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An Improved Screening Method for the Detection and Isolation of *Escherichia coli* 0157:H7 From Meat, Incorporating the 3M Petrifilm™ Test Kit - HEC - for Hemorrhagic *Escherichia coli* 0157:H7

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ABSTRACT

A screening method was devised incorporating a commercially available reactive disc blot ELISA for *Escherichia coli* 0157 antigen, into a cultural screening program for the isolation of *E. coli* 0157:H7 from meat and poultry products. The method includes the inoculation of a raw or cooked meat sample into an enrichment broth, incubation with shaking at 37°C for 6 to 8 h, followed by inoculation of 3M Petrifilm™ E. coli Count plates with dilutions of the enrichment culture. The Petrifilm plates were incubated at 42°C for 18 h and tested for the presence of the 0157 antigen. The enrichment cultures were reincubated static at 35°C after the initial shaken incubation. Isolation was attempted from the positive Petrifilm plates by both a direct picking and streaking method and by the 3M Prompt™ isolation method. Isolation also was attempted from the 24-h enrichment cultures by spread plating serial dilutions on 150 x 15 mm MacConkey sorbitol agar (MSA) and MSA with 5-bromo-4-chloro-3-indoxyl-ß-D-glucuronic acid cyclohexylammonium salt (BCIG). This fast and efficient screening procedure identifies negative and presumptive positive samples in 26-28 h. Isolation and confirmation of the presumptive positive isolates require an additional 3 to 4 d.

In 1982, there were two outbreaks of hemorrhagic colitis that were associated with the consumption of hamburgers. The causative organism of these outbreaks proved to be *Escherichia coli* 0157:H7 (*12,16*). Since then, this organism has been involved in a number of other instances of foodborne hemorrhagic colitis cases (*6,11*) and thus has become important to both clinical and food microbiologists. Clinicians have successfully made use of the organism's inability to ferment sorbitol to differentiate it from other *E. coli* in stool samples (*4,5,7*). Food microbiologists must use slightly different methods in order to isolate freeze- or heat-injured *E. coli*. These bacteria may be present in very low numbers in foods that contain large numbers as well as a wide variety of other microorganisms, many of which are sorbitol negative (*8,9*).

Food microbiologists, like clinicians, have used biochemical reactions on plating media (*4,9,10,14*) for isolation. In addition, some have looked for colonies using the specific 0157 somatic antigen or verocytotoxin antigens. This search for these specific antigens has been accomplished by the use of hydrophobic grid membrane filter-immunoblot (*2*) and hydrophobic grid membrane filter-enzyme-labeled antibody (*15*) procedures. Recently, Dr. Richard Matner (3M Company, St. Paul, MN) developed a commercially available immunoblot assay kit which detects the 0157 antigen in colonies from a mixed culture, grown on 3M Petrifilm™ E. coli Count plates. This method uses a reactive disc blot ELISA technique. This method also permits the isolation of suspect colonies from the Petrifilm for confirmation testing.

We have combined this immunoblot assay with our USDA cultural method to produce a fast and efficient screening procedure which identifies both negative and presumptive positive samples in 26-28 h. Isolation and confirmation of the presumptive positives can take an additional 3 to 4 d.

MATERIALS AND METHODS

Preparation of inoculated meat samples

A culture of *E. coli* 0157:H7 was grown overnight in trypticase soy broth (TSB; BBL, Cockeysville, MD) and spun down at 15,000 RPM for 15 min in a Sorvall RC-5B centrifuge. The supernatant was decanted, and the packed cells resuspended in Butterfield's Phosphate Diluent (BPD; 13) to give 50% light transmittance at 400 nm in a Bausch and Lomb Spectronic 20. Decimal dilutions to 10^{-6} were made in BPD. Retail ground beef balls, 25 gm each, were placed into individual sterile plastic bags and stabbed with a sterile pipet to produce holes leading to the center of the ball. Each sample was inoculated in the hole with 0.1 ml of a dilution of *E. coli* 0157:H7, and the hole squeezed closed. The samples were frozen for 3 to 7 d before analysis. Plate counts done on the decimal dilutions of the inoculum showed that samples inoculated with 0.1 ml of the 10^{-6} dilution routinely received an inoculum of 0.6 to 0.7 organisms/g. All uninoculated control samples were designated "A", and all inoculated samples were designated "B".

