Increased Sensitivity of the Rapid Hydrophobic Grid Membrane Filter Enzyme-Labeled Antibody Procedure for Escherichia coli O157 Detection in Foods and Bovine Feces

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Several strains of Escherichia coli O157:H7 artificially inoculated into vegetables and dairy products were recovered on hydrophobic grid membrane filters and enumerated by an enzyme-labeled antibody assay. The mean of the recoveries from 12 fresh vegetables was 108.8%, whereas that from 10 dairy products was 93.2%. Modified tryptic soy broth at 43°C with shaking at 100 rpm provided optimum recovery of the organism from meat, with a sensitivity of ≤1 CFU/g, which is 10 times more sensitive than direct plating. The method performed equally well with vegetable and dairy products. Tryptic soy broth, however, under the same conditions gave the best results for fecal samples. Of 22 symptomatic dairy cattle, reported as having positive Brucella titers when assayed with polyclonal antibodies, eight were found to contain E. coli O157 in their feces as demonstrated by the enzyme-labeled antibody assay by using monoclonal antibodies. This finding may explain some of the false-positive Brucella tests.

Escherichia coli O157:H7 has been implicated in foodborne outbreaks of hemorrhagic colitis throughout North America and in Europe (3, 11, 12, 15). Meat is the most common vector, and methods for isolating and identifying the organism from a meat product have been reported (17, 18). Recent outbreaks, however, have implicated vegetables and dairy products (7, 8). We therefore decided to examine the utility of our rapid hydrophobic grid membrane filter enzyme-labeled antibody (HGMF-ELA) method (18) for enumerating the organism in these foods. The procedure involves filtration of 1 ml of food homogenate through an HGMF and, after incubation, application of specific enzyme-labeled antibody; the antibody attaches to E. coli O157 cells, and with the addition of substrate and color development solution, a visible product is formed. Since hemorrhagic E. coli (O157:H7) is usually present in small numbers and the limit of detection of the HGMF-ELA method is 10 organisms per g, we investigated enrichment as a means to enhance recoverability and thereby lower the limit of detection of the E. coli organisms.

The monoclonal antibody does not react with Brucella spp. (18), whereas E. coli O157 polyclonal antibodies do cross-react with Brucella antigens (15). This might explain a number of false-positive tests that can occur during screening of dairy cattle for Brucella spp., and we resolved to test this hypothesis by examining stool specimens of cattle showing false-positive Brucella reactions.

MATERIALS AND METHODS

Bacterial strains. Seven strains of E. coli O157 from sources listed previously (18) were used for enumeration and recovery studies. Five of these, 760, H16, 059, 1122, and 19386, were the H7 serovar from different sources, and the other two strains, 3344 and 3199, were nonmotile; all produced verotoxin II or verotoxins I and II. Stock cultures were maintained on tryptic soy agar (TSA) (Difco Laboratories, Detroit, Mich.) at room temperature. Inocula for growth experiments consisted of stationary-phase cells, obtained by inoculating tryptic soy broth (TSB) (Difco Laboratories) with cells from the TSA slants and incubating them overnight at 35°C.

Naturally contaminated beef samples were obtained from meat counters of local retail grocers.

Monoclonal antibodies. Monoclonal antibodies to E. coli O157 and ascitic fluid were prepared by the method of Perry et al. (9).

Staining. The horseradish peroxidase-protein A-antibody complex ELA stain was used as described by Todd et al. (18).

Prefiltration of food samples. Disposable pipette-tip prefilters were tested for their effect on bacterial recovery from all the foods. The sterile foam filters are available from Richard Bracker Research Ltd., Ottawa, Canada, in bags of 25. The effect of prefiltration on the recovery of E. coli was tested by using a matched-pair t test to compare prefiltered and unprefiltered samples artificially contaminated with E. coli O157:H7.

