The optimization of isolation media used in immunomagnetic separation methods for the detection of *Escherichia coli* O157 in foods

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**Introduction**

*Escherichia coli* O157 is a major food-borne pathogen in humans causing increasing concern worldwide as the number of incidences continues to rise (Anon. 1995a). Clinical symptoms include vomiting and diarrhoea, haemorrhagic colitis and haemolytic uraemic syndrome (HUS), a cause of renal failure in children which can lead to long-term complications and death, which is now the single most common cause of acute renal failure in children in North America (Karmali 1989). Pathogenic bacteria causing such severe symptoms require sensitive and specific methods of detection.

A wide variety of foods have been implicated in *E. coli* O157 outbreaks, with many being of cattle and dairy product origin. Post-processing contamination of milk by *E. coli* O157 led to an outbreak resulting in the death of one person (O'Grady and Cole 1994). Infected cooked meat caused the death of 21 people in the central Scotland outbreak in 1996 (Ahmed and Dveyse 1999). There have been a number of cases in the USA where contaminated apple juice was shown to be the vehicle of infection (Beiser et al. 1993), and fermented, ready-to-eat meat products have also been associated with *E. coli* O157 (Anon. 1995b).

The infectious dose of *E. coli* O157 is estimated to be very low (Willshaw et al. 1994), thus further justifying the development of sensitive detection techniques. In recent years, immunomagnetic separation (IMS) methods (Chapman et al. 1994) have become widespread in food analysis, although initial isolation media were developed for the examination of faecal specimens. Problems were experienced by some workers when these protocols were used in food testing (Bolton et al. 1995), and, in particular, for processed foods where bacteria were physiologically damaged due to extremes of heat, desiccation, preservatives, high osmotic pressure or pH changes. Such micro-organisms can be sensitive to antimicrobial additions used to inhibit non-target bacteria and therefore, the choice and concentration of such additions is of vital importance to optimise target recovery.

The routine diagnostic laboratory uses a number of distinct steps in the IMS procedure, culminating in colony confirmation from a selective agar plate. This paper compares chemical composition and incubation tempera-
tours of the enrichment, and selective agar used in the IMS procedure to isolate both naturally-occurring and artificially-isolated E. coli O157 in foods. To show method sensitivity under extreme circumstances, the spiked studies used low numbers (<1 g⁻³) of physiologically-pressed target bacteria in the presence of high numbers of healthy background microorganisms. Foods of known contamination with E. coli O157 were studied, minced (ground) beef was tested initially, with subsequent validations performed on cheese, apple juice and peppercorn.

MATERIALS AND METHODS

Bacterial strains

Three separate cocktails of E. coli O157, each containing four or five different strains, were employed to minimize the influence of a typical single strain. The isolates were from food, environmental, and clinical sources from the Department's collection. Previous work (date, not presented) had shown them to be resistant to a range of antimicrobials (rifampicin, cefadroxil and potassium nitrite) used routinely in IMS methods.

Preparation of spiking cocktail

Isolates of E. coli were grown individually in nutrient broth at 37°C for 18 h prior to mixing 10 ml aliquots in a sterile container to form the cocktail. This was diluted in sterile phosphate-buffered saline (PBS) to a level of approximately 10⁶ m⁻¹.

Preparation and testing artificially-contaminated foods

All foods, for spiking studies, were initially confirmed as absent from naturally-occurring E. coli O157 using buffered peptone water supplemented with vancomycin. 8 mg Γ⁻¹ (BPW-V) enrichment at 42°C for 6 h, followed by IMS with beads (0.1 ml) placed equally onto two cellulose tetra-acetate sorbitol MacConkey agar (CTSMAC) plates. After incubation at 37°C for 24–18 h, presumptive E. coli O157 colonies (non-sorbitol fermenting) were tested using latex agglutination (Oxoid). Minced samples contained background E. coli white colony and peppercorn contained variable coliform levels. Apple juice contained no background micro-organisms. Minced beef was spiked separately with all cocktails, and other foods were spiked with a single cocktail only.

Aliquots (20 ml) of the diluted cocktail were added to 100 g retail minced beef to give approximate levels of 100 g⁻¹. The minced was then subjected to a number of freeze/thaw cycles (between -20 and +15°C) in order to reduce numbers and physiologically stress the bacteria. The time periods at +15°C were short (<4 h) to minimise bacterial growth. The number of survivors were calculated by the Most Probable Number method with each dilution enriched in BPW-V followed by IMS. Each subsequent plating on CTSMAC incubated at 37°C. Four freeze/thaw cycles typically gave a reduction to <1 g⁻¹, but if significantly above this number, the sample was subjected to one more cycle. Prior to testing spiked minced beef, samples were mixed with an equal weight of unsifted mince previously shown to contain high numbers (approximately 10⁶ g⁻¹) of micro-organisms (but containing no E. coli O157). Minced samples (200 g) were homogenised for 2 min in a stomacher (Don Whitley Scientific, Shipley, UK) in 800 ml PBS, and 50 ml volumes of supernatant fluid were added to 50 ml double strength enrichment broth under test. Replicate experiments were performed with all three cocktails. Pasteurized goat's cheese (50 g) was surface spread with 1 ml of cocktail containing approximately 10⁶ m⁻¹ and stored at 4°C for 2 days prior to analysis, thus simulating post-processing contamination. Testing of IMS enrichments was performed as for minced beef.

