Combined use of an immunomagnetic separation method and immunoblotting for the enumeration and isolation of *Escherichia coli* O157 in wastewaters

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ABSTRACT

C. GARCÍA-ALJARO, X. BONJOCHE AND A.R. BLANCH 2004. Aims: The detection of *Escherichia coli* O157:H7 in environmental samples is a human concern. The high persistence of this serotype in the environment suggests that contaminated animal wastewater could act as a potential reservoir. Nevertheless, the high levels of background microflora and cell damage because of environmental stress hamper the isolation of this pathogen without using enrichment methods. This study develops a method for the detection of *E. coli* and investigates its prevalence in animal and human wastewaters.

Methods and Results: Incubation of the sample for 1 h 30 min at 37°C in peptone water supplemented with vancomycin and cefsulodin, enhanced the recovery of bacteria whilst ensuring that no growth occurred. Subsequently, a combination of immunomagnetic separation, cefixime–tellurite–sorbitol MacConkey (CT–SMAC) plating and immunoblotting with specific O157 antibodies allowed the detection, enumeration and isolation of *E. coli* O157 strains in human, swine and cattle wastewaters, which presented values of 0\(\log_{10}\) ml\(^{-1}\) units, respectively. Some of the isolates carried genes coding for Shiga toxins, intimin and enterohemolysin.

Conclusions: *Escherichia coli* O157 is commonly present in animal and human wastewaters. The developed method reduced the high rate of false positives reported for other technical approaches.

Significance and Impact of the study: The confirmation of serotype by specific immunomethods is necessary to prevent false-positive detection and incorrect enumeration.

Keywords: enumeration, O157, sewage.

INTRODUCTION

*Escherichia coli* O157:H7 has been a concern for human health since it was first described as a human pathogen in 1982 (Riley *et al.* 1983). This serotype has been responsible for a large number of human outbreaks and continues to be a threat because of the severity of the symptoms associated with its infection. The variety of symptoms following *E. coli* O157:H7 infection range from haemorrhagic colitis to the development of haemolytic uraemic syndrome (HUS), mostly in children, and thrombotic thrombocytopenic purpura (TTP) in adults, although asymptomatic carriers have also been identified in the population during some outbreaks (Stephan and Untermann 1999).

The major virulence factors associated with *E. coli* O157:H7 are the production of one or two potent cytotoxins, the Shiga toxins (Stxs), Stx\(_{1}\) and/or Stx\(_{2}\) and Stx\(_{2}\) variants (c, d, e, f and g) (Melton-Celsa and O’Brien 1998; Schmidt *et al.* 2000; Leung *et al.* 2003), which have also been found in non-O157 strains, and the adherence factors, which allow the cell to adhere to epithelial cells and are responsible for the generation of attachment and effacing lesions. The best-known adherence factor is the intimin protein, encoded by the *eaeA* gene (Yu and Kaper 1992), which adheres to the Tir receptors encoded by a conserved chromosomal region, known as the locus of enterocyte effacement (LEE), which is
present in the majority of *E. coli* O157 strains (Nataro and Kaper 1998). This region also contains the genes coding for proteins involved in the release of the Tir proteins into the enterocyte. However, other adherence factors have been described in some LEE-negative strains associated with human disease, like the autoaglutinlinine protein encoded by the *saa* gene (Paton *et al.* 2001). Another important virulence factor is the plasmid pO157, which is found in nearly all O157 strains, as well as in some non-O157 STEC. This plasmid carries the *ehxA* gene coding for an enterohemolysin, which is different from the *z* haemolysin found in uropathogenic *E. coli* (Schmidt *et al.* 1995), and a serine protease (Esp P), which could contribute to the development of bloody diarrhoea (Brunder *et al.* 1997).