Statistical evaluation of enrichment conditions. Numerical values for the bacteria recovered under different conditions were converted to log_{10} values. However, owing to nonnormality of these data, nonparametric rank statistics by the method of distribution-free multiple comparisons based on Friedman rank sums (4) were carried out for data from pure-culture studies, artificially inoculated meat, and naturally contaminated meat (see Fig. 1). For this method, the seven strains of E. coli O157 in the pure culture, the artificially contaminated meat, and the four naturally contaminated meat samples were treated as replicates.

Food samples and bovine fecal specimens. For recovery and enumeration of E. coli O157 from foods, the following products were examined: fresh vegetables (beets, broccoli, cabbage, carrots, green beans, lettuce, mushrooms, onions, potatoes, potato salad, spinach, and yellow beans), dairy...
products (butter, cheese [blue Danish, Cheddar, cottage, cream, curds, mozzarella, processed slices] margarine, and raw milk); and regular ground beef. For purposes of data comparison, margarine is included with the dairy foods. The raw milk was obtained from Agriculture Canada; all other products were purchased from local retailers.

For surveys of meat, matched samples of neck muscle and feces from calves slaughtered at slaughterhouses in Canada and raw milk and feces from dairy cattle having a false-positive Brucella titer were supplied by Agriculture Canada. Thirteen samples of beef implicated in an outbreak of hemorrhagic colitis were obtained from the Western Region of the Health Protection Branch and stored at −18°C.

**Recovery of E. coli O157 strains added to vegetables and dairy foods.** A 10-g sample of each vegetable and dairy food was blended separately in 90 ml of peptone water as described for meat (5). Then, 100 to 200 CFU of stationary-phase E. coli O157 organisms was added per ml of homogenate. Each food was tested twice for each of the seven strains.

Controls consisting of 100 ml of peptone water were treated in the same way as the food samples to give the inoculum count. For all samples, aliquots (1 ml) were applied to HGMFs, incubated on HC agar, stained by the ELA assay, and counted as reported previously for meat (18). Counts were compared with the known levels of inoculum to give the relative recoveries. Aliquots of the food homogenates recovered from E. coli O157.

**Isolation of E. coli O157 strains from food and feces after enrichment.** For both artificially inoculated and naturally contaminated samples, 10 g of meat or feces was suspended in 90 ml of TSB or modified TSB (mTSB) and incubated for 16 to 20 h at 43 or 35°C with or without shaking. Modified TSB consists of (per liter) TSB (30 g), bile salts 3 (1.5 g) (Difco Laboratories), dipotassium phosphate (1.5 g), and novobiocin (20 mg) (Sigma Chemical Co., St. Louis, Mo.) (2). For artificially inoculated meats, between 10 and 30 stationary-phase E. coli O157 cells were added to the broths before enrichment, each broth being tested separately. Controls were run under identical conditions to the test broths, one set containing inoculated broths without meat and the other containing meat but no inoculum. For enrichment of vegetables and dairy products, approximately 30 E. coli O157 cells were added to 90 ml of TSB or mTSB containing 10 g of the food, which was then blended. After overnight incubation at 43°C with shaking, 1-ml aliquots of dilutions were filtered through HGMFs, incubated overnight, and then stained as described above.

**Verotoxin testing.** E. coli O157 strains were tested for verotoxin by using Vero cells as described by Konовалchuk et al. (6).

**Replication of organisms on HGMFs.** When required (for mushrooms and potato salad [Table 1] and for cream cheese [Table 2]), replication of HGMF growth units before staining was carried out by using the RP-10 Replicator (Richard Brancker Research Ltd.) (13).

### RESULTS AND DISCUSSION

**Utility of prefiltration.** Prefiltration of fibrous or clumped food suspensions was desirable to prevent bridging (joining of adjacent HGMF grid cells across the hydrophobic barrier by food particles). No statistically significant difference (P = 0.45 based on 87 pairs of observations) was found when a matched-pair t test was used to compare numbers of bacteria recovered from prefiltered and unprefiltered samples.