Apple juice (containing no preservative) was tested with both stressed and unstressed E. coli O157 to simulate recoveries from pre- and post-processing contamination. To stress the cells, the cocktail (10⁶ m⁻¹) was inoculated into 200 ml apple juice (pH 3.5) and stored at 4°C for 10 days, after which time the count had fallen to approximately 10⁶ m⁻¹. The survivors were further diluted and inoculated into fresh apple juice (502 ml into 200 ml) a final concentration of 10⁷ m⁻¹. Unsteriled cells of the same cocktail at similar numbers in apple juice were tested separately. Volatile (25 ml) spiked apple juice were added to 225 ml enrichment broths followed by IMS.

Comparison of enrichment broths and incubation temperatures

Enrichment broths were tested on modifications of BPW (Oxoid) with additions of vancomycin (8 mg Γ⁻¹), David Bull Laboratories), cefsulodin (0.05 mg Γ⁻¹), Wyeth.
Aytrey Research) and cefadroxil (10 mg L⁻¹, Sigma) (BPW-TCC). BPW with vancomycin alone (BPW-V), and with cefadroxil and minocycline concentrations reduced to one quarter strength (BPW-V + 1/4 C + 1/4 M), were also tested. Modifications of tryptone soya broth (Oxoid) and EC medium (Oxoid) were investigated (Table 1). Incubation temperatures tested included 30, 37, and 42 °C, and incubation times remained constant at 6 h. All broths were equilibrated at 20°C prior to food addition and incubation.

Immunomagnetic separation technique

Enrichment volumes (1 ml) were transferred in sterile Eppendorf tubes containing 0.2 ml Captiva™ O157 immunomagnetic beads (International Diagnostics Group, Bury, UK). The Eppendorf tubes were placed in a rack, mixed for 30 min, before discarding the supernatant fluid and re-superseding the beads (without the magnet) in wash buffer (PBS + 0.05% Tween 20). Two further washing stages were performed prior to transferring the beads (in 0.1 ml buffer) equally onto two selective agar plates incubated at 37°C for 16–18 h.

Selective agars

Those used (Table 2) included sorbitol MacConkey agar (SMAC, Oxoid), SMAC with additions (Matt Diagnostic) of cefalotin (C, 0.05 mg L⁻¹) and potassium tellurite (T, 2.5 µg L⁻¹) (CTSMAC), SMAC with one third strength CT additions and SMAC with two third strength CT additions. The performance of three chromogenic agars was investigated, including CHROMagarO157 (Oxid-Tech Diagnostics Ltd), Rhodes™ 37, 37°C12 and 42°C, and Don Whitley Scientific and SD-9 (QA Life Sciences). All were made according to the manufacturer's instructions and stored at chill temperatures for <7 days prior to use. All agars were incubated at 37°C except SD-39 which was tested at 42°C, and incubation times were approximately 16–18 h at which time presumptive target colonies could be distinguished.

<table>
<thead>
<tr>
<th>Enrichment medium</th>
<th>Colonies</th>
<th>Untreated</th>
<th>Stress</th>
<th>Untreated</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPW-VCC 37 °C</td>
<td>-</td>
<td>++</td>
<td>+ + + + +</td>
<td>++</td>
</tr>
<tr>
<td>nTSB + 1/4 C + 1/4 M</td>
<td>+</td>
<td>++</td>
<td>+ + + + +</td>
<td>++</td>
</tr>
<tr>
<td>BPW-V 43 °C</td>
<td>+ +</td>
<td>++</td>
<td>+ + + + +</td>
<td>++</td>
</tr>
</tbody>
</table>

Table 1 Recovery of spiked Escherichia coli O157 from minced beef by different IMS enrichments at different temperatures

<table>
<thead>
<tr>
<th>Enrichment medium</th>
<th>37 °C</th>
<th>40 °C</th>
<th>42 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPW-VCC*</td>
<td>++</td>
<td>+ +</td>
<td>++</td>
</tr>
<tr>
<td>BPW-V pH 7.4</td>
<td>++</td>
<td>+ +</td>
<td>++ ++</td>
</tr>
<tr>
<td>MTSSB + 1/4 C + 1/4 M</td>
<td>++</td>
<td>+ +</td>
<td>++ ++</td>
</tr>
<tr>
<td>EC medium + NMM*</td>
<td>++</td>
<td>+ +</td>
<td>++ ++</td>
</tr>
</tbody>
</table>

*BPW + vancomycin (8 mg L⁻¹) + cefadroxil (0.05 mg L⁻¹) + minocycline (10 mg L⁻¹).

Testing of naturally-contaminated foods

Samples of cheese and minced beef naturally contaminated with E. coli O157 were included in this study. The cheese was responsible for a small, localized outbreak in north east Scotland and the minced beef was obtained from routine surveillance. Both strains of E. coli O157 were VTEC negative, VT2 positive and were phage type 21/28. Samples were homogenized in PBS, the supernatant fluid was subdivided and added to equal volumes of double strength BPW-V and nTSB + N incubated at 42°C, and BPW-VCC incubated at 77°C, for 16–18 h. Immunomagnets were divided equally and plated onto CTSMAC and CHROMagarO157 plates.
RESULTS

Three criteria were used when comparing results in this study: the presence or absence of target pathogens; the volume of background flora and the numbers of recovered E. coli 0157.

