

Immunomagnetic separation of *Escherichia coli* O157:H7 from environmental and wastewater in South Africa

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Abstract

Recreational and drinking water supplies polluted with sewage have become an important source of *E. coli* O157:H7 infection. Immunomagnetic separation (IMS) has been extensively used for the isolation of *E. coli* O157:H7 from food and stool samples but not for samples such as wastewater. In this study the IMS method was used in combination with the *E. coli* O157:H7 selective media, immunoassays, biochemical tests and PCR, to assess the prevalence of *E. coli* O157:H7 in selected sewage and environmental water in South Africa. Environmental and wastewater were seeded with *E. coli* O157:H7 to determine the sensitivity and selectivity of the enrichment-IMS-selective agar method. Naturally occurring *E. coli* O157:H7 organisms were recovered from selected samples by means of IMS. The IMS concentrates were plated on three selective *E. coli* O157:H7 media. *E. coli* O157:H7 was detected in seeded sewage and river water samples with numbers as low as 1.2 cfu·ml⁻¹. The IMS procedure was used to investigate the prevalence of *E. coli* O157:H7 in randomly selected sewage and river water samples in South Africa. A total of 91 sewage- and 40 river water samples were tested and 17.6% and 20% yielded suspected *E. coli* O157:H7 colonies on CT-SMAC agar medium respectively. PCR was used to confirm the presence of genes coding for Shiga toxin-1 (Stx1), Shiga toxin-2 (Stx2), enterocyte attaching and effacing genes (*eaeA*) and enterohaemolysin (*hly*). Standard immunoassay kits specific for the O157 and H7 antigen and a biochemical indole test were used for further *E. coli* O157:H7 confirmation. Three colonies from one sewage sample (1.1 % of all sewage samples) agglutinated with anti-*E. coli* O157 and H7 antiserum and contained the genes coding for Stx2, *eaeA* and *hly*. None of the colonies isolated from the river water samples were positive for *E. coli* O157:H7. CT-SMAC proved to have limited *E. coli* O157:H7 selective capabilities from samples such as sewage with high bacterial counts. Seeded sample experiments indicated that IMS is a suitable method for isolating *E. coli* O157:H7 from samples with high bacterial interference and low numbers of *E. coli* O157:H7. Evidence has been presented that the enrichment-IMS-selective agar procedure substantially increased the sensitivity of *E. coli* O157:H7 isolation compared to direct plating of test samples onto selective agar generally practised in the past.

Keywords: *Escherichia coli* O157:H7, immunomagnetic separation, river water, sewage

Introduction

Escherichia coli O157:H7 is characterised by its ability to produce shiga toxins that are cytotoxic to monkey kidney (Vero) cells and human cervical cancer (HeLa) cells (Ismaili et al., 1995). *E. coli* O157:H7 produces a variety of clinical syndromes including bloody and non-bloody diarrhoea, haemorrhagic colitis (HC) and haemolytic uraemic syndrome (HUS) (Karmali et al., 1985). Infections caused by *E. coli* O157:H7 are recognised frequently, which resulted in an increased interest in the incidence and detection of this organism (Griffin and Tauxe, 1991; Boyce et al., 1995; Goldwater and Bettelheim, 1998; Nataro and Kaper, 1998). A considerable number of epidemiological, clinical and laboratory investigations have been carried out on *E. coli* O157:H7 infections (Nataro and Kaper, 1998). The failure of clinical laboratories to screen for this organism, with subsequent under-reporting of enterohaemorrhagic *E. coli* (EHEC) infections, complicates estimates on the burden of disease caused by *E. coli* O157:H7. The US Center for Disease Control (CDC) estimated the annual disease burden of *E. coli* O157:H7 in the United States to be more than 73 000 infections and as many as 61 deaths (CDC, 2001). *E. coli* O157:H7 is the pathogen most frequently isolated from stool

specimens that contain visible blood (Slutsker et al., 1997). The World Health Organisation (WHO) is particularly concerned about this because bloody diarrhoea is a major cause of morbidity and mortality among children in developing countries in the southern hemisphere, including South Africa (WHO, 1997).

