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Rapid Hydrophobic Grid Membrane Filter–Enzyme-Labeled Antibody Procedure for Identification and Enumeration of *Escherichia coli* O157 in Foods†

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An O-antigen-specific monoclonal antibody, labeled by horseradish peroxidase-protein A, was used in a hydrophobic grid membrane filter–enzyme-labeled antibody method for rapid detection of *Escherichia coli* O157 in foods. The method yielded presumptive identification within 24 h and recovered, on average, 95% of *E. coli* O157:H7 artificially inoculated into comminuted beef, veal, pork, chicken giblets, and chicken carcass washings. In food samples from two outbreaks involving *E. coli* O157:H7, the organism was isolated at levels of up to 10^3/g. The lower limit of sensitivity was 10 *E. coli* O157 per g of meat. Specific typing for *E. coli* O157:H7 can be achieved through staining with labeled H7 antiserum or tube agglutination.

Since 1982, *Escherichia coli* O157:H7 has been responsible for several outbreaks of hemorrhagic colitis in the United States, Canada, the United Kingdom, and Belgium (10, 17, 18, 20; B. Rowe, H. R. Smith, S. M. Scotland, and R. J. Gross, Abstr. Int. Symp. Workshop Verocytotoxin-Producing Infect., abstr. no. CEP-1, 1987). A more serious syndrome of hemolytic uremia has affected some of the cases, mainly in children (11, 13), and deaths have occurred particularly in nursing home outbreaks (5, 16).

Unfortunately, the organism is not easily isolated from food because it is usually present in low numbers and is accompanied by competitive microflora, including other strains of *E. coli*. Isolation and detection methods first used for *E. coli* O157:H7 depended upon plating of foods on selective *E. coli* media modified to allow the O157 strains to grow (incubation temperature of <45°C or lower bile salts concentration) and to demonstrate their typical biochemical reactions (inability to ferment sorbitol, lack of fluorescence in the presence of 4-methylumbelliferyl-β-D-glucuronide [MUG], and production of indole). Such media include sorbitol-MacConkey agar (9) and HC medium (21). Confirmation of isolates was made with O157 and H7 antisera. Other approaches have concentrated on verocytotoxins produced by this and other pathogenic *E. coli* serotypes, e.g., an immunoblot technique described by Doyle and Schoeni (8) and a DNA probe prepared by Levine et al. (12) for the detection of plasmids that mediate epithelial cell attachment of verocytotoxin-producing strains. Although the immunoblot and DNA probe methods are more sensitive than the direct plating techniques, they are labor intensive, require noncommercial reagents, and need several days before yielding results. Development of a highly specific monoclonal antibody (MAb) that binds the somatic antigen (M. B. Perry, D. R. Bundle, M. A. J. Gidney, and H. Lior, submitted for publication) offered the possibility of providing a rapid yet sensitive *E. coli* O157:H7 detection method. This MAb was developed following chemical studies of the structural interrelationships of the somatic antigens of *E. coli* O157 (15) and other gram-negative organisms with related O antigens.

The value of the hydrophobic grid membrane filter (HGMF) had already been demonstrated for the isolation of *E. coli* from food by the work of Doyle and Schoeni (8) for the detection of verocytotoxin-positive strains and by the work of Szabo et al. (21) for the enumeration of *E. coli* O157:H7. In addition, Cerquiera-Campos et al. (6) had developed a convenient enzyme-labeled antibody (ELA) staining procedure for salmonellae which was based on the HGMF. Therefore, we developed experiments to show how effective HGMFs were for isolation and enumeration of *E. coli* O157 from food with specific identification by MAbs.

MATERIALS AND METHODS

Bacterial strains. *E. coli* O157:H7 and other *E. coli* strains (Table 1) included in this study were obtained from H. Lior, Laboratory Centre for Disease Control, Health Protection Branch, Ottawa, Ontario, Canada; A. Borczyk, Reference Bacteriology Laboratories, Ontario Ministry of Health, Toronto, Ontario, Canada; and G. K. Morris and N. Strockbine, Enteric Bacteriology Section, Enteric Diseases Branch, Division of Bacterial Diseases, Centers for Disease Control, Atlanta, Ga. Other bacteria listed in Table 1 were selected from our own collection. Strains were maintained at room temperature on slants of Trypticase soy agar (Difco Laboratories, Detroit, Mich.). Inocula for growth experiments consisted of stationary cells. These were obtained by inoculating brain heart infusion broth (Difco Laboratories) with cells from the slants and incubating them overnight at 35°C.