<table>
<thead>
<tr>
<th>Food</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beets</td>
<td>103.4</td>
</tr>
<tr>
<td>Green beans</td>
<td>110.5</td>
</tr>
<tr>
<td>Yellow beans</td>
<td>109.2</td>
</tr>
<tr>
<td>Broccoli</td>
<td>109.4</td>
</tr>
<tr>
<td>Cabbage</td>
<td>124.8</td>
</tr>
<tr>
<td>Carrots</td>
<td>100.2</td>
</tr>
<tr>
<td>Lettuce</td>
<td>107.7</td>
</tr>
<tr>
<td>Mushrooms</td>
<td>52.5 (121.5)</td>
</tr>
<tr>
<td>Onion</td>
<td>115.3</td>
</tr>
<tr>
<td>Raw potato</td>
<td>115.7</td>
</tr>
<tr>
<td>Potato salad</td>
<td>80.4 (95.7)</td>
</tr>
<tr>
<td>Spinach</td>
<td>99.7</td>
</tr>
</tbody>
</table>

* Average recovery for seven strains tested twice each, inoculated separately into each food, and suspensions prefilted before filtering through HGMFs. The overall average recovery was 109.4%, including recoveries of replicates where applicable.

**Recovery after replicate plating from the original HGMF to another one.**

Although the disposable foam prefilters are relatively coarse (passage size, about 150 μm) and are unlikely to trap bacteria, they may have the opposite effect, concentrating suspended bacteria in the filtrate by reducing fluid volumes by the amount occupied by removable solids. Bacterial recoveries for foods yielding coarse debris tend to approach 110%, whereas recoveries for foods yielding finer debris, which tend to pass the prefiler, are ≤100% (Tables 1 and 2).

For some foods, such as milk, prefiltration may not be necessary. However, for consistency in these experiments, all food analyses included prefiltration. The prefilters are inexpensive and are easily fitted onto or removed from pipette tips.

**Recovery of E. coli O157 from vegetables.** Direct filtration of artificially contaminated vegetables through HGMFs with subsequent detection of E. coli O157 by the ELA procedure performed on overnight growth yielded an overall recovery of 108.8% of the organisms added to the foods (Table 1). Generally, recovery from vegetables posed no problem for the methodology used. The one striking exception occurred when mushrooms were analyzed (52.5% recovery), because a clouded residue on the HGMFs did not allow mushrooms to mask some ELA-positive grid cells. Although E. coli O157 was readily recoverable from raw potato (115.7%), recoveries dropped to 80.4% when potato salad was tested. Close scrutiny revealed that the ELA reaction itself, rather
than the growth of the organisms, was being inhibited. The source of this inhibition was not determined. To overcome masking and inhibition of the ELA reaction, it was necessary to replicate overnight HGMF growth from original direct platings to secondary HGMFs. After incubation of the secondary HGMFs, the ELA reactions performed on them showed >95% recovery, indicating that viable cells previously unstainable by the ELA procedure were transferred to the secondary HGMFs where they could be stained.

**Recovery of E. coli O157 from dairy products.** E. coli was recovered satisfactorily from all foods in the dairy category except for cream cheese and processed cheese. The intensity of the ELA reaction was greatly reduced in the cream cheese samples, resulting in lower apparent recoverability (82.5%). Replication onto secondary HGMFs for the ELA reaction, as described above for mushrooms and potato salad, allowed more intense staining.

Processed cheese gave the lowest recoveries (26.8%), a consequence of the very poor filterability of the homogenate; these results corroborate the data of Sharpe et al. (14). Filtration may be improved, however, by digestion with pronase (Sigma) or more economically with trypsin-Tween 80 as reported by Peterkin et al. (10).