Enrichment studies

An initial comparison of enrichment incubation temperatures at 30, 37 and 42°C indicated that 30°C gave significantly poorer target recovery after overnight incubation (data not presented). Incubation at 30°C was therefore omitted from subsequent investigations. A comparison of E. coli 0157 recoveries from ground beef by six different enrichment methods incubated at 37, 40 and 42°C is presented in Table 1. The results are representative of approximately 10 replicate experiments from a number of ground beef samples spaced sequentially with the three cocktails. Cocktails had no effect on the results shown in Table 1. Throughout the tests, BMP supplemented with vancomycin at 8 mg l⁻¹, with or without one quarter normal strength ceftazidime and cephalaxin, gave optimum recovery. BMP-V at pH 7.0 was superior to BMP-V at pH 6.0. While BMP with the full supplement of vancomycin, ceftazidime and cephalaxin showed minimal background flora, it consistently gave a poor or zero recovery of E. coli 0157. Modified TSBS proved to be slightly inferior to BMP-V at pH 7.0 at 42°C, but superior to EC broth + N. There was a marked difference between incubation temperatures with 42°C giving best, and 37°C giving poorer recovery. Bacteria, while these tests were poorly defined as being overgrown with background flora, making individual isolate recognition difficult. Of the media tested on MacConkey agar, CTSMAC proved superior, with minimal non-target growth.

Validation studies of IMS enrichment media in foods other than minced beef compared BMP-V (conventional IMS broth, mTSBS + N (propidium iodide) broth and BMP-V (autumus broth as shown in Table 2) clearly showed the poor performance of BMP-V, which failed to isolate E. coli O157 from cheese and the stressed E. coli O157 in peppers. The recovery of unstressed cells in peppers, sausages and unstressed E. coli O157 from apple juice, were low in numbers from BMP-V. No differences were observed in the performance of the other two enrichment except in the numbers of stressed cells recovered, which were slightly less from mTSBS+N. Replicate testing of cheese and peppers showed natural levels of cultured microflora, varying from 10⁵ g⁻¹ to 10⁶ g⁻¹, while apple juice contained <10⁵ cfu ml⁻¹.

Selective agars

Selective agars based on either SMAC or different chromogenic reactions were tested and are listed in Table 3. The food source did not have an effect on agar ratings. The agar with the best rating had high numbers of easily observed target bacteria, while those rated poorly were often completely overgrown with background flora, making individual isolate recognition difficult. Of the media tested on MacConkey agar, CTSMAC proved superior, with minimal non-target growth.

Table 3 Comparison of E. coli O157 selective agars for immunomagnetic separation

<table>
<thead>
<tr>
<th>Selective agar</th>
<th>Rating</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTSMAC*</td>
<td>+++</td>
</tr>
<tr>
<td>SMACI</td>
<td>++</td>
</tr>
<tr>
<td>SMAC + 1/3 CTJ</td>
<td>++</td>
</tr>
<tr>
<td>SMAC + 2/3 CTJ</td>
<td>+</td>
</tr>
<tr>
<td>CHROMagar*</td>
<td>+++</td>
</tr>
<tr>
<td>KO*</td>
<td>-</td>
</tr>
</tbody>
</table>

*Cefotaxime-tellurite modified MacConkey, vancomycin 40 µg ml⁻¹, potassium tellurite 25 µg ml⁻¹.

**Refridgerated MacConkey + xanthine and tellurite at the third normal strength.

***Refridgerated MacConkey + xanthine and tellurite at two thirds normal strength.

- CHROMagar supplemented O157 isolation medium.
- SRS Agar supplemented O157 isolation medium.
- CBIGA supplemented O157 isolation medium.

- < indicates gain recovery, ++, 20–50; ++++, >200; 100–200; +++, >50 restored colonies recovered.

- +++++ indicates gain recovery, >1000; ++++, >200 restored colonies recovered.

- **Bacteria, while these tests were poorly defined as being overgrown with background flora, making individual isolate recognition difficult. Of the media tested on MacConkey agar, CTSMAC proved superior, with minimal non-target growth.

Naturally-contaminated foods

Foods previously found to contain E. coli 0157 were tested by the three IMS enrichments. The number of replicate tests reduced the sample size available for testing. The results are presented in Table 4, which indicates the number of samples giving a presence or absence of the target pathogen.

BMP-V (2°C) gave the greatest number of positives and BMP-V, failed to isolate E. coli O157 from all samples. Modified TSBS + N (42°C) gave one positive from a cheese sample and zero from the minced beef samples.

DISCUSSION

When IMS first became commercially available, routine food testing laboratories regularly employed a sample enrichment in BMP-V:CC at 37°C, followed by bead

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Table 4 Testing naturally-contaminated foods for Escherichia coli O157 by three enrichment procedures

<table>
<thead>
<tr>
<th>Enrichment condition</th>
<th>Min−4</th>
<th>No. positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW-2 V C 37 °C</td>
<td>0/4</td>
<td>0/2</td>
</tr>
<tr>
<td>mTSB + N 42 °C</td>
<td>1/0</td>
<td>0/2</td>
</tr>
<tr>
<td>BW-F 42 °C</td>
<td>2/0</td>
<td>1/2</td>
</tr>
</tbody>
</table>

1050 mg/l peptone broth + bile salts (15 g l−1) + novobiocin (20 mg l−1).
Buffered peptone water (BPW) + uva-cynara (8 mg l−1) pH 7.0.

incubation on CTSMAC (Bell and Kayridakes 1998), as indicated by the immunobead manufacturers. However, some strains of E. coli O157 were shown to be sensitive to many of the antimicrobials used in this particular method (MacRae et al. 1997), and a wide range of enrichment conditions (media and temperature) were tested by a number of laboratories in order to improve recovery. Several enrichments were based on BPW, which is used widely to aid enrichment of physiologically-stressed microorganisms such as Salmonella spp. (Edel and Kampelmacher 1973). Bolten et al. (1992) recognized that the cefixime and cefsulodin supplements in the enrichment step were potentially inhibitory to some E. coli O157 and tested a range of reduced concentrations, resulting in an optimum BPW enrichment containing one quarter of the normal antimicrobials.