MAb. Female BALB/c mice (Charles Rivers Canada Inc., St. Constant, Quebec, Canada) were immunized with *E. coli* O157:H7 (LCDC 82-1933; NRCC 4125) phenol-killed cells (Perry et al., submitted). Spleen cells from two immunized mice were fused with the Sp2/O plasmacytoma cell line, and the resultant hybridomas were selected on the basis of an indirect enzyme-linked immunosorbent assay using purified lipopolysaccharide from *E. coli* O157:H7 (15). The speci-

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TABLE 1. ELA cross-reactions with O157 MAb

Organism and strain, phenotype, or description	No. of strains tested	Growth on HC medium	% of strains showing positive ELA reaction
Escherichia coli			
O157:H7	55	+	100
O157:H16	3	+	100
O157:H45	3	+	100
O157:H$^-$	2	+	100
O26	2	+	0
O138	1	+	0
O126	2	+	0
O128	1	+	0
O68	1	+	0
O111	1	+	0
Not O157:H7	13	+	0
MUG$^-$ Sor$^-$ Ind$^+$ (not O157:H7)[a]	4	+	0
MUG$^+$ (weak) Sor$^+$ (slow) Ind$^+$(not O157:H7)[a]	29	+	0
Escherichia hermannii	1	+	0
Proteus spp.	6	+	0
Citrobacter spp.	10	+	0
Enterobacter spp.	9	+	0
Pseudomonas spp.	4	+	0
Achromobacter spp.	1	−	NA[b]
Bordetella spp.	1	−	NA
Acinetobacter spp.	1	−	NA
Klebsiella spp.	1	+	0
Serratia spp.	2	+	0
Shigella spp.	2	+	0
Providencia spp.	1	+	0
Salmonella spp.			
Groups C,D,F	21	+	0
Group N	3	+	100
Yersinia enterocolitica 0:9	1	−	NA
Hafnea alvei	2	+	0
Staphylococcus aureus	12	+	0

[a] These strains were false-presumptives by direct plating on HC medium (21).
[b] NA, Not applicable.

ficity has been tested against *E. coli* O7, O116, and O157 and *Escherichia hermannii* (Perry et al., submitted).

Ascitic fluid. BALB/c mice were primed by intraperitoneal injection of 0.5 ml of 2,6,10,14-tetramethylpentadecane (Pristane [Sigma Chemical Co., St. Louis, Mo.]). One to two weeks later, they were injected with 10^6 hybridoma cells. After 8 to 10 days, ascitic fluid was tapped and stored at −20°C. Until it is available commercially from the Armand Frappier Institute, Montreal, Quebec, the ascitic fluid can be requested from G. Adams, Division of Biological Sciences, National Research Council, Ottawa, Ontario, Canada.

Foods. Ground beef, veal, pork chops, chicken giblets, and chicken carcass washings were used in recovery experiments; the foods were obtained from local retailers.

Pure culture tests. Stationary-phase cells were diluted to approximately 10 cells per ml in peptone water. Various volumes (usually 10 ml, but occasionally 100 ml) were filtered through HGMFs (ISO-GRID; QA Laboratories Ltd., Toronto, Ontario, Canada) which were incubated for 16 to 20 h at 43°C on HC medium (21); MUG, which was required in the original HC medium formulation, is not essential for this isolation method.

Recovery of *E. coli* O157:H7 strains. To 10 g of meat were added 90 ml of peptone water and 10^3 to 10^4 stationary-phase *E. coli* O157:H7 cells. The mixture was blended for 2 min, either in a Mason jar with an Osterizer blender at high speed or by a Stomacher 400 (Canadian Laboratory Supplies Ltd., Pointe Claire, Quebec, Canada). Aliquots (1 ml) were pipetted through disposable 100-μm-pore-size prefilters (19) (Richard Brancker Research Ltd., Ottawa, Ontario, Canada), dispensed in 10 ml of peptone water, and filtered through HGMFs by using the MF-10 Spreadfilter (Richard Brancker Research Ltd.). The membranes were incubated at 43°C for 16 to 20 h on HC agar (21). General principles of filtering were described previously by Sharpe and Peterkin (19), and detailed procedures can be found in the Health Protection Branch procedure MFHPB-26, available from Evaluation Division, Bureau of Microbial Hazards, Health Protection Branch, Tunney's Pasture, Ottawa, Ontario, Canada.