Media preparation

Modified EC broth with novobiocin (mEC+n; 10). Tryptone (Difco, Detroit, MI) 20 g/L, bile salts #3 (Difco) 1.12 g/L, lactose 5.0 g/L, K_2HPO_4 4.0 g/L, KH_2PO_4 1.5 g/L, NaCl 5.0 g/L, distilled water 1 L. If necessary the pH was adjusted to 6.9±0.1 before

autoclaving at 121°C for 15 min. A filter sterilized aqueous solution of sodium novobiocin (potency 890 µg/mg; Sigma N1628, St. Louis, MO) was added to the cooled medium to produce a final concentration of 20 mg sodium novobiocin/L.

Phenol red sorbitol agar + 4-methylumbelliferyl-ß-D-glucuronide (PRS-MUG). Phenol red broth base (Difco) was prepared according to manufacturer's instructions. To this was added 2% agar, 0.5% D-sorbitol, and 0.005% MUG (Biosynth International, Skokie, IL). The medium was adjusted to pH 6.8-6.9 and sterilized by autoclaving at 121°C for 15 min, tempered, and poured into 100 x 15 mm petri dishes (40 ml/plate).

Eosin methylene blue agar (EMB). Levine's formula EMB (BBL) was prepared according to manufacturer's instructions with extra agar added to bring the final agar concentration to 3%. The medium was sterilized by autoclaving at 121°C for 15 min, tempered, and poured into 100 x 15 mm petri dishes (15 to 20 ml/plate).

MacConkey sorbitol agar with 5-bromo-4-chloro-3-indoxyl-ß-D-glucuronic acid cyclohexylammonium salt (MSA-BCIG; 9). MSA (Difco 0079-17-7) was prepared according to manufacturer's instructions. The BCIG (Biosynth International) was added in solution by dissolving 0.1 gm in 2.5 ml of 95% ethanol - 0.5 ml of 1 N NaOH for each liter of medium. The complete medium was autoclaved at 121°C for 15 min, tempered, and poured into both 150 x 15 mm and 100 x 15 mm petri dishes. MSA agar without BCIG was also prepared.

Cultures

All inoculated meat samples were prepared with an *E. coli* 0157:H7 culture received from J. Wells of the Centers for Disease Control, Atlanta, GA. Other *E. coli* 0157:H7 cultures used were meat isolates from the Food Safety and Inspection Service (FSIS), USDA, stock culture collection. *E. coli* serotypes 07 and 0116 were obtained from the *E. coli* Reference Center, Pennsylvania State University. All *Salmonella* and *Yersinia* cultures were FSIS stocks, and the remaining cultures were obtained from the University of Maryland Microbiology Department stock culture collection.

Petrifilm™ Test Kit - HEC - for hemorrhagic* E. coli *0157:H7*

Petrifilm Test Kits (wash concentrate, substrate solution, conjugate, Petrifilm E. coli Count plates, reactive discs, mesh spacers, spreader, and instruction sheet) were obtained from the 3M Company, St. Paul, MN (Catalogue No. 6477). The immunoblot assay was performed according to the manufacturer's instructions.

Detection and isolation of E. coli *0157:H7 from meat*

Individual 25 g meat samples were placed into 225 ml of mEC+n broth in Stomacher 3500 bags (Tekmar). The samples were stomached 2 min in a Colworth Stomacher Blender 3500 (Tekmar) and incubated 6 to 8 h at 37°C on a rotary shaker at 100 RPM. A portion of each enrichment culture was then diluted 1:10 and 1:100 in BPD and the original culture reincubated at 35°C static, overnight. One ml of the 1:10 and 1:100 dilutions was used to inoculate Petrifilm E. coli Count plates. The Petrifilm plates were incubated at 42°C for 18 h. After incubation, the top plastic film of the Petrifilm plate was carefully raised. The guar adhered to the plastic film, leaving a well in the foam base. An individually labeled reactive disc was placed in this well and orientation lines were drawn from the disc onto the foam base. The film with the guar was carefully rolled down onto the reactive disc and allowed to remain in contact for 2 min. The reactive discs, separated by mesh spacers, were then placed in a beaker of wash solution; up to 20 discs were batched per beaker. The reactive discs were washed twice, then incubated in conjugate solution for 30 min at room temperature on a rotary shaker at 100 rpm. At the completion of this incubation time, the conjugate solution was decanted and the reactive discs were washed three times with wash solution. The washed discs were then incubated in substrate solution for 10 min on a rotary shaker at 100 rpm at room temperature. Tap water was added to stop the reaction, decanted, and replaced with fresh tap water. The reactive discs were removed from the beaker, spread on paper towels, and blotted dry.