The major route of transmission of *E. coli* O157:H7 is through consumption of contaminated food and water, although person-to-person transmission has also been documented (Meng and Doyle 1998). Most outbreaks have been associated with beef contamination during the slaughter processing and ingestion of undercooked meat. Cattle are the primary reservoir of this pathogen (Chapman *et al.* 1993), although it has also been found in other domestic and wild animals (Beutin *et al.* 1993; Wallace *et al.* 1997). Many infections are through environmental transmission following the faecal oral route. Outbreaks associated with ingestion of water from water supplies, as well as outbreaks associated with recreational waters, possibly contaminated with faecal wastes, have been documented (Swerdlow *et al.* 1992; Chalmers *et al.* 2000). The largest reported outbreak associated with water took place in 2000 in Canada, with more than 2000 people infected (Hrudey *et al.* 2003).

Various methods have been employed in the detection of *E. coli* O157:H7, including the use of selective media, immunological assays and DNA probes (Barrett *et al.* 1991; Zadik *et al.* 1993; Huck *et al.* 1995; Nataro and Kaper 1998; Paton and Paton 1998). The culture methods exploit the biochemical characteristics of O157:H7 strains, which do not produce β-D-glucuronidase and usually do not ferment sorbitol within 24 h. The agar medium most commonly used for isolation of *E. coli* O157:H7 is sorbitol MacConkey (SMAC) agar containing cefxime and tellurite (Zadik *et al.* 1993). Confirmation of the O157 serotype is routinely achieved by latex agglutination with specific anti-O157 antibodies or detection of O157 related genes such as *rfpO157* (Wang *et al.* 2002). However, this medium also permits the growth of most other *E. coli* strains present in humans, animals and food, especially non-toxigenic *E. coli* O157 of H-types other than H7. A method based on the separation of *E. coli* O157:H7 using anti-O157 antibody-coated magnetic beads and subsequent plating onto CT-SMAC has been described (Wright *et al.* 1994). The disadvantage of these methods is that they do not differentiate between toxigenic and stx-negative O157 Shiga toxin-producing *E. coli* (STEC). Moreover, they fail to detect some atypical strains of O157 STEC that ferment sorbitol and present β-D-glucuronidase activity (Gunzer *et al.* 1992). Another shortcoming is the cross-immunoreactivity found with some bacteria like *Escherichia hermanii*, *Salmonella* O30 group, *Hafnia alvei* and *Citrobacter freundii* (Borczyk *et al.* 1987; Shimada *et al.* 1992; Bettelheim *et al.* 1993; Nataro and Kaper 1998). Consequently, suspected colonies must be confirmed as *E. coli* O157 by biochemical identification.

Most quantification methods are based on quantitative PCR assays (Li and Drake 2001). However, the limit of detection is around 10^3 cells ml^-1 so they are not valid for samples in which low numbers of O157 strains are expected. Moreover, most procedures are based on a prior enrichment step, which can alter the original concentration present in the sample. In addition, most of them are designed for the analysis of food samples, in which low levels of interfering bacteria and PCR inhibitors are present (Hara-Kudo *et al.* 2000).

The aim of this work was to develop a methodology for the enumeration and isolation of *E. coli* O157 strains in municipal sewage and animal wastewater samples, and to evaluate whether these aquatic environments could act as reservoirs for pathogenic *E. coli* O157 strains. The approach, based on immunomagnetic separation (IMS) with magnetic beads coated with specific anti-O157 antibodies, culture on CT-SMAC agar and specific anti-O157 immunoblotting, allowed enumeration and selective isolation of *E. coli* O157 strains, which were further analysed for the presence of virulence markers.

**MATERIALS AND METHODS**

**Bacterial strains and media**

*Escherichia coli* O157:H7 [American Type Culture Collection (ATCC) no. 43888] and *E. coli* CN13 (ATCC no. 700609) were used as positive and negative controls, respectively. These bacteria were grown aerobically at 37°C for 24 h on Tryptic Soy Agar (TSA) or Tryptic Soy Broth (TSB; Difco, le Pont de Claix, France), to obtain the bacterial cultures.

**Sample collection and enumeration of bacterial indicators**

A total of 14 raw wastewater 1-l samples were collected from the influents of one municipal sewage treatment plant (400 000 inhabitants) and from two slaughterhouses (cattle and pig, respectively). Samples were taken aseptically and transferred to sterile containers, and then transported to the...
laboratory for further analysis. They were kept at 4°C and analysis of the samples was performed within 6 h of sampling.