In this study, BPW-V pH 7.0 and BPW-V + % C + C were the optimum enrichment media for the recovery of stressed E. coli O157 in minced beef. It is probable that cefixime and cefsulodin at this reduced level are below the concentrations that have an effect on target recovery. Doyle and Schoeni (1987) proposed the use of modified TS1B + N at 37°C, which in our study compared favorably with other media tested, although incubation at 42°C improved recovery. However, it proved slightly inferior to BPW-V pH 7.0 and BPW-V + % C + C. Szabo et al. (1990) tested TS1B at 35 and 43°C, and found the addition of bile salts and novobiocin at the higher temperature improved recovery of E. coli O157 in foods containing competing microorganisms. Bolten et al. (1995) tested mTSB at 37 and 42°C alone in the BP with different additions and found mTSB at 42°C to be optimum. This medium, incubated at 41.5°C, was proposed by Scottor et al. (2000) and has been accepted as a draft International Standard by the International Organization for Standardization; it is widely used by a number of laboratories in the UK. The recommended medium of the US Department of Agriculture is a modified version of EC medium with novobiocin (Olver and Rask 1989) but in our study, it proved inferior to BPW-V pH 7.0.

Originally designed to enumerate coliforms in water and sludge, EC media contain bile salts and are therefore more selective than BPW-V, and possibly, more inhibitory to sub-lethally damaged microorganisms.

This study demonstrates the importance of enrichment incubation temperature. Temperature elevation above 37°C increased the number of samples testing positive for E. coli O157 and also increased the number of target bacteria recovered (Table 1). This was independent of enrichment composition and was possibly due to the higher temperature being inhibitory to some of the natural microbial flora in the tests. In the majority of cases, there were fewer non-target bacteria on plates from 42°C enrichment.

Further experiments were performed in other foods associated with E. coli O157 contamination (Table 2). A zero recovery from cheese by BPW-V (37°C) was somewhat surprising as good recovery was observed from the other two enrichments. The numbers of E. coli O157 spiked into cheese were slightly lower than in pepperoni, but the degree of physiological stress was assumed to be minimal (2 days of storage at 4°C). The use of BPW-VCC also failed to recover stressed E. coli O157 from pepperoni, and it is assumed that cells were damaged to the extent that the high antimicrobials concentration did not allow sufficient recovery in the 6 h incubation time. BPW-VCC did recover untargeted bacteria from pepperoni, supporting this theory. BPW-VCC was found to recover both stressed and unstressed E. coli O157 from apple juice, and perhaps the degree of stress with this product was less damaging than other methods applied in this work. The difference between BPW-V and mTSB + N lay in their ability to recover damaged bacteria in apple juice and pepperoni; fewer E. coli O157 were recovered from the mTSB + N enrichment. This difference may be attributed to the different damaged organisms by the presence of bile salts in TS1B, or to the superior recovery of such cells in the less nutritious peptone water (Edel and Kampelmacher 1973). All results indicate that optimum enrichment contains minimum antimicrobials, particularly from those with known activity against Gram-negative microorganisms such as cefixime and cefsulodin (Lambert and O'Grady 1992).

When Zadil et al. (1993) introduced oxacillin and tellurite additions to improve E. coli O157 selectivity, the majority of isolation procedures in the UK used SMAC. The introduction of oxacillin and tellurite into SMAC has made the observation of target pathogens clearer by reducing background flora. However, MacRae et al. (1997) showed that a small but significant number of E. coli O157 isolates exhibited partial inhibition in the presence of such antimicrobials, and suggested that the use of such additions may contribute to foods being incorrectly labelled as free from E. coli O157. It must be stressed that all strains of

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E. coli 0157 used in this study were not sensitiveto aztreonam and sul-fite and therefore, CTSMAC gave excellent results when compared with SMAC, with a lower number of concentrations of the two antimicrobials (Table 3). CTSMAC alone will not detect the presence of norbrolb-positive enterotoxigenic strains and therefore, only rarely in the UK but it is believed to be due to the fact that they do not pose a significant threat to human health in the future. Other European countries, such as Germany (Blessen et al., 2009), isolate norbrol-positive strains on a regular basis, which necessitates the use of an alternative selective plating medium. A number of sensitive enterotoxigenic strains were identified and these are based on chromogenic reactions to distinguish the target colony from background flora. Tsai et al. (2010) concluded that the Rainbow™ and a modified colony-methylene blue agar gave best recovery from heat-stressed E. coli 0157 in ground beef. The present study found Rainbow™ to be superior to a alternative chromogenic agar (having less background flora) and indeed, showed better recovery of target cells compared with CTSMAC. CHROMAgar™ 0157 was similar to performance on CTSMAC. In addition to the discrimination of E. coli 0157 from other enterocytes and food-associated microflora, CHROMAgar™ 0157 can be used to identify serogroups O111 and O103 (Bettelheim 1999), while Rainbow™ can distinguish serogroups O111/H8 and O26:H11 from E. coli 0157 (Bettelheim 1998). Quality Life Sciences. E. coli 0157 medium (SD-39) gave poor results in this study (incubated at 42°C), with a low number of concordant identifications of E. coli 0157. However, it must be stated that the manufacturers recommend incubation at 44°C, a temperature higher than that used for this study. Some workers (Dodd and Sadoon, 1994) have reported partial or complete inhibition of E. coli 0157 at this elevated temperature. Based on the results of this study, the present study used E. coli 0157 in a plate laboratory to isolate and characterize IMS beads equally well as CTSMAC and as a chromogenic agar. The current methodological choice is CHROMAgar™ 0157. The success of this study using yellow cockails, it was not possible to identify the individual component strains recovered after enrichment and plating, and therefore it exists that the media may not be optimal for all strains used.