**Effect of enrichment conditions on bacterial recoveries.** The results indicate that the HGMF-ELA procedure has utility not only with meats, but also with vegetable and dairy products. Unfortunately, the lower limit of detectability by the direct procedure is 10 organisms per g of food. Owing to suspected low levels of E. coli O157 naturally occurring in foods, especially meats (18), it was considered appropriate to investigate the use of an enrichment procedure to amplify very low E. coli O157 levels. TSB and mTSB (2) were compared at 43 and 35°C for their ability to enrich E. coli O157 in meat. The results for the pure culture, artificially contaminated meat, and naturally contaminated meat are shown in Fig. 1.

In pure culture, E. coli O157 is best recovered in TSB at 35°C (shaking or stationary) or in TSB or mTSB at 43°C (shaking only) (Fig. 1a). Although ranking of the treatments showed that mTSB at 43°C (stationary only) had the lowest recovery, statistical analysis showed that all other mTSB methods were roughly equivalent and, together with TSB at 43°C (stationary only), were the least preferred methods.

Figure 1b shows recoveries of E. coli O157 from artificially contaminated meat held under the same conditions as the pure culture. Shaking of samples in mTSB and to a lesser extent in TSB at 43°C gave highest recoveries; mTSB at 35°C with or without shaking was least suitable for recovery.

Figure 1c shows that mTSB at 35°C without shaking was inhibitory for the recovery of E. coli O157 from naturally contaminated ground beef. However, mTSB at 43°C with shaking gave the highest recoveries of E. coli O157.

These results suggest that the organism is a poor competitor. Although it grows most quickly in TSB when in pure broth culture at 35°C, introduction of food samples with inherent competitive flora could limit E. coli O157 growth. The effect of bile salts and novobiocin present in mTSB combined with the elevated incubation temperature (43°C) may provide a necessary advantage for growth of the E. coli O157.

For recovery of E. coli O157 from mushrooms, potato salad, butter, margarine, processed cheese, and cream cheese, both TSB and mTSB gave comparable results at 43°C with shaking. When fewer than 30 E. coli O157 cells were inoculated into 100 ml of a 10^{-1} dilution of these foods in either broth, growth to 10^6 to 10^8 was observed, equal to or surpassing that seen with meat. The ELA staining procedure performed on HGMFs of 10^{-2} and 10^{-6} dilutions of the enrichment broths yielded color reactions which were as clear and intense as pure culture controls. Enrichment, then, provides an alternate solution to the problem of inhibited ELA staining when assaying mushrooms, potato salad, butter, margarine, and cream cheese and effectively circumvents the poor filterability issue inherent in processed cheese analyses.

**Enrichment of naturally contaminated meat.** The enrichment method used in all further meat testing was mTSB broth with shaking at 43°C. In Table 3, results are given for direct and enriched detection of E. coli O157 in beef associated with a food poisoning incident. These samples had been analyzed by the direct HGMF-ELA procedure in January 1988 (18) and frozen until a comparison of direct and enriched procedures could be carried out in September 1988. Some die-off of cells occurred in the 9-month storage period, and the advantage of using enrichment for recovery of small numbers is obvious.

**Survey of veal meat and stools.** Analysis of veal neck muscle samples and corresponding fecal specimens from 300 carcasses revealed that six of the veal samples and five of the fecal samples contained E. coli O157. However, in none of the tests was an animal positive for both veal and stool, indicating that 11 of the 300 carcasses (3.7%) harbored E. coli O157 and that 6 of these were probably contaminated with feces from other animals. These isolates were detectable only after the samples were enriched; direct plating produced negative results. All E. coli O157-positive isolates were H7 negative.

**Enrichment of bovine stool specimens.** We compared TSB and mTSB at 43°C with shaking for 86 stool specimens. Five TSB specimens contained E. coli O157, whereas no positive results were obtained with mTSB. Therefore, although mTSB is preferred for food, TSB seems to be more effective for fecal specimens and was used in all analyses of stools.
TABLE 3. Isolation of E. coli O157:H7 from frozen meat samples implicated in an outbreak of hemorrhagic colitis by direct count and enrichment