Replication of organisms on HGMFs. Since the ELA staining procedure kills most bacterial cells, each primary membrane was copied before staining by using an HGMF Replicator (Richard Brancker Research Ltd.). With this instrument, only seconds are needed to transfer inocula in correct register from incubated HGMFs to the 1,600 grid cells of secondary HGMFs on HC medium.

Preparation of HRP-protein A-antibody complexes. For each HGMF requiring staining, 10 μl of ascitic fluid was dispensed in 1 ml of Tris-buffered saline (TBS; 20 mM Tris–500 mM NaCl [pH 7.5]) containing 1% gelatin and mixed with 3 μl of horseradish peroxidase (HRP)-protein A conjugate (Bio-Rad Laboratories, Richmond, Calif.). The solution was stirred for 1 h at 4°C to give the HRP-protein A-MAb complex. For ELA staining, this complex was diluted by adding 10 ml of TBS (pH 7.5) with 1% gelatin for each 10 μl of ascitic fluid present. This gave a final dilution of ascitic fluid of 1:1,000. For the discrimination of group N *Salmonella* spp., 0.1 ml of Spicer-Edwards EN antiserum complex (Difco) was stirred with 0.07 ml of HRP-protein A conjugate in 1 ml of 1% gelatin-TBS for 1 h at 4°C and then increased to 40 ml with TBS. This is a modification of the procedure described by Cerquiera-Campos et al. (6).

ELA stain on HGMF. After incubation overnight at 43°C, inoculated HGMFs were immersed in distilled water for 10 min with gentle agitation (60 rpm) to remove visible colony growth. The washed HGMFs were then transferred to a blocking solution (3% gelatin-TBS) for 30 min. After blocking, the HGMFs were immersed in the HRP-protein A-MAb complex for 60 min with constant shaking (60 rpm). The HGMFs were dipped into distilled water to remove excess antibody, rinsed two times (7 min each) in a solution of TBS containing 0.05% Tween 20, immersed in HRP color development solution (60 mg of 4-chloro-1-naphthol [Sigma]) which had been dissolved in 20 ml of ice-cold methanol, and added to 100 ml of TBS (pH 7.5) containing 60 μl of 30% H_2O_2 (6) for 15 to 30 min. Finally, the HGMFs were rinsed in distilled water. ELA-positive colonies were visible as purple dots. These were counted by eye or automatically by the MI-100 HGMF Interpreter (Richard Brancker Research Ltd.).

RESULTS AND DISCUSSION

Specificity of the MAb. Pure cultures of *E. coli* O157:H7 (55 strains), O157:H$^-$ (2 strains), and O157:H16 and O157-H45 (3 strains each) and three group N *Salmonella* strains reacted similarly to HRP-conjugated MAb, as evinced by the appearance of purple dots in HGMF grids (intense black dots on HGMFs in Fig. 1B). None of the other strains of organisms tested gave this reaction. In particular, organisms such as *E. hermannii* and *E. coli* MUG$^-$ Sor$^-$ Ind$^+$ (Table 1)