Water-borne transmission of *E. coli* O157:H7 has been reported from both recreational water and contaminated drinking water (Swerdlow et al., 1992; Keene et al., 1994; ProMed, 2000). One of the most recent outbreaks of *E. coli* O157:H7 occurred in the water supply system of the small farming community of Walkerton, Ontario in Canada in May 2000 when six people died and more than 2000 people fell ill (ProMed, 2000). The high number of enterohaemorrhagic *E. coli* organisms isolated from the faeces of patients (Takeda, 1997), has led to the concern that these organisms, especially *E. coli* O157:H7, could pose a significant health risk when sewage leaks into water supplies.

Food-borne transmission of *E. coli* O157:H7 is another important source of infection in humans (Griffin, 1995). The most common vehicle of transmission is through the ingestion of faecally contaminated meat products (Griffin, 1995). Cattle are the main reservoirs of *E. coli* O157:H7, although it has been isolated from other animals such as chickens, pigs and sheep (Griffin and Tauxe, 1991; Griffin, 1995; Müller et al., 2002). A variety of food sources other than meat products have been implicated in the transmission of *E. coli* O157:H7: raw cow's milk and cheese, pasteurised milk, mayonnaise, apple cider, fruit and vegetables (Besser et al., 1993; Griffin, 1995; McCarthy, 1998; Nataro and Kaper, 1998).

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Once *E. coli* O157:H7 is introduced into the community through food or water it can be transmitted from person to person (Paton and Paton, 1998). Person-to-person transmission occurs in day-care centres, nursing homes or where there is close contact between individuals (Karmali, 1989; Griffin, 1995). The modes of transmission for sporadic *E. coli* O157:H7 infections appear to be similar to those for outbreaks (Griffin, 1995). Three cases of laboratory-acquired *E. coli* O157:H7 infection have been reported (Ostroff et al., 1989; Booth and Rowe, 1993; Burnens et al., 1993).

None of the previous water-borne transmission studies (Swerdlow et al., 1992; Keene et al., 1994; Ackman et al., 1997) used IMS for concentrating *E. coli* O157:H7 from water sources. Direct plating of water samples on *E. coli* O157 selective media was used to detect *E. coli* O157:H7 in these cases. This study focused on the use of the enrichment-IMS-selective agar method for the isolation of *E. coli* O157:H7 from river water and sewage samples. The presence of *E. coli* O157:H7 was confirmed with molecular and biochemical techniques.

Materials and methods

Bacterial strains

Shiga toxin 2 (Stx2)-positive strain *E. coli* O157:H7 (ATCC 43889) and Stx2-negative strain *E. coli* O157:H7 (ATCC 43888) were used as Stx2 positive and negative controls respectively (Muniesa and Jofre, 1998). Stx1-positive *E. coli* C600 and Stx1-negative *E. coli* C600 were used as Stx1 positive and negative controls. Prof. J. Jofre from the University of Barcelona, Spain, supplied all *E. coli* control cultures.

Sewage and river water

Sewage and environmental water samples (500 ml each) were collected weekly over a period of one year (September 1998 to August 1999). Sewage sampling sites included Daspoort, Zeekoegat and Baviaanspoort water purification plants near Pretoria, Gauteng, South Africa. River water samples were collected from the Levuvhu River in the Northern Province, Pienaars and Apies Rivers, situated near Pretoria, Gauteng, and Klip River (south of Johannesburg, Gauteng) in South Africa. Samples were kept at 4°C to 10°C and examined within 24 h after collection.

Immunomagnetic separation (IMS) of *E. coli* O157:H7

The effectiveness of the immunomagnetic separation (IMS) method for the selective recovery of *E. coli* O157:H7 from food and stool specimens has been well established (Wright et al., 1994; Tomoyasu, 1998). Dynabeads® anti-*E. coli* (Dynabeads anti-*E. coli* O157; Dynal, Oslo) are made of uniform, superparamagnetic, polystyrene beads with adsorbed and affinity purified anti-*E. coli* O157 antibodies covalently bound to the bead particle surfaces. These magnetic antibody-coated beads are incubated with the pre-enrichment culture to allow the target bacteria to bind onto it. The IMS concentrates are plated on selective media and suspected colonies were confirmed with molecular and biochemical techniques.