<table>
<thead>
<tr>
<th>Sample</th>
<th>Direct count/g* in:</th>
<th>Enrichment* +ELA( )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Jan 1988</td>
<td>Sept 1988</td>
</tr>
<tr>
<td>Ground beef (no. 1)</td>
<td>6.2 x 10^3</td>
<td>6.2 x 10^3</td>
</tr>
<tr>
<td>Ground beef (no. 2)</td>
<td>5.1 x 10^3</td>
<td>2.4 x 10^3</td>
</tr>
<tr>
<td>Stewing beef (no. 1)</td>
<td>1.0 x 10^2</td>
<td>ND</td>
</tr>
<tr>
<td>Stewing beef (no. 2)</td>
<td>1.1 x 10^3</td>
<td>ND</td>
</tr>
<tr>
<td>Stewing beef (no. 3)</td>
<td>6.4 x 10^2</td>
<td>ND</td>
</tr>
<tr>
<td>Stewing beef (no. 4)</td>
<td>2.2 x 10^2</td>
<td>ND</td>
</tr>
<tr>
<td>Roast</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Flank steak</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Tenderloin</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Cutlet (no. 1)</td>
<td>1.1 x 10^2</td>
<td>ND</td>
</tr>
<tr>
<td>Cutlet (no. 2)</td>
<td>2.5 x 10^3</td>
<td>1.1 x 10^3</td>
</tr>
<tr>
<td>Rib steak (no. 1)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Rib steak (no. 2)</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Isolates confirmed as O157:H7 with H7 antisera.
* Lower limit of detection, 10^3.
* Tested September 1988, after meat had been frozen for 9 months.
* Tested January 1988, as reported by Todd et al. (18).
/ ND. Not detected.

Perhaps the use of mTSB for these samples created too toxic an environment for E. coli O157. An increased concentration of bile salts from both mTSB and the added fecal material may have suppressed growth, as previously suggested (17).

E. coli O157 as a cause of false-positive Brucella reactions

In serological testing for Brucella spp. in cattle, false-positive results are sometimes encountered where no Brucella spp. are isolated (16). Caroff et al. (1) earlier reported the cross-reactivity of E. coli O157 antigens with Brucella abortus antibodies. Therefore, stools and milk from some dairy cattle which showed false-positive test results in an Agriculture Canada Brucella screening program were analyzed in our laboratory for E. coli O157 by using the monoclonal antibody-ELA assay on TSB-enriched samples. None of the milk samples had demonstrable E. coli O157, but feces from 8 of 22 of these animals (36.4%) showed E. coli O157 contamination.

Conclusion

In conclusion, the rapid HGMF-ELA procedure is useful in quantifying E. coli O157 in vegetables and dairy products. Used as a screening method, it at least demonstrates the absence of E. coli O157 at the level of 10 to 20 cells per g of vegetable or dairy product within 1 day. The exception is processed cheese, which is nonfilterable.

For a qualitative indication of E. coli O157 in meat, enrichment in mTSB at 43°C with agitation at 100 rpm gave the highest recoveries, albeit not significantly different from the other treatments except mTSB at 35°C. The limit of sensitivity of the enrichment procedure (≤ 1 CFU/g) is an improvement over that for the direct count (10 CFU/g [14]). This is important since samples containing <10 CFU/g have been implicated epidemiologically in food-borne outbreaks of hemorrhagic colitis (Table 3). The enrichment procedure is also effective in supporting rapid growth of E. coli O157 in vegetables and dairy foods. Enrichment of bovine stool specimens is better achieved in TSB than in mTSB when incubated at 43°C with shaking. When the method was used in a survey of veal carcasses (meat and stool samples), the contamination rate was 3.7%. In an examination of fecal samples from false-positive Brucella cattle the rate of contamination by E. coli O157 was almost 10 times greater (36.4%), indicating that many of these reactions may be caused by the presence of E. coli O157.

ACKNOWLEDGMENT

We appreciate the assistance of J. Speirs, Bureau of Microbial Hazards, for verotoxin testing of E. coli O157 isolates.

LITERATURE CITED