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A Research Note

A Screening Method for the Isolation of *Escherichia coli* 0157:H7 from Ground Beef

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ABSTRACT

A screening method was developed for the isolation of *Escherichia coli* 0157:H7 from raw ground beef. Suspensions at a 1:10 dilution of beef were made in a modified EC broth with novobiocin (mEC+n; EC broth with 1.12 g/L instead of 1.5 g/L Bile salts #3 and novobiocin at 20 mg/L). The samples were macerated in a Stomacher for 2 min and either shaken at 37°C (100 RPM) for 6 h, or incubated static at 35°C for 24 h. Appropriate dilutions of the cultures were then spread plated on 150x15 mm plates of MacConkey sorbitol agar (MSA). The MSA plates were incubated at 42°C overnight. A set of two plates consisting of a deep (40 ml/plate) phenol red sorbitol agar plate with 4-methylumbelliferyl ß-D-glucuronide (PRS-MUG), and a Levine EMB agar plate with added agar for a final concentration of 3%, were gridded into 12 numbered sections. Sorbitol negative colonies were picked from the MSA plates, spread on the appropriate section of the EMB, and stabbed into the corresponding section on the PRS-MUG plate. Those cultures that were sorbitol negative and MUG negative on PRS-MUG and were typically *E. coli* on EMB were confirmed biochemically and serologically. By this procedure 0157:H7 was isolated from 5 of 10 meat samples inoculated at 0.6 organisms/g, and 10 of 10 samples at the 5/g level using the 6 h shaken method. With the 24 h static incubation method, 0157:H7 was isolated from 8 of 10 samples at the 0.6/g level and 10 of 10 at the 5/g level. Thirteen strains of 0157:H7 inoculated at levels between 0.4 and 0.6/g were tested and 9 of the 13 were isolated with the 6 h method, and 13 of the 13 with the 24 h method. The method is reliable and simple enough to be used in large screening programs.

Cases of hemorrhagic colitis, bloody diarrhea, and hemolytic uremic syndrome caused by *Escherichia coli* 0157:H7 have been associated with the consumption of ground beef *(10,11,12)*, and ground beef is the suspected vehicle in other cases. Furthermore, dairy cattle are a probable reservoir of the organism *(2,3,13)*. Nevertheless, the organism has seldom been isolated from ground beef. We have developed an isolation method for this organism that is appropriate for the efficient screening of large numbers of beef samples.

The AOAC procedure for *E. coli* enumeration calls for growing the organism in EC broth at 45.5°C. This method is unsuitable for serotype 0157:H7 which will not grow, or grows poorly, in EC broth at that temperature. However, two characteristics of the serotype can be exploited to differentiate it from other *E. coli* serotypes: its inability to ferment sorbitol (or to do so very slowly) and the absence of ß-glucuronidase. It has been reported that 80.3% of *E. coli* are sorbitol positive *(5)* and approximately 97% have ß-glucuronidase *(7)*.

Our procedure uses a modification of EC broth as an enrichment medium, MacConkey sorbitol agar (MSA) plates incubated at 42°C for colony isolation, and a gridded two-plate system consisting of a plate of phenol red sorbitol agar containing 4-methylumbelliferyl ß-D-glucuronide (PRS-MUG), and a plate of Levine eosin methylene blue agar (EMB). In this way we differentiate sorbitol negative, ß-glucuronidase negative (detected with MUG) *E. coli* from other isolates in a beef sample.

MATERIALS AND METHODS

Inoculation of meat samples

A culture of *E. coli* 0157:H7 was grown overnight in Trypticase Soy Broth (TSB, BBL) 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 *(14)* (BPD) to give 50% light transmittance at 400 nm in a Bausch and Lomb Spectronic 20. Decimal dilutions to 10^{-6} were made in BPD. Ground beef balls weighing 25 g, placed in individual sterile plastic bags, were 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 one week 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 approximately 13 cells for an inoculation level of about 0.5/g, and those inoculated from the 10^{-5} dilution received about 5/g.

Media

Modified EC broth with novobiocin (mEC+n). Tryptone (Difco) 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, and distilled water 1 L. If necessary the pH was adjusted to 6.9 ± 0.1 before autoclaving at 121°C for 15 min. After the medium cooled, a filter sterilized aqueous solution of sodium novobiocin (potency 890 µg/mg) (Sigma) was added to produce a final concentration of 20 mg/L. Modified EC broth (mEC) was prepared as above omitting the novobiocin.

MacConkey sorbitol agar (MSA). 1% D-sorbitol was added to MacConkey agar without sugar (Difco) and prepared according to manufacturer's instructions, tempered, and poured into 150x15 mm petri dishes (approximately 80 ml/plate).