Municipal sewage

Settled sewage samples as well as settled sewage samples (100 ml) seeded with *E. coli* O157:H7 (1.2×10^0 to 1.2×10^3 cfu·ml⁻¹) were inoculated (100 µl of each sample) and enriched in 50 ml of

buffered peptone-saline water (Oxoid, CM509) supplemented with Vancomycin (8 mg·l⁻¹), Cefixime (0.05 mg·l⁻¹) and Cefsulodin (10 mg·l⁻¹) (VCC) antibiotic solution (MAST® Diagnostics) to inhibit the growth of gram-positive organisms as well as *Aeromonas* and *Proteus* spp. The sewage enrichment broth suspensions were incubated in a shaker incubator (Hub-O-Mat) for 6 h at 37°C while rotating at 100 r·min⁻¹.

River water

River water samples as well as river water samples (100 ml) seeded with *E. coli* O157:H7 (1.2×10^0 to 1.2×10^3 cfu·ml⁻¹) were filtered (100 ml of each sample) through 0.45 µm Gelman GN-6 Metricel filter membranes (Prod no. 66191), placed into, incubated and enriched in VCC-supplemented buffered peptone-saline water (Difco) for 6 h at 37°C.

Aliquots (1 ml) of the pre-enriched samples with the addition of 20 µl of Dynabead® suspension were incubated at room temperature for 10 min with continuous mixing. This step was performed to allow the O157-specific antibodies coated onto the beads to bind to the target bacteria. The bead-bacteria complexes were separated using a magnetic particle concentrator, Dynal® MPC-M for 3 min (Dynal, Oslo). After discarding the supernatant and resuspending the bead-particles in a phosphate-buffered saline-Tween, pH 7.0 (PBS-Tween) (Sigma) solution, the process was repeated four times (Dynal® product brochure, 1995). The final bead-bacteria complexes were resuspended in 100 µl washing buffer (PBS-Tween).

Isolation of *E. coli* O157

After immunomagnetic separation, 10 µl and 50 µl volumes of each sewage and river water IMS concentrates were transferred to *E. coli* O157 selective media. The *E. coli* O157:H7 selective media used in this study were Cefixime-tellurite Sorbitol-MacConkey agar (CT-SMAC) (Oxoid), CHROMagar O157 (Dynal®) and Rainbow agar O157 (Biolog). *E. coli* O157:H7 strains produced typical colourless colonies on CT-SMAC, red/pink colonies on CHROMagar O157 and grey/black colonies on Rainbow O157 agar after 24 h of incubation at 37°C.

All suspect colonies were subcultured on CT-SMAC to confirm its non-sorbitol fermenting properties. Non-sorbitol fermenting colonies were examined for the presence of the genes coding for Stx1, Stx2, enterohaemolysin and *eaeA*. A loopful of these colonies was dispersed in 500 µl ultra-high quality (UHQ) water and boiled at 99°C for 10 min without further treatment to obtain bacterial DNA for amplification with PCR (Sambrook et al., 1989).

Oligonucleotide primers (Sigma-Genosys Ltd.) specific for Stx1 (VT1), Stx2 (VT2), EHEC 1 and 2 (*eaeA* primers) and EHEC P1 and P2 (enterohaemolysin plasmid primers) were used in the polymerase chain reaction (PCR) (Pollard et al., 1990; Gannon et al., 1992; Fratamico et al., 1995) (Table 1). Each 90 µl PCR reaction mixture contained: 10 µl of Mg-free 10x amplification buffer (Promega); 6 µl of 25 mM MgCl₂ (Promega); 2 µl of 10 mM dNTP (Promega); 100 pmol of VT1 and VT2 primers; 2.5 U of Taq DNA polymerase (Promega) and 10 µl of template DNA. An additional PCR was performed with the same PCR reaction mixture for the detection of the *eaeA* (100 pmol EHEC1 and EHEC2 primers) and enterohaemolysin (100 pmol EHEC/P1 and 100 pmol EHEC/P2 primers) genes. The mixtures were overlaid with one drop of sterile mineral oil and placed in an automated thermocycler (Hybaid). The PCR cycle consisted of an initial 5 min DNA