Chromocult® coliform agar (Merck, Darmstadt, Germany) was used for the enumeration of total coliforms and E. coli after incubation at 37°C for 24 h. Fecal coliforms were enumerated on m-FC agar (Difco), after incubation at 44.5°C for 24 h. All bacterial indicators were enumerated by the membrane filtration method using 0.45 μm pore size membranes (Millipore, Mosheim, France) according to standard methods (Anon. 1998).

Enumeration of Escherichia coli O157 in sewage and wastewater samples

In order to recover cells that could be injured and inhibit the growth of the non-O157 strains that may be present in the samples, two different recovery systems were assessed. One hundred millilitres of an autoclaved wastewater sample seeded with a 10^2 CFU ml^-1 hundred millilitres of an autoclaved wastewater sample was enumerated on m-FC agar (Difco), after incubation at 44.5°C for 24 h. All bacterial indicators were enumerated by the membrane filtration method using 0.45 μm pore size membranes (Millipore, Mosheim, France) according to standard methods (Anon. 1998).

Escherichia coli O157-specific colony immunoblotting

The enumeration of E. coli O157 was confirmed by specific colony immunoblotting using anti-O157 antibodies (Oxoid, Basingstoke, UK). The colonies were transferred onto a nitrocellulose membrane (Hybond ECL; Amersham Pharmacia Biotech, Bucks, UK) for subsequent immunodetection of the O157 antigen by placing the membrane onto the plate and careful removal. Cells were fixed on the membrane with TBS buffer (10 mmol l^-1 Tris, 0.15 mol l^-1 NaCl) and saturated 3 mm Whatman paper (Whatman, England). The membrane was then incubated for 30 min at 37°C. Later, the membrane was blocked with TBS-3% Bovine Serum Albumin (BSA; Sigma, St Louis MO, USA) for 1 h, and washed with TBS-1% BSA for 10 min with agitation. Incubation with the E. coli O157-specific antibody, 1:1000 in TBS-Tween solution (TBS-0.5% Tween 20) was performed for 1.5 h. Membranes were washed three times for 5 min each in TBS-Tween. The second incubation with alkaline phosphatase-conjugated anti-rabbit IgG antibody (Sigma) at 1:1000 in TBS-Tween solution was performed for 1.5 h. Later, the same three washing steps described above were repeated. The membrane was incubated in a solution of 100 mmol l^-1 Tris–HCl, 100 mmol l^-1 NaCl for 5 min. Twenty microlitres of NBT-BCIP (Roche Diagnostics, Mannheim, Germany) was added to 1 ml of the solution and the membrane was incubated in the dark until a blue colour indicating a positive reaction was observed in the positive control. To stop the reaction the membranes were washed in distilled water and air-dried. Colonies showing a positive signal were isolated from the original plate and further characterised.

PCR analysis for detection of the rfb and fliC genes

Colonies showing a positive signal in the immunoblot were isolated and tested for the presence of the rfb gene encoding the O157 antigen and the fliC gene encoding the H7 fimbriae antigen by the PCR.

PCRs were performed with a GeneAmp PCR system 2400 (Perkin–Elmer, PE Applied Biosystems, Barcelona, Spain). DNA template was prepared directly from two colonies of each strain suspended in 50 μl of double-distilled water and heated to 96°C for 10 min prior to addition to the reaction mixture. The reaction mixture consisted of 2 mmol l^-1 MgCl_2, 10× buffer (provided by the manufacturer), 200 μmol l^-1 dNTPs, 2 U Taq DNA polymerase (Eppendorf, Hamburg, Germany), 0.3 μmol l^-1 of each primer (Table 1) and 2 μl of the extracted DNA. The final volume was adjusted to 25 μl with sterile double-distilled water. PCR conditions used in this study have been described...
previously (Wang et al. 2002). Five microlitres of each PCR product was analysed by agarose (1%) gel electrophoresis and bands were visualized by ethidium bromide staining.