A presumptive positive test was indicated by the presence of dark gray to black spots on the reactive discs. This signal frequently appeared as small circles since the reaction tended to follow the outline of the gas bubble associated with the *E. coli* colony on the Petrifilm.

Isolation of *E. coli* 0157:H7 from the samples was then attempted by two methods, our USDA cultural method (*9,10*) and the 3M recommended Prompt™* isolation method. For the cultural method, decimal dilutions were made of the reserved 24-h mEC+n enrichment cultures to 10^{-6} in BPD. Spread plates, using 0.1 ml of the 10^{-4}, 10^{-5}, and 10^{-6} dilutions, were made on 150 x 15 mm MSA or MSA-BCIG agar plates for each sample and incubated at 42°C overnight. A set consisting of one PRS-MUG and one EMB plate was identically labeled and gridded into 12 numbered sections for each set of MSA or MSA-BCIG plates. Sorbitol negative colonies (white) from the MSA plates and sorbitol negative, BCIG negative colonies (white) from the MSA-BCIG plates were picked. Each colony selected was spread in the center of a section of the EMB plate and stabbed in the corresponding section on the PRS-MUG plate. When possible, 12 colonies were picked from the primary plating medium of each sample. These plates were incubated at 35°C overnight and then examined for sorbitol fermentation and MUG reactions on PRS-MUG, and appearance on EMB. Those isolates that were sorbitol negative (no color change), MUG negative (no fluorescence under UV light), and had the typical dark purple growth with a green metallic sheen on EMB were tested with the Oxoid *E. coli* 0157 Latex Test. Presumptive positive isolates were then confirmed with tube biochemical tests and an H7 agglutination test. Cultures that were not H7 were serotyped by the *E. coli* Reference Center, Pennsylvania State University.

For the 3M Prompt isolation method, we matched the reactive disc to its Petrifilm plate. Three dark gray to black spots and the orientation lines on the reactive disc were darkened with a felt tip marker, and the disc was then placed on a light box. The Petrifilm plate was placed on top of the reactive disc and the orientation lines on the Petrifilm plate were brought into alignment with those on the reactive disc. The darkened spots were now directly under the areas that caused the reaction. These areas were marked on the top plastic film with a felt tip marker. The top plastic film was raised and placed, guar side up, on a hard surface. A BBL Prompt wand (BBL Prompt™ Inoculation System/Tubes, BBL 26306) was used to remove the guar from the three marked areas. The wand was placed in a 1 ml Prompt tube, vortexed for 60 sec, and 100 ml, 10 ml, and 1 ml were spread plated on 100 x 15 mm MSA or MSA-BCIG plates. The plates were incubated at 42°C and typical colonies were picked and identified as described above. If *E. coli* 0157:H7 was not isolated, we returned to the plates and picked colored colonies (red, purple, or blue) to attempt to identify the source of the signal.

When pure cultures were tested, they were grown in TSB, diluted in BPD, and plated directly onto the Petrifilm E. coli Count plates.

RESULTS AND DISCUSSION

Initially five pure culture *E. coli* 0157:H7 meat isolates

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were tested with the 3M blot ELISA procedure. All five gave positive reactions on the reactive discs, with many of the spots following the outline of the gas bubble on the Petrifilm.

Next, ground beef samples inoculated with *E. coli* 0157:H7 were analyzed. Nine packages of ground beef were obtained, each from a different retail establishment, and 25 gm inoculated (0.7 *E. coli* 0157:H7/gm), and uninoculated control samples were prepared as previously described. The samples were enriched in mEC+n and the resulting cultures tested with the 3M blot ELISA procedure. Isolation of 0157:H7 was attempted from all samples (both inoculated and the uninoculated controls) on MSA plates. The Prompt isolation method was not used in this experiment. Instead, colonies were picked with a needle from the marked positive areas of the Petrifilm guar and streaked onto MSA plates. The results (Table 1) show that the 3M blot ELISA procedure detected 0157 antigen in all except one of the inoculated samples but also in two of the uninoculated samples. A sorbitol positive 0157:H38 *E. coli* was isolated from one of the uninoculated samples (35A), but the cause of the signal from the other uninoculated sample (39A) could not be determined. *E. coli* 0157:H7 was isolated from four of the 9 inoculated samples by the cultural method, and from three of the inoculated samples by picking from the guar. In no case was there cultural isolation without a positive 3M blot ELISA.