Two foods naturally contaminated with E. coli 0157 were available in sufficient quantity for replicate testing during the course of the work. The levels of E. coli 0157 in the two foods (as enumerated by MPN) were found to be at <10^3 CFU/g, but their physiologically state was unknown. Although both mixed beef and cheese were supplied by the retailer, at fresh product, their history was unclear. For example, the beef may have been frozen prior to sale and the cheeses could have been stored for an extended period (several months) before purchase, both of which could result in sub-lethally damaging target bacteria. Results showing such differences between enrichment short that stressed E. coli 0157 could have been present in all samples. The use of optimum IMS enrichment media as described has enabled this laboratory to isolate and confirm the presence of low numbers of sub-lethally damaged E. coli 0157 from a number of foods. In the majority of tests, there was a high background level of micro-organisms which did not interface with recognition of the target bacteria. A protocol is proposed for enriching in IPPV-V at 42°C for 6 h, and plating immediately on CTSMAC and CHROMAgar™ 0157, to improve E. coli 0157 isolation from foods.

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The authors are grateful to the Scottish Executive (Rural Affairs Department) who wholly funded this work and to M-Therm Diagnostics Ltd and Ken Wadley Scientists who donated the chromogenic agars.

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Aliquots (20 ml) of the diluted cocktail were added to 100 g retail minced beef to give approximate levels of 100 g⁻¹. The mix was then subjected to a number of freeze/thaw cycles (between -20 and +15°C) in order to reduce numbers and physiologically stress the bacteria. The time periods at +15°C were short (<4 h) to minimize bacterial growth. The number of survivors were calculated by the Most Probable Number method with each dilution enriched in BPW-V for 42°C followed by IMS and subsequent plating on CTSMAC incubated at 37°C. Four freeze/thaw cycles typically gave a reduction to <1 g⁻¹, but if significantly above this number, the sample was subjected to one more cycle. Prior to testing spiked minced beef, samples were mixed with an equal weight of unsterile mince previously shown to contain high numbers (approximately 10⁷ g⁻¹) of micro-organisms (but containing no E. coli O157). Mince samples (200 g) were homogenized for 2 min in e-Stomacher (Dow Whitley Scientific, Skiplay, UK) in 800 ml PBS, and 50 ml volumes of supernatant fluid were added to 50 ml double strength enrichment broth under test. Replicate experiments were performed with all three cocktails.

Post-treatment plate count (50 g) was surface spread with 10 ml of cocktail containing approximately 10⁶ ml⁻¹ and stored at 4°C for 2 days prior to analysis, thus simulating post-processing contamination. Testing of IMS enrichments was performed as for minced beef.

Apple juice (containing no preservative) was tested with both stressed and unstressed E. coli O157 to simulate recoveries from pre- and post-processing contamination. To stress the cells, the cocktail (10⁵ ml⁻¹) was inoculated into 200 ml apple juice (pH 3.5) and stored at 4°C for 10 days, after which the count had fallen to approximately 10⁴ ml⁻¹. The survivors were further diluted and inoculated into fresh apple juice (100 ml into 200 ml) a final concentration of 10⁴ ml⁻¹. Unstressed cells of the same cocktail at similar numbers in apple juice were tested separately. Volumes (25 ml) of spiked apple juice were added to 225 ml enrichment broths followed by IMS.

Pepperoni (pre-sliced, 100 g) was spiked separately with stressed and unstressed E. coli O157 to simulate recoveries from pre- and post-processing contamination. Sliced cells were produced by inoculating (1 ml) of the cocktail (10⁵ ml⁻¹) into 200 ml high salt (NaCl, 12.5 g/l), low pH (4.5), low temperature (5°C) broth (McCarthy et al. 1998). Counts fell by 2 log numbers over a 10-day period. The pepperoni slices (100 g) were dipped (20 x) in 100 ml cocktail (10⁷ ml⁻¹), giving a final concentration of 10⁶ g⁻¹, and stored at 4°C for 18 h prior to testing as for minced beef. Numbers of unstressed E. coli O157 in apple juice and pepperoni were expected to approximately the same as in stressed E. coli O157 assays.

Comparison of enrichment broths and incubation temperatures

Enrichment broths were tested on modifications of BPW (Oxoid) with additions of vancomycin (3 mg ml⁻¹, David Bull Laboratories), cefoxitin (0.05 mg ml⁻¹, Wyeth-
Ayers Research) and cefadolin (10 mg g⁻¹, Sigma) (BPW-YCC). BPW with vancomycin alone (BPW-V), and with cefadolin and vancomycin concentrations reduced to one quarter strength (BPW-V + 1/4 C + 1/4 V) were also tested. Modification of tryptone soya broth (Oxoid) and EC medium (Difco) were investigated (Table 1). Incubation temperatures tested included 30, 37, 40 and 42°C, and incubation times remained constant as 6 h. All broths were equilibrated at 20°C prior to food addition and incubation.

