 ml of each culture was added to 20 μl magnetic beads and IMS performed as above using two washes in PBS with Tween-20 0·002% v/v, beads being surface spread as above onto CT-SMAC and incubated for 18 h at 37 °C. In each case sorbitol non-fermenting colonies were confirmed as *E. coli* O157 as above and the number of colonies of this and other organisms counted. Experiments were performed in duplicate.

Specificity of the IMS detection system

Twelve different strains of *E. coli* O157 previously isolated from bovine faeces or beef carcass meat [4] (Table 1) were each grown overnight at 37 °C in nutrient broth and c.f.u./ml were estimated as above. Dilutions of each of the broth cultures were prepared in nutrient broth and added to 10 g portions of minced beef, previously thawed and at 4 °C, to give samples containing approximately 20 *E. coli* O157 per 10 g; an uninoculated 10 g portion of beef was used as a control. Each 10 g portion of beef was added to 90 ml of BPW-VCC and enrichment culture and IMS performed as above.

In addition, 1 ml of enrichment culture of beef inoculated with strain P1446 was added to 20 μl of Dynabeads anti-*E. coli* O157 and to 20 μl of magnetic beads coated with an unrelated antibody (Dynabeads anti-CD8, Dynal, Oslo). IMS was performed as above and beads finally resuspended in 100 μl PBS; c.f.u./ml of both *E. coli* O157 and other organisms in this suspension were estimated by a conventional serial dilution method with surface spreading of dilutions on to SMAC medium. Experiments were performed in duplicate.

Examination of milk samples and milk filters

Milk samples (279 forestream milk samples from individual cattle and 12 samples of milk from the bulk storage tank) and filter washings (two sets obtained by thorough rinsing of the filter immediately after use in 50 ml of sterile buffered peptone water) were collected between mid-May and mid-August 1993, from a farm possibly linked to a cluster of human cases of *E. coli* O157 infection [5]. 25 ml of milk or filter washing was added to 225 ml BPW-VCC and 100 ml of milk from the bulk tank was added to 900 ml of BPW-VCC and incubated for 6 h at 37 °C. after which direct subculture and IMS were performed as above. Sorbitol non-fermenting colonies were tested by latex agglutination and positive colonies were confirmed as VT$^+$ *E. coli* O157 by agglutination to titre with antiserum to *E. coli* O157, cell culture assay for VT, DNA hybridization using polymerase chain reaction-generated DNA probes for genes encoding VT_1 and VT_2 and plasmid

Table 2. *Effect of volume of magnetic beads (at 2·5 mg/ml) and tube pre-treatment on isolation of* E. coli *O157*

	Mean % recovery of *E. coli* O157	
Bead volume (μl)	Untreated tube	Siliconized tube
0	0	0
5	32	35
10	67	60
20	97	92

analysis, all as described previously [4, 10]. Strains were also referred for phage typing to the Laboratory for Entric Pathogens, Central Public Health Laboratory, Colindale, London.

RESULTS

Optimizing immunomagnetic separation of E. coli *O157*

Effect of bead volume and tube pre-treatment

Optimum recovery (97% of inoculum) was achieved using 20 μl of magnetic beads (Table 2). In the absence of magnetic beads *E. coli* O157 was not recovered from either untreated or siliconized microtubes (Table 2).

Reduction of non-specific binding of organisms to beads

Results are shown in detail in Table 3. Most of the non-O157 *E. coli* were removed from the beads in the first two washes and increased removal was achieved only at concentrations of Tween-20 of $\geqslant$ 0·2% v/v. At concentrations of Tween-20 of $\geqslant$ 0·02% v/v the number of *E. coli* O157 appearing in the wash supernates gradually increased.

Isolation of E. coli *O157 from minced beef*

The samples of pooled minced beef had a TVC of approximately 10^8 c.f.u./ml; *E. coli* O157 was not isolated from uninoculated beef by either method. Following enrichment culture, *E. coli* O157 was isolated from the inoculated samples by direct subculture only at an initial inoculum of 2000 c.f.u/10 g. Using IMS the organism was isolated from an initial inoculum level of 20 c.f.u./10 g (Table 4), but with some variation between duplicate experiments.

Specificity of the IMS technique

All 12 strains of *E. coli* O157 were recovered by the IMS technique. Non-specific binding of *E. coli* O157 to beads coated with an unrelated antibody was not observed (Table 5).

Isolation of E. coli *O157 from milk samples*

E. coli O157 was not isolated from milk from the bulk storage tank or from washings from the filters, but was isolated from 2 (0·7%) of 279 forestream milk samples from individual cattle. Both isolates were obtained only by IMS and were

Table 3. *Removal of* E. coli *O157 and non-O157* E. coli *by washing of beads*

Tween-20 (% v/v)	Wash no.	Ratio *E. coli* O157/non-O157*
0·0002	1	−/+++
	2	+/+++
	3	+/++
	4	+/+
0·002	1	−/+++
	2	+/+++
	3	+/+
	4	+/+
0·02	1	+/+++
	2	+/+++
	3	+/++
	4	++/+
0·05	1	−/+++
	2	+/+++
	3	+/+
	4	++/+
0·1	1	+/+++
	2	+/+++
	3	++/++
	4	++/+
0·2	1	+/+++
	2	+/+++
	3	++/+++
	4	+++/+++

* −, not detected; +, < 10 c.f.u.; ++, 10–100 c.f.u.; +++, > 100 c.f.u.