The above experiment was repeated with another nine meat samples, again each from a different commercial establishment. This time the Prompt isolation method was used along with both MSA and MSA-BCIG agars as the plating media (Table 2). The 3M blot ELISA procedure detected 0157 antigen in eight of the 9 inoculated samples, and in one of the 9 uninoculated samples. In no case was there cultural isolation without a positive 3M blot ELISA result. Comparison of the isolation procedures showed the USDA cultural isolation on MSA-BCIG to be the most reliable, with the isolation of the organism from eight of the 9 inoculated samples. The cultural method using MSA isolated from six of the 9 samples. The addition of BCIG to MSA detects ß-glucuronidase positive microorganisms. Since *E. coli* 0157:H7 is ß-glucuronidase negative, the medium becomes more differential (*9*). However, using the Prompt method of isolation from the Petrifilm guar, *E. coli* 0157:H7 was recovered from five of the 9 samples on MSA and four of the 9 samples on MSA-BCIG.

A survey of 101 uninoculated meat samples was done, comparing the 3M blot ELISA procedure, the cultural isolation method on MSA, and the Prompt isolation method from presumptive positive Petrifilm plates. We tested 30 bob veal kidneys, 26 ground beef samples, 10 cooked hamburger patties, 10 raw chicken samples, 20 pork sausages, and 5 ground pork samples. The 3M blot ELISA results showed 93 samples negative and 8 presumptive positive for 0157 antigen. All samples were negative by the USDA cultural method. Of the 8 positive 3M blot ELISA samples, two were false positives containing sorbitol positive, 0157 positive, *E. coli* (isolated by the Prompt method). One of these was serotype H43 and the other H16. The remaining six 3M blot ELISA positive samples were negative by both the cultural and Prompt isolation methods. The cause of the positive signals could not be determined. The 0157:H43 and 0157:H16 *E. coli* were isolated from pork sausages. The other suspect samples included 1 bob veal kidney, 2 ground beef samples, 1 chicken wing, and 2 ground pork samples.

The ability to isolate *E. coli* 0157:H7 from mixed cultures is dependent upon the number and character of the

TABLE 1. *Detection of* E. coli *0157:H7 from inoculated and control ground beef samples by the 3M blot ELISA and comparison of isolation by the direct pick method from Petrifilm guar with the USDA cultural isolation method on MSA.*

Sample[3]	APC[1]	MSA 35C[2]	3M Blot ELISA	Isolation method	
				Direct pick from guar (MSA)	Cultural (MSA)
33A	1.4×10^6	6.6×10^3	-	-	-
33B			+	+	+
34A	2.0×10^4	1.8×10^4	-	-	-
34B			+	-	-
35A	9.7×10^4	6.0×10^4	+	-	-*
35B			+	-*	+
36A	5.0×10^5	4.0×10^5	-	-	-
36B			+	+	-
38A	2.3×10^3	1.8×10^2	-	-	-
38B			+	-	-
39A	6.9×10^4	5.8×10^3	+	-	-
39B			+	-	+
40A	2.2×10^5	3.5×10^4	-	-	-
40B			+	+	+
41A	6.4×10^2	7.0×10^1	-	-	-
41B			+	-	-
42A	1.2×10^5	1.1×10^5	-	-	-
42B			-	-	-

*Isolation of a sorbitol positive *E. coli* 0157:H38.

[1]Aerobic plate counts (APC) done on plate count agar of meat samples after freezing.

[2]Total counts on MSA at 35°C of meat samples after freezing.

[3]"A" samples are controls. "B" samples are inoculated with 0157:H7 at the 0.7 organisms/gm level.

TABLE 2. *Detection of* E. coli *0157:H7 from inoculated and control ground beef samples by the 3M blot ELISA and comparison of the Prompt isolation method with the USDA cultural method on both MSA and MSA-BCIG.*

Sample	APC[1]	3M Blot ELISA	Isolation method: Prompt MSA	Prompt MSA-BCIG	Cultural MSA	Cultural MSA-BCIG
43A	2.1×10^5	+	-	-	-	-
43B		+	+	-	+	+
44A	1.6×10^6	-	-	-	-	-
44B		+	-	-	-	+
45A	1.5×10^6	-	-	-	-	-
45B		+	-	-	+	+
46A	9.6×10^5	-	-	-	-	-
46B		+	+	+	+	+
47A	1.3×10^6	-	-	-	-	-
47B		+	+	+	+	+
48A	3.1×10^5	-	-	-	-	-
48B		+	-	-	+	+
49A	1.9×10^6	-	-	-	-	-
49B		-	-	-	-	-
50A	2.7×10^5	-	-	-	-	-
50B		+	+	+	+	+
51A	1.8×10^4	-	-	-	-	-
51B		+	+	+	-	+