TABLE 1 Primer sequences and predicted sizes of PCR amplified products for the detection of EHEC O157, the haemolysin plasmid and Stx (VT)-specific genes of <i>E. coli</i> O157:H7				
Primer	Oligonucleotide sequence (5'-3')	Target(s)	Size of amplified product (base pairs)	Reference
VT1a VT1b	GAAGAGTCCGTGGGATTACG AGCGATGCAGCTATTAATAA	Stx 1	130	Pollard et al. (1990)
VT2a VT2b	TTAACCACACCCACGGCAGT GCTCTGGATGCATCTCTGGT	Stx2	346	Pollard et al. (1990)
EHEC 1* EHEC 2*	CAGGTCGTCGTGTCTGCTAAA TCAGCGTGGTTGGATCAACCT	<i>eaeA</i>	1087	Gannon et al. (1993)
EHEC/P1# EHEC/P2#	ACGATGTGGTTTATTCTGGA CTTCACGTCACCATACATAT	60-MDa plasmid	166	Fratamico et al. (1995)
* = EHEC genes specific for <i>E. coli</i> O157 # = Haemolysin plasmid Primers from Sigma-Genosys Ltd. London Road, Papisford, Cambridgeshire, CB2 4EF, UK				

Figure 1

The detection of Stx1(130 bp product) and Stx2 (346 bp product) amplicons of *E. coli* O157:H7 isolates from sewage (Daspoort Water Purification Plant, Pretoria, South Africa) using gel electrophoresis.

Lane 1: 100 bp ladder

Lane 2: *E. coli* colony 1 from Daspoort sewage west intake

Lane 3: *E. coli* colony 2 from Daspoort sewage west intake

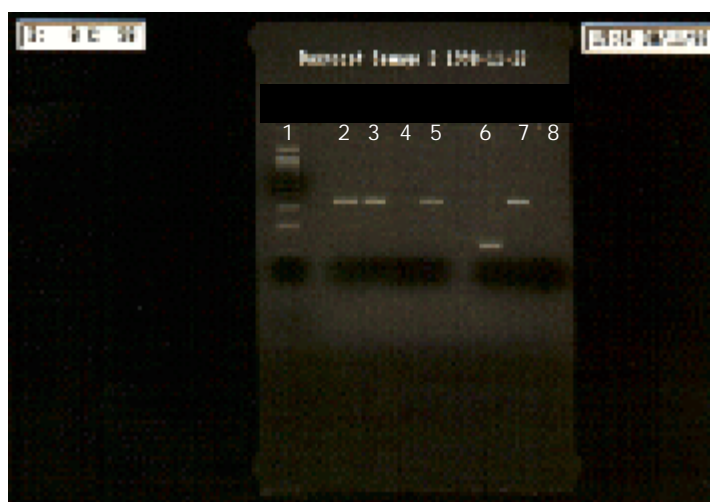
Lane 4: *E. coli* colony 3 from Daspoort sewage west intake

Lane 5: *E. coli* colony 4 from Daspoort sewage west intake

Lane 6: Stx1 positive control (130 bp)

Lane 7: Stx2 positive control (346 bp)

Lane 8: Negative control



denaturation cycle at 94°C followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min (Pharmacia LKB Thermocycler). The amplicons (20 µl aliquots from each amplification) were detected by gel electrophoresis using a 2% agarose (SeaKem® LE) gel suspension stained with ethidium bromide (Sigma). A 100 base pair DNA molecular size marker (Promega) was used. The amplified products were visualised by UV-transillumination (UVP -Transilluminator) and the image was captured using the UVP Image store 5000 gel documentation system (Fig. 1).

Suspect *E. coli* O157:H7 colonies isolated from all selective media (CT-SMAC, Rainbow agar O157 and CHROMagar O157) were individually tested for agglutination using a commercial *E. coli* O157 slide agglutination kit with antisera against *E. coli* O157 (Mast Assure, Mono Factor O157, code:M12030). In addition, all colonies were biochemically confirmed as *E. coli* by their

ability to produce indole from tryptophan using Kovac's reagent (ISO, 2001).