LPS profiles and immunoblotting

The presence of the O157 antigen was confirmed by extraction of LPS followed by immunoblotting and analysis with the Singlepath® O157 detection kit (Merck).

Extraction of LPS was performed using the proteinase K method (Hitchcock and Brown 1983). Ten microlitres of the extracted LPS were electrophoresed in duplicate using denaturing polyacrylamide gels (Laemmli 1970). Electrophoresis was performed using a Mini-Protean II Dual Slab Cell (Bio-Rad, Richmond, CA, USA). One gel was silver stained (Tsai and Frasch 1982) to study LPS patterns and the other was used for Western blotting to analyse O157 profiles. For this purpose, the second gel was electrotransferred with a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad) onto nitrocellulose membranes (Hybond ECL; Amersham Pharmacia Biotech) in a solution containing 25 mmol l⁻¹ Tris, 192 mmol l⁻¹ glycine and 20% methanol. The gel was run at 150 V for 1 h, according to the manufacturer’s instructions. Later, membranes were processed by immunoblotting with anti-O157 antibodies (Oxoid), following the colony immunodetection protocol described above.

Additionally, the strains were tested with the Singlepath® O157 detection kit (Merck), which allows detection of O157 antigen, according to the manufacturers instructions.

Phenotypic characterization

The isolated strains were phenotypically characterised by biochemical fingerprinting using the PhenePlate® system (PhP-Plate Microplates Technique AB, Stockholm, Sweden). PhP-RE plates were used and inoculated according to the manufacturer’s instructions. Later, clustering analyses using the unweighted pair-group method analysis (UPGMA) method were performed with the biochemical profiles obtained according to the Simpson’s Diversity Index (Hunter and Gaston 1988). Representative isolates were selected from those showing the highest mean similarity and the highest minimum similarity with respect to the other serotypes belonging to the same type (Kühn et al. 1991). Then, these strains were identified using the API 20E commercial gallery (BioMérieux, La Balme, France). Additionally, sequencing of the 16S rRNA gene was performed to identify one strain, which could not be identified with the API 20E gallery.

Sorbitol fermentation was confirmed by inoculating Purple Bromocresol Broth tubes containing 1% D-Sorbitol (Sigma) with the strain to be tested, and incubating at 37°C for 24 h. Sorbitol fermentation resulted in acidification of