### Immunomagnetic separation technique

Table 1 Summary of opuntia Erichia coli O157 from minced beef by different IMS enrichments at different temperatures

<table>
<thead>
<tr>
<th>Enrichment medium</th>
<th>37°C</th>
<th>40°C</th>
<th>42°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPW-YCC*</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>BPW-V pH 7.0</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>BPW-V pH 6.0</td>
<td>+</td>
<td>NT</td>
<td>+</td>
</tr>
<tr>
<td>BPW-V + 1/4 C + 1/4 V</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MTSS + N</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>EC medium + N</td>
<td>+</td>
<td>NT</td>
<td>+</td>
</tr>
</tbody>
</table>

*BPW + vancomycin (8 mg g⁻¹) + cefadolin (0.05 mg g⁻¹) + cefadolin (10 mg g⁻¹).*  
Thioliferous peroxide (BPW) = vancomycin (8 mg g⁻¹) pH 7.0.  
BPW + vancomycin (8 mg g⁻¹) + cefadolin (0.0125 mg g⁻¹) + cefadolin (5 mg g⁻¹).  
Tryptone soya broth + bile salts (15 g l⁻¹) + neomycin (20 mg l⁻¹).  
*EC medium + neomycin (20 mg l⁻¹).*  
NT, not tested. ±, <10 on agar recovery; +, >10-48; ++, >48-260; ++++, >260-1000; +++++, >1000. >250 target colonies recovered. Colony numbers are representative of several replicate experiments.

### Selective agar

The sterile test (Table 2) included sorbitol MacConkey agar (MAC, Oxoid), SMAC with additions (Mast Diagnostics) of cefadolin (C, 0.05 mg l⁻¹) and potassium tellurite (T, 2.5 mg l⁻¹) (CTSMAC), SMAC with one third strength CT additions and SMAC with two third strength CT additions. The performance of three chromogenic agar was investigated, including CHROMAgar O157 (M-Tech Diagnostics Ltd), Rambach AG, O157, 21/02 (Des Whitney Science), and SD-39 (QPA Life Sciences). All were made according to the manufacturer's instructions and stored at chill temperature for ≤7 days prior to use. All agar were incubated at 37°C, except SD-39 which was tested at 4°C, and incubation times were approximately 16-18 h at which time presumptive target colonies could be distinguished.

### Testing of naturally-contaminated food

Samples of cheese and minced beef naturally contaminated with *E. coli* O157 were included in this study. The cheese was responsible for a small, localized outbreak in north-east Scotland and the minced beef was obtained from routine surveillance. Both strains of *E. coli* O157 were VTEC negative, VTT positive and were phage type 21/28. Samples were homogenized in PBS, the supernatant fluid was subdivided and added to equal volumes of double strength BPW-V and MPSSNeat incubated at 4°C and BPW-YCC incubated at 37°C, for 16-18 h. Immunomagnetic beads were divided equally and plated onto CMSAC and CHROMAFLX O157 agar.

### Table 2 Comparisons of Erichia coli O157

<table>
<thead>
<tr>
<th>Enrichment media and incubation temperatures from different foods</th>
<th>Apple juice</th>
<th>Peppermint</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPW-V YCC 37°C*</td>
<td>++++++++</td>
<td>+++</td>
</tr>
<tr>
<td>MPSSNeat + B 37°C</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>BPW-V YCC 42°C*</td>
<td>++++++++</td>
<td>+++</td>
</tr>
</tbody>
</table>

*BPW + vancomycin (8 mg g⁻¹) + cefadolin (0.05 mg g⁻¹) + cefadolin (10 mg g⁻¹).*  
Tryptone soya broth + bile salts (15 g l⁻¹) + neomycin (20 mg l⁻¹).  
Thioliferous peroxide (BPW) = vancomycin (8 mg g⁻¹) pH 7.0.  
+, Indicate zero recovery; ++, >10-48; ++++, >48-260; +++++, >260-1000; ++++++, >1000. >250 target colonies recovered.

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RESULTS

Three criteria were used when comparing results in this study: the presence/absence of target pathogen, the volume of background flora and the numbers of recovered E. coli O157.

Enrichment studies

An initial comparison of enrichment incubation temperatures at 30, 37, and 42°C indicated that 30°C gave significantly poorer target recoveries after overnight incubation (data not presented). Incubation at 30°C was therefore omitted from subsequent investigations. A comparison of E. coli O157 recoveries from minced beef by six different enrichment broths incubated at 37, 40, and 42°C is presented in Table 1. The results are representative of approximately 40 replicate experiments from a number of minced beef samples spiked separately with the three cocktails. Cockshut comparison had no effect on the ratings shown in Table 1. Throughout the tests, BPW supplemented with vancomycin at 8 mg L⁻¹, with or without one quarter normal strength colistin and rifampicin, gave optimum recovery. BPW-Y at pH 7.0 was superior to BPW-V at pH 6.6. While BPW with the full supplement of vancomycin, colistin and rifampicin showed minimal background flora, it consistently gave a poor or zero recovery of E. coli O157. Modified TSB proved to be slightly inferior to BPW-V at pH 7.0 at 42°C, but superior to EC broth + N. There was a marked difference between incubation temperatures at 42°C giving best, and 37°C giving poorest recovery.