Table 4. *Detection of* E. coli *O157 by two methods after enrichment culture in BPW-VCC*

Initial inoculum of *E. coli* O157/10 g beef	Ratio of *E. coli*/background organisms on duplicate plates*			
	DS		IMS	
2000	+/+++	+/+++	+++/+	+++/+
200	−/+++	−/+++	+++/+	++/+
20	−/+++	−/+++	−/+	+/+
2	−/+++	−/+++	−/+	−/+

*DS, direct subculture to CT-SMAC: IMS, immunomagnetic separation and culture on CT-SMAC; −, not detected; + < 10 c.f.u.; ++, 10–100 c.f.u.; +++, > 100 c.f.u.

Table 5. *Non-specific adherence* E. coli *O157 to magnetic beads coated with specific and unrelated antibodies*

Coating of beads	Mean recovery rate (c.f.u./ml)	
	E. coli O157	Background organisms
Anti-O157	$2{\cdot}8 \times 10^3$	30
Unrelated antibody	20	30

confirmed as *E. coli* O157 by agglutination to titre with *E. coli* O157 antiserum: they produced VT as determined by cell culture, hybridized with a DNA probe for VT_2 but not with one for VT_1, harboured a single 92 kb plasmid and were of phage type 2.

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DISCUSSION

Cattle have been identified as a reservoir of VT$^+$ *E. coli* O157 in Canada [11], the USA [2, 12, 13] and the UK [3–5, 14]. However, only two outbreaks of infection by the organism in the UK have, to date, had microbiologically confirmed food sources. An outbreak in Sheffield in May 1993 was linked to the consumption of untreated milk [5] and an outbreak in Gwent later in the same year was linked to beefburgers (Professor S. R. Palmer, personal communication). Sensitive methods are therefore needed for detecting VT$^+$ *E. coli* O157 which may be present in food and environmental samples in only small numbers.

Standard methods for the selective isolation of *E. coli* from food have used growth at 44 °C as a selective factor. However, VT$^+$ *E. coli* O157 has an upper temperature limit for growth of 41 °C in selective *E. coli* broth [15] and grows only poorly at temperatures higher than this in non-selective media [16]. Standard methods are therefore ineffective for the isolation of VT$^+$ *E. coli* O157 from food, and specific methods are needed. VT$^+$ *E. coli* O157 do not ferment sorbitol, whereas most other *E. coli* do, and SMAC medium has become widely used for their isolation. However, SMAC medium relies entirely on differential sugar fermentation and does not select VT$^+$ *E. coli* O157 from other *E. coli* or sorbitol non-fermenting genera and therefore lacks sensitivity. Improvements to the selectivity of SMAC [7, 8] have resulted in increased sensitivity of isolation of *E. coli* O157 from faecal samples, but are not sufficiently sensitive for isolation of small numbers of the organisms from food and environmental samples.

IMS has been shown to be more sensitive than direct culture for isolation of *E. coli* O157 from artificially mixed cultures and inoculated meat samples [17, 18], but before combining the technique with our enrichment culture we addressed problems encountered in these studies. Fratamico and colleagues [17] reported 36–60% recovery of *E. coli* O157 by IMS from suspensions in PBS; the recovery rate of 97% described in this report is higher, but may have been influenced by the fact that the separations were carried out in enrichment medium, which may have allowed continued growth of the organisms during the 30 min separation period. Both previous studies [17, 18] found non-specific binding of organisms other than *E. coli* O157 to both beads and tubes to be a problem and used washing procedures in PBS with foetal bovine serum 1% v/v [17] or physiological saline [18] and silicon treated tubes in attempts to overcome this. Adherence of *E. coli* O157 or background organisms to either untreated or siliconized tubes was not found in the present study; although speculative, this may have been due to the use of polypropylene tubes rather than the glass tubes used previously [18]. Additionally it was found that non-specific binding of organisms could be reduced, but not eliminated, by incorporation of the detergent Tween-20 into the PBS washing solution. The optimal concentration for removal of background organisms, but not *E. coli* O157, was found to be in the range of 0·002–0·05% v/v.

Enrichment culture in BPW-VCC and subculture to SMAC supplemented with cefixime 0·05 mg/l and rhamnose 0·5% w/v has been used previously for isolating *E. coli* O157 from beef carcasses [4] but in the present study direct subculture of BPW-VCC to CT-SMAC proved to be insensitive as an initial inoculum of only 2000 c.f.u./10 g beef could be detected. Inclusion of the IMS step in the isolation procedure enhanced this sensitivity about 100-fold to a detection limit of

20 c.f.u./10 g beef; this is similar to the level of sensitivity reported by Fratamico and colleagues [17]. Using the IMS technique *E. coli* O157 was isolated from 2 of 279 forestream milk samples from individual cattle. Neither strain was isolated by direct subculture of the enrichment medium to CT-SMAC, confirming the enhanced sensitivity of the IMS method for isolation of *E. coli* O157 from food.

Other immunological methods have been described for the detection of *E. coli* O157 including enzyme immunoassay [19, 20] and immunoblot techniques [21, 22]. Although sensitive, these methods can be time-consuming, technically demanding, expensive and prone to give positive results that cannot be confirmed by culture. The polymerase chain reaction has also been described as a sensitive method for detecting *E. coli* O157 in food [23] but it is technically demanding, expensive, requires confirmation by culture and also detects VT^+ *E. coli* of serogroups other than O157; such strains may be present in up to 17% of beef and milk samples [24–26] and their significance in relation to human illness is unclear. In contrast, IMS is rapid, technically simple, specific for *E. coli* O157 and, most importantly, yields an isolate of the organism that may be useful in epidemiological studies.

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