[1]APC - Aerobic plate count done on plate count agar of meat samples after freezing.

background microflora. This is particularly true when testing raw meat samples which may contain 10^6 microorganisms per gram. To resuscitate and amplify *E. coli* 0157:H7 in a meat sample, it is usually necessary to enrich the sample in a selective medium. While the mEC+n enrichment broth is not entirely selective for *E. coli* 0157:H7, it does provide sufficient growth advantages for *E. coli* organisms to be useful in combination with the 3M blot ELISA. A single *E. coli* 0157:H7 bacterium growing on the Petrifilm E. coli Count plate will produce a detectable signal in the 3M blot ELISA unless the signal is obscured by excessive background microflora. Therefore preenriching the sample for 6-8 h is sufficient to generate detectable levels of 0157:H7 even when the initial numbers are less than one per gram. This short preenrichment step also ensures that the background microflora will be held to a minimum for that particular sample.

In order to confirm a presumptive positive 3M blot ELISA result, it is necessary to isolate the organism from the culture medium. In some cases excessive background microflora found in raw meat samples may prevent the recovery of *E. coli* 0157:H7 on streak plates and on Petrifilm plates, particularly after preenrichment. This may have contributed to the failure to isolate *E. coli* serotype 0157 from several of the suspect samples signaled by the 3M blot ELISA. The number of confirmed presumptive positives may increase when cooked meat products or other products containing a lower level of endogenous microflora are tested.

Since polyclonal *E. coli* 0157 antibodies have been reported to cross react with *Brucella* species, *Francisella tularensis, Salmonella* group N, *Pseudomonas maltophilia, Vibrio cholerae, Yersinia enterocolitica* 0:9 (*1*) and *E. coli* 07 and 0116 (*3*), we decided to test a variety of organisms with the 3M Test Kit. *Salmonella* cultures from every lettered "O" group (A-Z) as well as "O" groups 29, 51, 52, 54, 55, 57, and 59 were tested. Strains of groups A, D, and 29 did not grow on the Petrifilm at 42°C. Of all the *Salmonella* cultures tested, only the group "N" strain gave a positive reaction on the 3M blot ELISA. Three cultures of *Y. enterocolitica* 0:9 would not grow on the Petrifilm at 42°C. We then tested 12 other cultures that would grow on the Petrifilm at 42°C: *Acinetobacter calcoaceticus, Citrobacter freundii, Enterobacter cloacae, E. aerogenes, Klebsiella pneumoniae, K. pneumoniae* ATCC 13883, *Hafnia alvei, Proteus vulgaris, Pseudomonas aeruginosa, Serratia marcescens, E. coli* 07, and *E. coli* 0116. All gave negative results with the 3M blot ELISA.

From this work, it appears that the 3M Petrifilm Test Kit - HEC - for Hemorrhagic *E. coli* 0157:H7 is a reliable negative screen for the 0157 antigen. No false negatives were obtained when compared with our USDA cultural method. The 3M blot ELISA procedure appears to be at least as good as the USDA cultural method. By using this Test Kit, most of the negative samples can be identified in 26-28 h, leaving a small number of presumptive positive samples for confirmation testing.

In our survey, 92% of the samples would have been eliminated by the 3M Test Kit. A summary of the results shows a false negative rate of 0 and a false positive rate of 2%. In addition, 6% of the positive 3M blot ELISA results were culturally unconfirmed suspect samples. If the 3M blot ELISA negative samples in our survey had been discarded, we would have eliminated the work involved in diluting 93 samples, plating these samples on 279 MSA (MSA-BCIG) plates, picking a maximum of 1116 colonies to both 93 PRS-MUG and 93 EMB plates, as well as the time and cost involved in making these media.

Although the Prompt method did successfully isolate sorbitol positive and/or ß-glucuronidase positive 0157 organisms signaled by the 3M blot ELISA, the USDA cultural method for the isolation of 0157:H7 was found to be the more reliable isolation procedure.

It appears that the most efficient use of the 3M Petrifilm Test Kit in a screening program for *E. coli* 0157:H7 would be to: a) enrich samples in mEC+n at 37°C for 6 to 8 h on a rotary shaker; b) inoculate the Petrifilm E. coli Count plates and incubate at 42°C for 18 h; c) reincubate enrichment cultures static at 35°C overnight; d) test the growth on the Petrifilm plate with the 3M blot ELISA; e) discard any 3M blot ELISA negative samples; f) attempt isolation from the 3M blot ELISA presumptive positive samples by both the Prompt and USDA cultural methods; and g) confirm isolates biochemically and serologically.

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