Results

Assessment of the sensitivity of the enrichment-IMS-selective agar procedure revealed that *E. coli* O157:H7 colonies were recovered from sewage and river water samples with average counts of seeded *E. coli* O157:H7 as low as 1.2 cfu·ml⁻¹. The enrichment procedure increased average counts of *E. coli* O157:H7 in seeded sewage samples from 1.2 to 45 cfu·ml⁻¹ (3 650%) and in seeded river water samples from 1.2 to 72 cfu·ml⁻¹ (5 900%). In the case of samples seeded with higher numbers of *E. coli* O157:H7 the percentage increase in counts of *E. coli* O157:H7 accomplished by enrichment was lower (Table 2). In the case of sewage seeded with *E. coli* O157:H7 to average counts of 1 200 cfu·ml⁻¹, the percentage

TABLE 2 Assessment of the sensitivity of the enrichment-IMS-selective agar method using testson seeded samples				
Seeded samples	Count of seeded <i>E. coli</i> O157:H7 cfu·ml ⁻¹			
	Before Enrichment	After Enrichment	Percentage Increase (%)	Presence-absence after IMS
Sewage	1 200	1 740	45	+
	120	170	42	+
	12	52	333	+
	1.2	45	3650	+
River water	1 200	2 950	146	+
	120	890	641	+
	12	140	1 067	+
	1.2	72	5900	+

Enrichment was on CT-SMAC Agar. Counts are averages of tests carried out in triplicate.
E. coli O157:H7 was detected in all seeded samples.
+ = Present
- = Absent

increase was only 45%.

After the above determination of the sensitivity of the enrichment-IMS-selective agar method, the procedure was applied in studies on the incidence of naturally occurring *E. coli* O157:H7 in selected samples. A total of 91 sewage and 40 river water samples was examined. Suspect *E. coli* O157:H7 colonies were isolated from 16 sewage (17.6%) and 8 river water (20%) samples using the three selective media. No *E. coli* O157:H7 colonies were isolated when sewage and river water were directly plated on the selective media due to interfering growth and overwhelming numbers of non-pathogenic strains of *E. coli*. Three colonies isolated on CT-SMAC from one sewage sample of the Daspoort west intake (Table 2) agglutinated with the anti-*E. coli* O157 antisera, tested positive for indole using Kovac's reagent and contained the genes coding for Stx2, *hly* and *eaeA* after PCR. These three isolates imply that 6.25% of suspect *E. coli* O157:H7 colonies isolated from sewage were confirmed to be *E. coli* O157:H7, and that *E. coli* O157:H7 colonies were isolated from 1.1% of sewage samples analysed. No suspect *E. coli* O157:H7 colonies were isolated from any of the 40 river water samples analysed.

Although not confirmed by statistically meaningful results, CT-SMAC proved the agar medium of choice for the selective cultivation of *E. coli* O157:H7. The choice is predominantly based on the morphology and colour of suspect colonies, and the extent to which their detection was obscured by background growth.

Discussion

Evidence has been presented that the enrichment-IMS-selective agar procedure substantially increased the sensitivity of *E. coli* O157:H7 isolation compared to direct plating of test samples on selective agar. Comparative tests revealed that the enrichment step increased counts of *E. coli* O157:H7 seeded into samples of sewage and river water by up to 5 900% (Table 2). The higher percentage increase in counts of *E. coli* O157:H7 in samples seeded with low

numbers of the organism, is probably due to normal population dynamics and maximum numbers of organisms attainable in a steady-state culture. This phenomenon serves the objectives of the enrichment procedure because initially low numbers of *E. coli* O157:H7 require higher levels of enrichment for detection. Since *E. coli* O157:H7 colonies were recovered from all seeded samples, the lowest numbers of the organisms detectable in the samples concerned have unfortunately not been established. Determination of the lowest number of *E. coli* O157:H7 detectable would require tests in which samples are seeded with numbers of *E. coli* O157:H7 even lower than those in Table 2. However, the results indicated that *E. coli* O157:H7 would be detectable when present in numbers as low as 1.2 cfu·ml⁻¹. Although not confirmed by statistically meaningful results, the CT-SMAC agar medium seemed to yield the best results of the three media used for the selective cultivation of *E. coli* O157:H7 bacteria. In addition, CT-SMAC agar was less expensive than Rainbow Agar O157 and CHROMagar O157.