Table 1 Primer oligonucleotides used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Nucleotide sequence</th>
<th>Target sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stx1-a</td>
<td>TCTCAGTGCGGTCTTCTATG</td>
<td>stx1</td>
<td>Wang et al. (2002)</td>
</tr>
<tr>
<td>Stx1-b</td>
<td>TACCCCTCAACTGCTAATA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stx2-a</td>
<td>GCCGTTTATTTGCATATTGC</td>
<td>stx2</td>
<td>Wang et al. (2002)</td>
</tr>
<tr>
<td>Stx2-b</td>
<td>TACCCCTCAACTGCTAATA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stx2c-a</td>
<td>GCCGTTTATTTGCATATTGT</td>
<td>stx2c</td>
<td>Wang et al. (2002)</td>
</tr>
<tr>
<td>Stx2c-b</td>
<td>AGTACTCTTTTCCGGGCCACT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stx2d-a</td>
<td>GTTAAATTTTGGTCTTCAAGTGATAT</td>
<td>stx2d</td>
<td>Wang et al. (2002)</td>
</tr>
<tr>
<td>Stx2d-b</td>
<td>CAGCAATCTGAACTTGACG</td>
<td></td>
<td></td>
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<tr>
<td>Stx2e-a</td>
<td>ATGAGGTGTATAGTTAAGGGA</td>
<td>stx2e</td>
<td>Wang et al. (2002)</td>
</tr>
<tr>
<td>Stx2e-b</td>
<td>AGCCCATATAATATTCTCGT</td>
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<td></td>
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<tr>
<td>Stx2f-a</td>
<td>GTGTCTCTAGCATATATTGCAG</td>
<td>stx2f</td>
<td>Wang et al. (2002)</td>
</tr>
<tr>
<td>Stx2f-b</td>
<td>CATGATTAAATCTGAAACAGAAA</td>
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<tr>
<td>209F</td>
<td>GTTTATTTTCTGTGATATCT</td>
<td>stx2g</td>
<td>Leung et al. (2003)</td>
</tr>
<tr>
<td>781R</td>
<td>GTAATAAGCCGTACAGTA</td>
<td>cwaA</td>
<td>Wang et al. (2002)</td>
</tr>
<tr>
<td>EAE-a</td>
<td>AATGTTTAATGCTGTGATAGTGG</td>
<td>eaeA</td>
<td>Wang et al. (2002)</td>
</tr>
<tr>
<td>EAE-b</td>
<td>GCCCTTCATCATCATTTCGCTTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HlyA-a</td>
<td>AGCCTGCAAGGTGCGGCTCTG</td>
<td>chxA</td>
<td>Wang et al. (2002)</td>
</tr>
<tr>
<td>HlyA-b</td>
<td>TACGGGATTGCTGCAAGTTCAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>27-F</td>
<td>AGAGTTTGTACCTTGCGTCTACG</td>
<td>16S rRNA</td>
<td>Weisburg et al. (1991)</td>
</tr>
<tr>
<td>1492-R</td>
<td>TACGGTTACCTTGATTACGAC</td>
<td>enterobacteria</td>
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</tr>
<tr>
<td>RfbE-a</td>
<td>CATACGGTGAGGTGGATAGG</td>
<td>rfbO157</td>
<td>Wang et al. (2002)</td>
</tr>
<tr>
<td>RfbE-b</td>
<td>ATTCCTCTCTTCTCGCCTG</td>
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<td></td>
</tr>
<tr>
<td>FlIC-a</td>
<td>TACCAGCGGAAAAGCAACTCC</td>
<td>fliC17</td>
<td>Wang et al. (2002)</td>
</tr>
<tr>
<td>FlIC-b</td>
<td>GTTCGGGCAAGCTTATTGATACC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
the media with the corresponding change in colour from blue to yellow.

Additionally, β-D-glucuronidase activity was assessed by inoculating the corresponding strain in 250 µl of PBS with a β-glucuronidase tablet (Diatabs, Rosko, Denmark) and incubating for 4 h or overnight at 37°C. The appearance of a yellow colour was considered a positive result.

Sequencing of 16S rRNA gene

Sequencing was performed with the ABI PRISM Big Dye III terminator cycle sequencing ready reaction kit (PE Applied Biosystems) in an ABI PRISM 3700 DNA analyzer (PE Applied Biosystems) according to the manufacturer’s instructions with the primers listed in Table 1. All sequences were performed in duplicate.

Nucleotide sequence analysis for homologous DNA sequences were performed in the EMBL and GenBank database libraries were performed with the WISCONSIN PACKAGE, version 10.2, Genetics Computer Group (GCG), Madison, WI, USA. BLAST analyses were done with the tools available at http://www.ncbi.nlm.nih.gov. Multiple sequence alignment was performed with the software MULTALIN, version 5.4.1 (Corpet 1998).

Analysis of virulence factors

Detection of stx genes, stx1, stx2 and stx2 variants (c, d, e, f and g), the eaeA gene coding for the intimin protein, and the gene coding for the enterohaemorrhagic haemolysin, ehxA, was carried out by PCR as described above with the primers described in Table 1. The positive controls used in the PCR included E. coli O157:H7 (ATCC no. 43984) (stx1, stx2, eaeA, and ehxA positive), E. coli O91:H21 (stx2cha and stx2cob positive, which are subtypes of stx2d), E. coli O174:H11 (stx2d positive) and E. coli ‘FAC9’ (stx2 positive) (Bertin et al. 2001), E. coli ONT (stx2g positive) (Morabito et al. 2001) and one E. coli environmental isolate (S86; stx2g positive), whilst the negative control was E. coli DH5α, which does not carry any of these virulence genes.