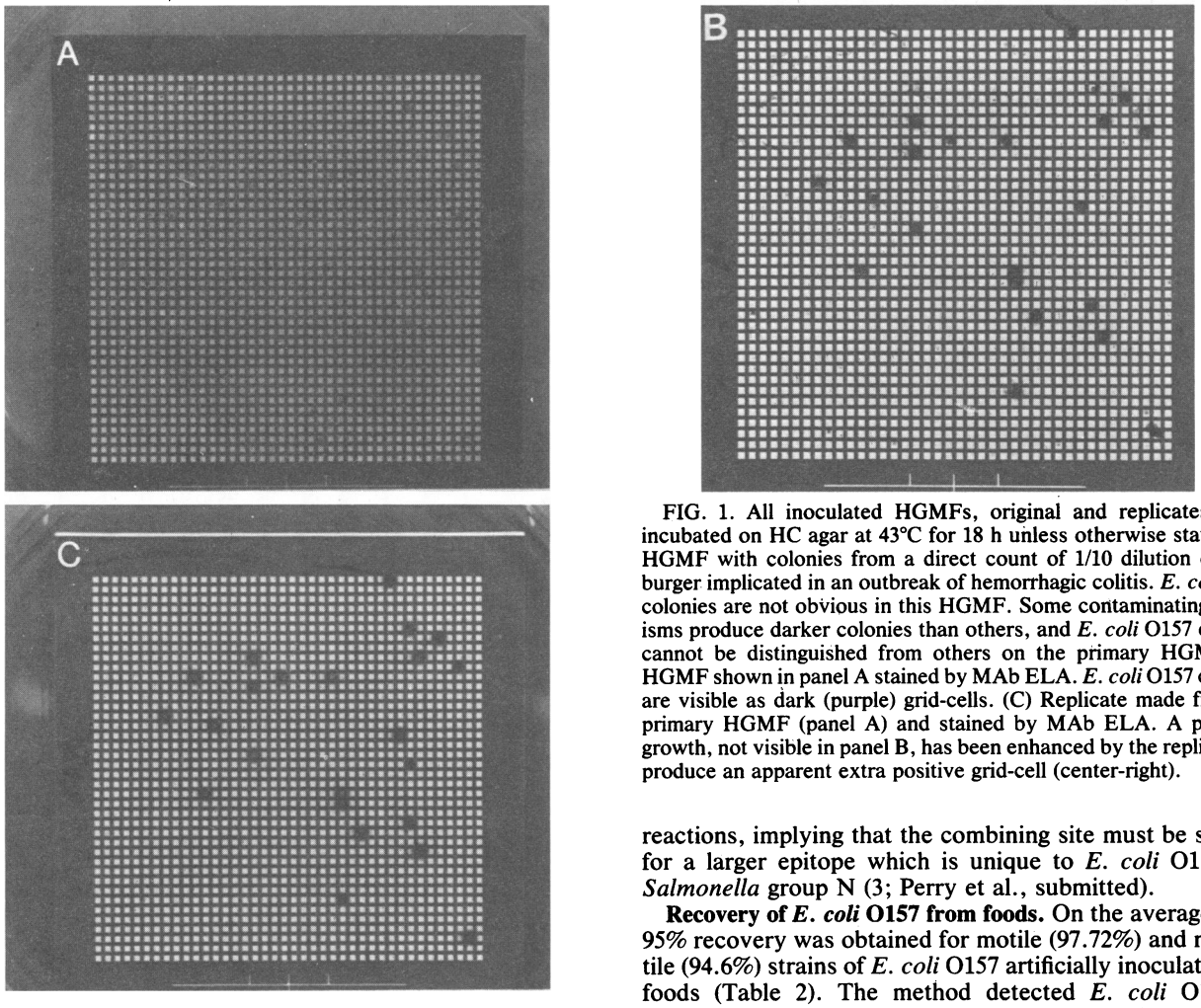

FIG. 1. All inoculated HGMFs, original and replicates, were incubated on HC agar at 43°C for 18 h unless otherwise stated. (A) HGMF with colonies from a direct count of 1/10 dilution of hamburger implicated in an outbreak of hemorrhagic colitis. *E. coli* O157 colonies are not obvious in this HGMF. Some contaminating organisms produce darker colonies than others, and *E. coli* O157 colonies cannot be distinguished from others on the primary HGMF. (B) HGMF shown in panel A stained by MAb ELA. *E. coli* O157 colonies are visible as dark (purple) grid-cells. (C) Replicate made from the primary HGMF (panel A) and stained by MAb ELA. A pin-point growth, not visible in panel B, has been enhanced by the replicator to produce an apparent extra positive grid-cell (center-right).

which yielded false-positives in other identification schemes (1, 21) did not react. These results confirm the specificity of the monoclonal (4) in comparison to the nonspecific O157 polyclonal antiserum which cross-reacts with *Brucella abortus*, *Brucella melitensis*, *Yersinia enterocolitica* serogroup 0:9, *Salmonella* group N, and *Pseudomonas maltophilia* 555 (2–4, 7, 14). The common epitope responsible for these cross-reactions is the rare sugar 4-amino-4,6-dideoxy-D-mannose present in the cell wall lipopolysaccharide (14). The MAb described here does not exhibit these cross-reactions, implying that the combining site must be specific for a larger epitope which is unique to *E. coli* O157 and *Salmonella* group N (3; Perry et al., submitted).

Recovery of *E. coli* O157 from foods. On the average, over 95% recovery was obtained for motile (97.72%) and nonmotile (94.6%) strains of *E. coli* O157 artificially inoculated into foods (Table 2). The method detected *E. coli* O157:H7 organisms satisfactorily even when the HGMFs were saturated with competing microorganisms because relatively few cells need be present on the membrane to produce a visible reaction. However, since the ELA reaction kills bacterial cells, HGMFs should be replicated before staining. Further identification of H7 or other serotypes of O157 can be done directly on replicate filters as described below or from picks taken from the HGMFs. The efficacy of the replication procedure is shown in Fig. 1.