The higher efficiency of the enrichment-IMS-selective agar procedure for the recovery of naturally occurring *E. coli* O157:H7 in sewage and river water would appear to be supported by the isolation of at least one *E. coli* O157:H7 organism from a sewage sample in this study. In comparison, the survey described by Müller and colleagues (2001) using conventional plating of test samples on selective media, failed to recover the pathogen from any of the samples analysed.

Enterohaemorrhagic *E. coli* bacteria have been isolated from sewage in Germany by direct plating of test samples onto selective media (Höller et al., 1999). *E. coli* O157:H7 has been isolated from patient stool specimens as well as samples of meat products and milk associated with infections (Nataro and Kaper, 1998). However, as far as can be established this is the first report on the isolation of *E. coli* O157:H7 from sewage in South Africa, and the first application of the enrichment-IMS-selective agar procedure for the isolation of *E. coli* O157:H7 from wastewater anywhere in the world.

One out of 16 samples (6.3%) of suspect *E. coli* O157:H7 were confirmed as *E. coli* O157:H7. This confirms the shortcomings of the agar media for the selective cultivation of *E. coli* O157:H7. The three *E. coli* O157:H7 colonies isolated from the same sewage sample were probably the offspring from the same original organism which multiplied during the initial enrichment stage. This is confirmed by identical features such as the toxicity factors that they carried (Table 3). Shortcomings of media for the selective cultivation of *E. coli* O157:H7 in test samples with heavy background growth such as sewage and river water have been reported by other researchers (Bettelheim, 1998). Bettelheim (1998) pointed out that the black colonies of *E. coli* O157:H7 were difficult to distinguish on Rainbow Agar O157 in the presence of large numbers of other *E. coli* colonies of different colours. These observations call for further improvement of methods for the selective cultivation of *E. coli* O157:H7 in the presence of large numbers of wild type *E. coli* and other bacteria capable of growing on the selective media. Resistance to the antibiotics used for the suppression of bacteria other than *E. coli* O157:H7 may largely be accountable for the problem. One solution may therefore be to find antimicrobial agents which more efficiently suppress background growth and strains of *E. coli* other than *E. coli* O157:H7.

TABLE 3
Isolation of *E. coli* O157:H7 from selected samples of sewage and river water

Samples	Total number of samples	Number of isolates (percentage of total)		Toxicity factors		
		Suspect isolates	Confirmed isolates	Stx2	Hly	eaeA
Sewage	91	16 (17.6 %)	1 (1.1 %)	1	1	1
River water	40	0	0	0	0	0
Total	181	16 (8.8 %)	1 (0.6 %)	1	1	1

Despite shortcomings of the growth media for the selective cultivation of *E. coli* O157:H7, evaluation of the sensitivity of the enrichment-IMS-selective agar procedure in seeding experiments confirmed that it was capable of detecting numbers of *E. coli* O157:H7 as low as 1 cfu·ml⁻¹ in any of the sewage and river water samples analysed (Table 2). These results confirmed that *E. coli* O157:H7 bacteria did occur in low numbers in the samples under investigation.

Data reported on the isolation of at least one *E. coli* O157:H7 organism from sewage and results of the seeding experiments, confirmed that a procedure is available for the relatively sensitive isolation of *E. coli* O157:H7 from a variety of environmental samples with heavy background growth and large numbers of wild type *E. coli* and related bacteria. This enrichment-IMS-selective agar technique could be applied for purposes such as analysis of samples related to *E. coli* O157:H7 infections. The enrichment-IMS-selective agar procedure developed in this study is superior to methods used in the past, but there is potential for further improvement. This refers in particular to the final step for the selective cultivation of *E. coli* O157:H7 isolates. Selection may be improved by using alternative antimicrobial agents which more efficiently suppress interfering background growth and wild type *E. coli*. The development of a multiplex PCR for the simultaneous detection of Stx1, Stx2, *eaeA* and the enterohaemolysin plasmid would improve the time efficiency of the molecular detection method and should be investigated.

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