Table 2. Enumeration of bacterial indicators and Escherichia coli O157 in municipal sewage and wastewater samples

<table>
<thead>
<tr>
<th>Origin</th>
<th>n TC</th>
<th>FC</th>
<th>EC</th>
<th>CT-SMAC</th>
<th>Immuno O157</th>
<th>Putative Confirmed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cattle wastewater</td>
<td>4·7 (0·3)</td>
<td>4·7 (0·3)</td>
<td>4·5 (0·5)</td>
<td>1·7 (0·6)</td>
<td>1·0 (0·7)</td>
<td>54</td>
</tr>
<tr>
<td>Pig wastewater</td>
<td>2·5 (0·2)</td>
<td>5·8 (0·2)</td>
<td>5·5 (0·4)</td>
<td>0·8 (0·2)</td>
<td>0·4 (0·2)</td>
<td>4</td>
</tr>
<tr>
<td>Human sewage</td>
<td>8·6 (0·3)</td>
<td>5·3 (0·3)</td>
<td>5·2 (0·2)</td>
<td>&gt;2·7</td>
<td>0·2 (0·2)</td>
<td>9</td>
</tr>
</tbody>
</table>

Enumeration data shown as log10 (CFU + 1) ml⁻¹ units. 
n, number of samples; TC, enumeration of total coliforms; FC, enumeration of faecal coliforms; EC, enumeration of E. coli; CT-SMAC enumeration of colonies growing after IMS and CT-SMAC incubation; Immuno O157, enumeration of colonies showing positive signal after specific O157 immunoblotting.

The ability to produce the Shiga toxin proteins, Stx1 and Stx2, was tested in the strains encoding any of the stx genes using the Duopath VT® detection kit (Merck), according to Park et al. (2003). Briefly, the strains to be tested were cultured for 6 h in modified CAYE broth (Merck), and then 180 µl of the culture was treated with 20 µl of Polymyxin B solution and further incubated for 10 min at 37°C. A volume of 160 µl of the mixture was dispensed into the circular sample port of the test device. Results were observed after a 20 min room temperature incubation.

RESULTS

Recovery of sub-lethally damaged cells

Incubation of 100 ml of the sample in 50 ml of peptone water supplemented with vancomycin (8 mg l⁻¹) and cefsulodin (10 mg l⁻¹) for 1 h 30 min at 37°C with agitation allowed recovery of the initial cells without altering the original composition of the sample. Consequently, this was the procedure for recovery that was applied on this study. Incubation of the sample for more than 1 h 30 min was discarded because it resulted in an increase of the population. In contrast, the other enrichment procedure assessed, peptone water supplemented with vancomycin (8 mg l⁻¹), cefsulodin (10 mg l⁻¹) and cefsuloxime (1 mg l⁻¹), was discarded because the use of cefsuloxime at that concentration significantly decreased the growth of the control strain, E. coli O157:H7.

Enumeration of O157 strains

All the samples studied presented heavy growth of the different bacterial indicators analysed (Table 2). However, there were differences in the number of strains capable of growing on CT-SMAC agar after immunomagnetic separation with anti-O157 antibody-coated paramagnetic beads (presumptive E. coli O157 strains) in samples from human or animal origin. In human sewage samples the number of presumptive E. coli O157 was higher than 2 log10 units, whilst in animal wastewater samples this number was lower.
However, in human samples the number decreased significantly after the O157-specific immunoblotting (confirmed O157 isolates), resulting in a reduction of more than $2 \log_{10}$ units. A reduction of between 0.4 and 0.7 $\log_{10}$ units was observed in the animal wastewater samples (Table 2).

A high percentage of the isolated strains (78%) were confirmed as *E. coli* O157 by LPS extraction and specific O157 immunoblotting. Additionally, the expression of the O157 LPS antigen was analysed by PCR in these strains, showing a positive band for amplification of the O157 *rfb* gene in all of them. The commercial O157 detection kit also gave positive results for all of these strains.