Phenol red sorbitol agar + MUG (PRS-MUG). Phenol red broth base (Difco) was prepared according to manufacturer's instructions. Two percent agar was added and heated to dissolve; to this was added 0.5% D-sorbitol and 0.005% MUG (Sigma). The resulting pH was 6.8-6.9. The medium was sterilized by autoclaving at 121°C for 15 min, tempered and poured into 100x15 mm petri dishes (40ml/plate to make deep dishes).

Eosin methylene blue agar (EMB). Levine's formula EMB (BBL) was prepared according to manufacturer's instructions. Enough extra agar was added to bring the concentration to 3% agar.

Stock cultures

Fourteen stock cultures were received from J. Wells and N. Strockbine of Centers for Disease Control, Atlanta, GA. Most of the developmental work was done with the one isolate from meat, the remainder were clinical isolates.

Isolation method

A 25g meat sample was placed in 225 ml of mEC+n in a Stomacher 3500 bag. The sample was blended 2 min in a Stomacher 3500(Colworth). It was then incubated either for 6 h at 37°C on a rotary shaker with a 1 inch diameter circular orbit at 100 RPM, or static for 24 h at 35°C. After incubation, the 6 h shaken culture was diluted to 10^{-2} in 9-ml BPD dilution blanks. One tenth of a ml of the undiluted culture and 0.1 ml of each dilution were plated on to MSA plates (3 plates/sample), spreading the liquid over the agar surface with a sterile glass spreading rod. Cultures of overnight incubated mEC+n broth were spread from the 10^{-4}, 10^{-5}, and 10^{-6} dilutions. The MSA plates were incubated at 42°C overnight. A set of PRS-MUG and EMB plates were identically labeled and gridded into 12 numbered sections for each sample. Sorbitol negative colonies (white) from the MSA plates were picked and spread in the center of a section on the EMB plate and then stabbed in the corresponding section on the PRS-MUG plate. The use of a magnifying glass attached to a fluorescent light was beneficial when picking colonies from the MSA plates. Twelve colonies were picked and the plates incubated overnight at 35°C. The plates were examined for sorbitol fermentation, the presence of ß-glucuronidase, and typical *E. coli* growth. The cultures that were sorbitol negative, MUG negative, and typical *E. coli* were confirmed on triple sugar iron agar (TSI), sorbitol and cellobiose fermentation broths, tryptone broth for indole, methyl red-Voges-Proskauer (MR-VP) broth, Simmon's citrate agar, and lysine and ornithine decarboxylase broths. Cultures conforming to the biochemical pattern of *E. coli* 0157:H7 were then serotyped.

RESULTS AND DISCUSSION

In the development of this method, our attention first focused on the plating medium. MacConkey agar containing sorbitol (MSA), rather than lactose, has long been in general use for the isolation of this serotype and has proved useful, particularly in clinical samples *(1,6,8,9)*. Our observations however, showed that with meat, more than just this medium was necessary to repress the competitive flora relative to the number of desired organisms. Towards this end we tested various temperatures of incubation and found that while serotype 0157:H7 exhibited no significant difference in viability on MSA at 35 and 42°C, the microbial flora of various meat samples was greatly reduced at 42°C. We compared streak plates and spread plates for efficiency in isolation of the organism and found spread plates to be superior.

In working with inoculated meat samples, we found that some sorbitol fermenting strains would not develop the red color on crowded MSA plates at 42°C, and thus appeared to be sorbitol negative. To identify these fermenters, and to identify the MUG negative isolates, the PRS-MUG plate incubated at 35°C, was added to the method. We found that a substantial number of the sorbitol negative - MUG negative isolates picked from the MSA plates were not *E. coli,* therefore, we added the EMB companion plate to the PRS-MUG plate to give a presumptive identification of *E. coli* isolates. Occasionally an isolate picked from the MSA plate would prove to be a *Proteus* that exhibited swarming on the EMB plate. We found that by adding agar to 3%, this swarming could be eliminated.

Because our laboratories work primarily with samples shipped to arrive frozen, we needed an enrichment broth to aid in the recovery of freeze injured cells. Szabo et al. *(15)* reported that bile salts #3 at 0.112% in the presence of 0.5% NaCl would allow the full recovery of *E. coli* 0157:H7 at 44.5°C while inhibiting non-enteric bacteria. Doyle et al. *(4)* reported the addition of novobiocin to be advantageous. We therefore changed the formula of EC broth, reducing the bile salts and adding novobiocin.