Validation studies of IMS enrichment media in foods other than minced beef compared BPW-VCC (conventional IMS broth), mTSB + N (prepared IS0 broth) and BPW-V (optimum both as shown in Table 1). The results (Table 2) clearly indicate the poor performance of BPW-VCC, which failed to isolate E. coli O157 from cheese and the strained E. coli O157 from pepperoni. The recoveries of transmigrated cells in pepperoni, had strained and stressed E. coli O157 from apple juice, were low in number from BPW-VCC. No differences were observed in the performance of the other two environments compared.3 Strained cells were recovered, which were slightly less from mTSB+N. Replica testing of cheese and pepperoni showed natural levels of microbial flora, varying 10⁶ cfu g⁻¹ and 10⁷ g⁻¹, while apple juice contained <10³ cfu ml⁻¹.

Selective agars

Selective agars based on either SMAC or different chromogenic reactions were tested and are listed in Table 3. The food sources do not have an effect on agar ratings. The agar with the best rating had high numbers of easily observed target bacteria, while those rated poorly were often completely overgrown with background flora, making individual isolate recognition difficult. Of the media based on MacConkey agar, CTSMAC proved superior, with minimal background growth. Two chromogenic broths showed good recovery of E. coli O157 with low background microflora. Rainbow™ was slightly superior to CHROMagar™, having an almost complete absence of background flora. In the majority of cases, 32–39 failed to isolate the target which was totally obscured by a lawn of competing enteric bacteria. Extended incubation periods (18–24 h) often resulted in overgrowth by background flora.

Naturally-contaminated foods

Foods previously found to contain E. coli O157 were tested by three IMS enrichments. The number of replicate tests reflects the small sample size available for testing. The results are presented in Table 4, which indicates the number of samples yielding a presence or absence of the target pathogen. BPW-V (42°C) gave the greatest number of positives and BPW-VCC failed to isolate E. coli O157 from all samples. Modified TSB + N (42°C) gave one positive from a cheese sample and zero from the minced beef sample.

DISCUSSION

When IMS tests became commercially available, routine food testing laboratories regularly employed a sample enrichment in BPW-VCC at 37°C, followed by bas...
Table 4 Testing naturally-contaminated foods for E. coli O157 by three enrichment procedures

<table>
<thead>
<tr>
<th>Enrichment condition</th>
<th>Choice</th>
<th>Minus</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW-PG-V 37°C</td>
<td>No +</td>
<td>0/6</td>
</tr>
<tr>
<td>mTSB + N 42°C</td>
<td>1/6</td>
<td>0/2</td>
</tr>
<tr>
<td>BW-V 42°C</td>
<td>2/6</td>
<td>1/2,</td>
</tr>
</tbody>
</table>

*BW-PG + vancomycin (8 ng g⁻¹) + coline (015 g g⁻¹) +
  cefotaxime (10 mg l⁻¹). |
†Tryptone soy broth + bile salts (1:5 g l⁻¹) +
  novobiocin (20 mg l⁻¹). |
‡Thermolabile peptide water (BW-PG + vancomycin (8 ng g⁻¹) pH 7:4.

Incubation on CTS-MAC (Bell and Kyriakides 1998), as
indicated by the immunobead manufacturers. However,
some strains of E. coli O157 were shown to be sensitive
to many of the antimicrobials used in this particular method
(MacRae et al. 1997), and a wide range of enrichment
conditions (media and temperatures) were tested by a
number of laboratories in order to improve recovery.
Several enrichments were based on BW-PG, which is used
widely to aid enrichment of physiologically-stressed micro-
organisms. In some cases, such as Salmonella spp. (Edel and
Kampelmacher 1973), Boltom and Schonig (1975) recognized
that the cefoxime and cefulodin supplements in the enrichment step were poten-
tially inhibitory to some E. coli O157 and tested a range of
reduced concentrations, resulting in an optimum BW-PG
enrichment containing one quarter of the normal amni-
acridine strength.

In this study, BW-PG-V pH 7.0 and BW-PG-V + C + C were
the optimum enrichment media for the recovery of
stressed E. coli O157 in minced beef. It is probable that
cefoxime and cefulodin at this reduced level are below
the concentrations that have an effect on target recovery. Doyle
and Schonig (1978) proposed the use of modified TSB + N at
37°C, which in our study compared favourably with other
media tested, although incubation at 42°C improved recov-
ery. However, it proved slightly inferior to BW-PG-V pH 7.0
and BW-PG-V + C + C. Sklazar et al. (1996) tested TSB at
35 and 45°C, and found the addition of bile salts and
novobiocin at the higher temperature improved recovery of
E. coli O157 in foods containing competing micro-orga-
nisms. Boltom et al. (1995) tested mTSB at 37 and 42°C
and found mTSB at 42°C to be optimum. This medium, incubated at 41-35°C,
was proposed by Scorti et al. (2000) and has been accepted as a
draft International Standard by the International
Organization for Standardization; it is widely used by a
number of laboratories in the UK. The recommended
medium of the US Department of Agriculture is a modified
version of EC medium with novobiocin (Kowald and Rice
1989) but, in our study, proved inferior to BW-PG-V pH 7.0.

Originally designed to enumerate coliforms in water and
shellfish, EC media contain bile salts and are therefore more
selective than BW-PG-V, and possibly, more inhibitory to sub-
lethally damaged micro-organisms.