**Characterization of the O157 strains isolated**

A total of 72 strains were isolated and subjected to biochemical fingerprinting with the PhenePlate® system. The diversity index was 0.94 and clustering analyses showed two main clusters (Fig. 1). A total of 28 representative strains were selected. All of them were identified as *E. coli* with the commercial API 20E strip except one strain isolated from pig wastewater, which was confirmed as *E. coli* after sequencing of the gene coding for the 16S rRNA. A total of 14 strains presented the classical phenotype of *E. coli* O157:H7, resulting in no fermentation of sorbitol within 24 h and the absence of $\beta$-D-glucuronidase activity. All of the strains showing this phenotype were isolated from cattle. In contrast, the three strains isolated from human sewage were positive for both reactions. The strain that could not be identified by the API 20E strip was also positive for both tests, and the other two strains isolated from pig wastewater were positive for sorbitol fermentation but negative for the $\beta$-D-glucuronidase test. The other eight strains were also positive for both reactions.

None of the strains showing $\beta$-D-glucuronidase activity and fermentation of sorbitol carried genes coding for any of the virulence factors analysed. In contrast, those typical *E. coli* O157:H7 strains (negative for $\beta$-D-glucuronidase and sorbitol fermentation) carried the *stx*₂, *ehxA*, and *eaeA* genes, and 36% also carried the *stx*₁ gene. The distribution of these genes among the different strains is summarized in Table 3.

The production of Stx₁ and Stx₂ protein was tested in those strains carrying the genes coding for some of these proteins. All of the strains carrying the different genes were capable of producing the toxin except one, which carried the *stx*₂ gene without observable production of the toxin protein (Table 3).

**DISCUSSION**

Several methods have been described for the detection and isolation of *E. coli* O157:H7 over the last two decades. Most are based on the detection of two clonal biochemical characteristics, the inability to ferment sorbitol and the absence of $\beta$-D-glucuronidase activity (Zadik *et al.* 1993; Bettelheim 1998). However, since the first outbreaks of *E. coli* O157:H7, which ferment sorbitol and present $\beta$-D-glucuronidase activity, were reported in Germany (Karch and Bielaszewska 2001) the need to develop other methods, not exclusively based on detecting the biochemical phenotype, emerged. It should be noted that although *E. coli* O157:H7 is the most frequent serotype isolated from humans, it is not the most common serotype isolated from animals or food (Lindqvist *et al.* 1998). However, the *E. coli*
Table 3 Characterization of the *Escherichia coli* O157 strains isolated in this study, which were selected as representatives after biochemical phenotyping

<table>
<thead>
<tr>
<th>O157 strains</th>
<th>n</th>
<th>Sorb</th>
<th>Stx1</th>
<th>Stx2</th>
<th>eaeA</th>
<th>ehxA</th>
<th>rfbO157</th>
<th>βGluc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Typical</td>
<td>14</td>
<td>–</td>
<td>5</td>
<td>14</td>
<td>14</td>
<td>14</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>Atypical</td>
<td>13</td>
<td>+</td>
<td>0</td>
<td>ND</td>
<td>0</td>
<td>ND</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>+</td>
<td>0</td>
<td>ND</td>
<td>0</td>
<td>ND</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

N, number of strains; Sorb, Sorbitol fermentation; β-Gluc, β-D-glucuronidase activity at 24 h; stx1 and stx2, stx genes; Stx1 and Stx2, detection of Stx protein production; eaeA, intimin gene; ehxA, enterohemorrhagic hemolysin gene; rfbO157 and βGluc, genes coding for the biosynthesis of the O157 somatic and the H7 flagellar antigens, respectively; ND, not determined.

*All strains possessed the stx2 variant gene. None of the other stx2 variants were observed.

O157 serotype has received the most attention because of the number of outbreaks in which it has been implicated and the number of people infected during an outbreak.

One of the most commonly used methods for the detection of the O157 serotype is immunomagnetic separation, followed by plating of the samples on CT-SMAC (Wright et al. 1994). Although this method is used for the detection of this pathogen in food, its efficacy has been questioned because of the high number of false positives (Fujisawa et al. 2000; Ehlers et al. 2003). These can be caused by strains able to grow in the presence of cefixime and tellurite, making the isolation of the O157 serotype difficult, and also strains which cross-react with the O157 antigen (Borczyk et al. 1987; Shimada et al. 1992; Bettelheim et al. 1993).