To test the ability of this broth to support the growth of 0157:H7, tubes of mEC, mEC+n, and TSB were inoculated with the organism at a level of 16 cells/ml and incubated 18 h at 35, 42, and 45.5°C. The cultures at 35 and 42°C were incubated in a warm air incubator. The cultures at 45.5°C were incubated in a water bath. Plate counts were made on Plate Count Agar (PCA, Difco). At 45.5°C we observed no growth in mEC with or without novobiocin and very low growth in TSB (Table 1). At 42 and 35°C the counts in mEC and TSB were essentially the same, but the addition of novobiocin produced a decrease of approximately 95% at 42°C and a decrease of approximately 69% at 35°C when compared to TSB. These results showed that if novobiocin was to be used, it must be used at 35°C.

We reasoned that our broths would also contain beef from the sample which would act as a nutrient. To simulate this condition we added 1% beef extract to mEC+n and compared that growth to that occurring in mEC+n and

TABLE 1. *Counts per ml of* E. coli *0157:H7 inoculated at a level of 16 cells/ml after 18 h incubation in mEC, mEC+n and TSB at 35, 42, and 45.5°C.*

Temp. (C)	mEC	mEC+n	TSB
45.5	0	0	9.8×10^2
42	9.5×10^8	5.2×10^7	9.5×10^8
35	1.0×10^9	2.8×10^8	9.1×10^8

TSB when inoculated with 0.5 organisms/ml. Using a 6 h incubation on the shaker at 37°C, we found plate counts of 1.9×10^2 for mEC+n, 4.5×10^2 for mEC+n+1% beef extract, and 3.3×10^3 for TSB. Since the beef extract doubled the growth in mEC+n we then attempted isolation studies from beef.

Thirteen different packages of dairy cow ground beef were obtained and inoculated with 0157:H7 to produce samples containing 0.6/g or 5/g. Aerobic plate counts were done on uninoculated samples concurrently with the suspension of the meat sample into the broth. The isolation method previously described was followed. The results (Table 2) showed that isolation of *E. coli* 0157:H7 was accomplished from 5 of the 10 samples at the 0.6/g level using the 6 h shaken incubation of mEC+n, and from 8 of the 10 samples using the 24 h static incubation. When samples inoculated at the 5/g level were tested, isolation was accomplished from all 10 of the samples with both methods.

To test whether a variety of strains of 0157:H7 could be isolated by this method, we inoculated 25 g samples from a package of dairy cow ground beef with 13 stock cultures to give inoculation levels of 0.4/g to 0.6/g. The aerobic plate count of this meat was 9.0×10^3/g. All samples were analyzed as previously described. Nine of the 13 strains were isolated by the 6 h shaken method, and 13 of the 13 strains were isolated by the 24 h static method.

The biochemical tests chosen for the confirmation of the isolates included TSI to test lactose or sucrose fermentation, sorbitol as a final fermentation check, the IMViC reactions to confirm *E. coli*, cellobiose fermentation to distinguish *E. coli* from *E. hermanii (1)*, and lysine and ornithine decarboxylase as recommended by Haldane et al. *(6)* to reduce the number of sorbitol negative, MUG-negative *E. coli* that need to be serotyped.

We believe that this is a practical method for screening large numbers of meat samples as well as samples involved in disease outbreaks. The 6 h shaken method, though slightly less reliable than the 24 h static method, has the advantage of reducing the analysis time by a day and requires that only 2 dilutions be prepared rather than 6, however, it would be prudent to use both methods in a foodborne outbreak investigation.

This method is designed only to isolate *E. coli* 0157:H7. Though there are a number of other serotypes that produce the verocytotoxin (Shiga-like toxin) associated with this organism, this is the only verocytotoxin producing serotype that has been involved in foodborne outbreaks in this country.

TABLE 2. *Isolation of* E. coli *0157:H7 from ground beef inoculated at the 0.6/g and 5.0/g levels using both the 6 h shaken (sh) and 24 h static (st) mEC+n enrichment methods.*

0.6/g			5.0/g		
APC*	6 h sh	24 h st	APC	6 h sh	24 h st
3.4×10^3	-	+	4.2×10^3	+	+
4.0×10^3	+	+	5.1×10^3	+	+
7.3×10^3	+	+	3.3×10^5	+	+
3.3×10^5	+	+	6.4×10^3	+	+
2.1×10^3	-	+	6.7×10^2	+	+
4.3×10^2	+	+	4.5×10^3	+	+
5.2×10^3	-	-	3.1×10^5	+	+
2.2×10^4	-	-	3.2×10^4	+	+
2.1×10^4	+	+	1.8×10^5	+	+
3.4×10^5	-	+	6.2×10^3	+	+

*APC - aerobic plate count per gram.

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incubation time is of 5 d, the 5 d/7°C ADM proved to be as rapid and is the fastest method for enumeration of raw milks inoculated with lactic acid bacteria.

The ADM has the added advantages of requiring less time to perform than the SM and of being less expensive (*17*). It is therefore well suited for quality control units in dairy plants and government facilities.

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