This study demonstrated the importance of enrichment
incubation temperature. Temperature elevation above
37°C increased the number of samples testing positive for
E. coli O157 and also increased the number of false
bacteria recovered (Table 1). This was independent of
enrichment composition and was possibly due to the higher
temperatures being inhibitory to some of the natural
microbial flora in the foods tested. In the majority of cases,
there were fewer non-target bacteria on plates from 42°C
enrichments.

Further experiments were performed in other foods
associated with E. coli O157 contamination (Table 2). A
zero recovery from cheese by BW-PG-V (37°C) was
somewhat surprising as good recovery was observed from
the other two enrichments. The numbers of E. coli O157
spiked into cheese were slightly lower than in pepperoni, but
the degree of physiological stress was assumed to be minimal
(2 days of storage at 4°C). The use of BW-PG-V also failed
to recover stressed E. coli O157 from pepperoni, and it is
assumed that cells were damaged to the extent that the high
ammonium concentration did not allow sufficient recovery
in the 6 h enrichment time. BW-PG-V did recover unstressed
target bacteria from pepperoni, supporting this
theory. BW-PG-V was found to recover both stressed and
unstressed E. coli O157 from apple juice, and perhaps the
degree of stress with this product was less damaging than
other methods applied in this work. The difference between
BW-PG-V and mTSB + N lay in their ability to recover
damaged bacteria in apple juice and pepperoni; fewer E. coli
O157 were recovered from the mTSB + N enrichment.
This difference could be due to the presence of bile salts in
TSB, or to the superior recovery of such cells in the less
nutritious pepperoni water (Edel and Kampelmacher 1973). All results
indicate that optimum enrichment is achieved with mini-
mum antimicrobial addition, specifically from those with
known activity against Gram-negative micro-organisms such as

When Zadik et al. (1993) introduced cefoxime and tellurite
additions to improve E. coli O157 selectivity, the majority of
isolates produced in the UK used SMAC. The intro-
duction of cefoxime and tellurite into SMAC has made the
observation of target pathogens clearer by reducing back-
ground flora. However, MacRae et al. (1997) showed that a
small but significant number of E. coli O157 isolates
frequently exhibited partial inhibition in the presence of such anti-
microbials, and suggested that the use of such additions may
contribute to foods being incorrectly isolated as free from
E. coli O157. It must be stressed that all strains of

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E. coli O157 used in this study were not sensitive to colitoxin and citrulline and histidine, CTSMAC gave excellent results when compared with SMAC, with or without reduced concentration of the two amnonibiotics (Table 3). CTSMAC alone will not detect the presence of sorbitol-positive E. coli O157. There are currently isolated only rarely in the UK but it would be wise to assume that they do not pose a significant threat to human health in the future. Other European countries, such as Germany (Baudt 1999), isolate sorbitol-positive strains on a regular basis, which underlines the use of an alternative selective plating medium. A number of selective agars based on alternatives to sorbitol MacConkey are available commercially, and most are based on chromogenic reactions to distinguish the target colony from background flora. Taeoinsa et al. (1998) concluded that Rainbow™ and a modified colitoxin/ histidine blue agar gave recovery from heat-stressed E. coli O157 in ground beef. The present study found Rainbow™ to be superior to alternative chromogenic agars (having less background flora) and indeed showed better recovery of target cells compared with CTSMAC. CHROMagar™ O157 was equal in performance to CTSMAC. In addition to the discrimination of E. coli O157 from other enterics and food-associated microflora, CHROMagar™ O157 can be used to identify serotypes O111 and O103 (Bettelheim 1998a), while Rainbow™ can distinguish serotypes O111:H8 and O26:H11 from E. coli O157 (Bettelheim 1998b). Quality Life Sciences & E. coli O157 medium (SE-39) gave poor results in this study (incubated at 42°C, with a few numbers of sorbitol-positive strains giving positive identification of E. coli O157. However, it must be stressed that the manufacturers recommend incubation at 44°C, a temperature which is not recommended as a food safety measure in this work. Some workers (Dale and Schoeni 1994) have reported partial or complete inhibition of E. coli O157 at this elevated temperature. Based on the results of this study, the present method based on E. coli O157 as the only target organism to distribute IMS beads equally between CTSMAC and a chromogenic agar. The current economical choice is CHROMagar™ O157. Throughout this study using spiked cocktails, it was not possible to identify the individual component strains recovered after enrichment and plating, and the possibility that the data may not be optimal for all strains used.

Two foods naturally contaminated with E. coli O157 were available in sufficient quantities for replicate testing during the course of this work. The levels of E. coli O157 in these two foods (as enumerated by MPN) were found to be low at <10^2 MPN, but their physiological state was unknown. Although ground beef and cheese were supplied by the retailer at fresh produce, their history was unclear. For example, the beef may have been frozen prior to sale and the cheese could have been stored for an extensive period (several months) before purchase, both of which could result in sub-lethally-damaged target bacteria. Results showing such differences between enrichments suggest that expressed E. coli O157 could have been present in all samples. The use of optimum IMS enrichment media as described has enabled this laboratory to isolate and confirm the presence of low numbers of sub-lethally-damaged E. coli O157 from a number of foods. In the majority of tests, there was a high background level of micro-organisms which did not interfere with recognition of the target bacteria. A protocol is proposed for enriching in RPMI-V at 42°C for 4 h, and plating immunobeads on CTSMAC and CHROMagar™ O157, to improve E. coli O157 isolation from foods.

ACKNOWLEDGEMENTS

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