In this study, samples from human sewage and animal wastewater were analysed for the presence of *E. coli* O157. As shown in Table 2, these samples presented high microbial loads: over 5 log10 (CFU + 1) ml\(^{-1}\) units for the faecal bacterial indicators analysed. The presence of interfering strains accounted for the high microbial counts after IMS and CT-SMAC plating. However, this number was considerably reduced after the specific O157 immunoblotting described here. Hence, the number of presumptive O157 strains after immunoblotting dropped by around 2 log10 (CFU + 1) ml\(^{-1}\) in the case of the human samples, which contained the most interfering strains. In the animal wastewater samples the number of interfering strains was lower, but there was also a reduction of between 1 and 2 log10 (CFU + 1) ml\(^{-1}\) units. Some of the strains that grew on this medium were characterized as *Klebsiella pneumoniae*, *Enterobacter* spp. or other *E. coli* (data not shown). Similar results were obtained when isolating *E. coli* O157 from radish sprouts, in which a high microbial background was observed (Fujisawa et al. 2000).

As previously reported, bacteria present in aquatic environments are exposed to various stress factors, which can cause cell damage (Terzieva and McFeters 1991). This phenomenon, along with the low number of pathogens, makes their isolation difficult on the routinely used selective media (Willshaw et al. 1994). Several isolation methods have been described for *E. coli* O157 from various samples, most of which incorporate an enrichment step, making them ineffective for quantification purposes. Other methods are based on quantitative PCR (Li and Drake 2001), but their use is limited by a minimum concentration of 10\(^3\) cells ml\(^{-1}\).

Although this shortcoming can be avoided by a concentration step of the samples, it is also limited by the presence of PCR inhibitors in the samples. In this study, a method based on immunomagnetic separation, plating the sample onto CT-SMAC, and specific immunoblotting, was effective for the detection and enumeration of *E. coli* O157, irrespective of their biochemical characteristics. It was thus possible to detect both fermenting and non-fermenting sorbitol strains as well as strains with β-D-glucuronidase activity. The methodology applied in this study could solve some of the limitations found with IMS in certain samples. A previous study found that cattle excreted *E. coli* O157 ranging from 10\(^2\) and 10\(^5\) CFU g\(^{-1}\) (Zhao et al. 1995), but in another study it was impossible to recover the paramagnetic beads used to analyse bovine faecal samples (Parham et al. 2003), probably because of the composition of the sample. The enumeration of *E. coli* O157 obtained in the present study suggests that environmental samples such as animal wastewaters are reservoirs of this pathogen.

The characterization of the different O157 strains isolated showed that 50% belonged to *E. coli* O157:H7 and the other 50% were O157 but did not carry the βGluc gene. These *E. coli* O157:H7 are presumed to be pathogenic in humans because they present the typical characteristics of this serotype and carry the genes coding for the virulence factors (eaeA, ehxA and stx2). Additionally, some of them also carried the stx1 gene. These results are consistent with the results obtained by Omisakin et al. (2003), in which a total of 44 bovine faecal samples taken at slaughter were analysed. All of them carried the stx2 gene, 11% also carried the stx1 gene, and 89% harboured the eaeA gene. Our results showed that those *E. coli* O157 that did not carry the H7 βGluc gene also lacked the main virulence genes studied, suggesting that these strains were not pathogenic. Chapman et al. 2003).
(1997) obtained similar results in four O157 strains isolated from pigs, which did not carry either the stx genes or eaeA gene and did not carry the H7 flaC gene.

Consequently, the isolation or enumeration of putative pathogenic E. coli O157 strains cannot be based only on the use of IMS methods combined with selective plating on CT-SMAC. The confirmation of serotype by specific immunomethods and the presence of virulence factors by specific gene detection and/or detection of toxin production is necessary. Otherwise, false-positive detection and incorrect enumeration can be obtained.

The surveillance of this pathogen in the environment is of particular interest to prevent epidemiological threats in the population.

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