Testing of method with food-borne illness samples. The ELA method was tested on meat implicated in two outbreaks of human illness. In outbreak no. 1, in June 1987, 15

TABLE 2. Percent recovery from foods artificially inoculated with *E. coli* O157 strains[a]

Food	% Recovery[b]								
	Motile strains (H7)						Nonmotile strains (H⁻)		
	H16	059	760	1122	19386	Mean for motile strains	3199	3344	Mean for nonmotile strains
Beef	91	93	107	98	95	96.8	90	93	91.5
Veal	98	93	89	95	100	95.0	92	100	96.0
Chicken wash	97	94	96	106	99	98.4	98	86	92.0
Pork	85	125	81	124	80	99.0	88	98	93.0
Chicken giblets	110	75	100	92	120	99.4	101	100	100.5

[a] Recoveries are averages of 3 recoveries per strain per food.
[b] The total mean recovery was 97.7% for motile strains and 94.6% for nonmotile strains.

TABLE 3. Isolation and enumeration of *E. coli* O157 from food-borne disease in beef samples[a]

Outbreak no.	Sample	Count/g
1	Hamburger patties	1×10^2
2	Ground beef sample 1	6.2×10^3
	Ground beef sample 2	5.1×10^3
	Stew sample 1	1.0×10^1
	Stew sample 2	1.1×10^2
	Stew sample 3	6.4×10^2
	Stew sample 4	2.2×10^2
	Roast	ND[b]
	Flank steak	ND
	Tenderloin	ND
	Cutlet sample 1	1.1×10^2
	Cutlet sample 2	2.5×10^3
	Rib steak sample 1	ND
	Rib steak sample 1	ND

[a] All isolates were confirmed as O157:H7 by testing with H7 antiserum.
[b] ND, Not detected.

of 90 residents of a nursing home in Vegreville, Alberta, Canada, suffered from bloody diarrhea and severe cramps. *E. coli* was isolated from 10 of the 15 stool specimens. Two deaths subsequently occurred which may have been related to the infection. No staff were found to carry the organism, and the source was probably through direct consumption of contaminated meat. No meat served in early June remained for analysis, but some ground beef from the same shipment had been made into hamburger patties for future servings. These were recovered from frozen storage. For analysis of these patties, 10 g was blended in 90 ml of peptone water as described in Materials and Methods; 10^2 *E. coli* O157 per g was found in the meat analyzed (Table 3).

In outbreak no. 2, in November and December 1987, four members of a family in Burnaby, British Columbia, Canada, became ill from consuming baby beef obtained from one supplier. *E. coli* O157:H7 was subsequently recovered from their feces. The remaining 13 portions of meat were removed from the freezer and analyzed as described above. *E. coli* O157:H7 (confirmed with antisera) was found in 8 of the 13 samples at levels ranging from 1.0×10^1 to 6.2×10^3 per g (Table 3). Although other competitive organisms were in the meat, the main organism present was *E. coli* O157:H7. These analyses indicate that the ELA-HGMF method is capable of rapid isolation and enumeration of *E. coli* O157 at a level of 10/g in naturally contaminated meats.

Utility of the procedure. Used as a screening test, the procedure reliably demonstrates the absence of *E. coli* O157 at a level of 10 cells per g of food within one day. If a positive reaction is obtained, the presence of group N salmonellae cannot be excluded and a further overnight growth of the HGMF replicate, followed by a similar ELA procedure using labeled Spicer-Edwards EN complex antisera (6) to identify any group N *Salmonella* spp. present, should be done. Flagellum typing should be performed either from picks taken from the HGMF or directly on the replicate filter by using labeled N antisera in a HRP-protein A-antibody complex as described in the Materials and Methods section with anti-H7 or other H antisera substituting for the MAb. In our experience, however, Difco H7 antiserum is not strong enough to give a dark enough reaction, and it may be preferable, at this stage, to use a standard tube agglutination method.

The sensitivity of this HGMF method (10 *E. coli* O157 per g) is not as great as the more laborious research enrichment method of Doyle and Schoeni (1.5 *E. coli* O157:H7 per g [8]). However, the sensitivity may be improved by enriching cultures in Trypticase soy-novobiocin broth (8) before filtering through the HGMFs, if counts are not desired.

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