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Development of a Medium for Differentiation between *Escherichia coli* and *Escherichia coli* O157:H7

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ABSTRACT

A new medium (*Escherichia coli* O157:H7 medium: EOH) was developed for differentiation between *E. coli* and *E. coli* O157:H7. The EOH medium was compared with sorbitol MacConkey agar (SMAC), which is the most popular medium to enumerate *E. coli* O157:H7. Several combinations of 35 dyes were evaluated to develop the new medium. Indigo carmine (0.03 g/liter) and phenol red (0.036 g/liter) were found as the best combination for differentiation between *E. coli* O157:H7 and *E. coli* and added to the basal agar medium (SMAC medium excluding neutral red and crystal violet) for EOH medium. On the dark blue EOH medium, *E. coli* produced a yellow color with clear zone, whereas *E. coli* O157:H7 produced a red color without clear zone. For differentiation between *E. coli* and *E. coli* O157:H7, EOH has much better potential than SMAC. Furthermore, the red color produced by normal *E. coli* in SMAC may mask the light gray color produced by *E. coli* O157:H7, whereas the yellow color with clear zone did not mask the red color without clear zone in the EOH medium. The recovery numbers of *E. coli* O157:H7 from inoculated ground beef, pork, and turkey were not significantly different between SMAC and EOH media ($P > 0.05$). The recovery rates of heat- and cold-injured *E. coli* O157:H7 also were not significantly different ($P > 0.05$).

Escherichia coli O157:H7 has emerged recently as a recognized foodborne pathogen *(3, 4).* It was implicated as a cause of food poisoning *(12).* Since then, more than 16 documented foodborne disease outbreaks have been associated with *E. coli* O157:H7 *(9).* It may be present in foods of animal origin *(4).* Hemorrhagic colitis, hemolytic uremic syndrome, and thrombocytopenic purpura are the principal manifestations of illness *(12, 13). E. coli* O157:H7 is causing significant public health problems in the world *(10)* and now is listed among the most undesirable bacteria in foods *(1).* Food industries, state and federal regulatory agencies, and governments continuously attempt to deal with the food safety issue through research, evaluation, and regulations *(16, 17).*

Several methods have been developed for isolation of *E. coli* O157:H7 from food and clinical samples based on the knowledge that it is an enteric bacterium, unable to ferment sorbitol or produce β-glucuronidase *(4, 17).* As a component of these methods, a number of selective and differential plating media have been developed for *E. coli* O157:H7 *(15).* Sorbitol MacConkey agar (SMAC; Difco Laboratories, Detroit, Mich.) is used routinely as a selective, differential plating medium *(7, 8, 11).* However, the color changes are not strongly differentiable. Sometimes the red color produced by *E. coli* can cover the nearly colorless *E. coli* O157:H7 colonies, so they can be missed *(5).* Recently, Fung et al. *(5)* developed 202 medium. In this greenish blue medium, *E. coli* produced a yellow color, whereas *E. coli* O157:H7 produced a green color. Even though the differentiation between *E. coli* and *E. coli* O157:H7 in 202 is much better than that in SMAC, the differences from the original medium are not strong *(2).* Therefore, a new medium that can differentiate better between *E. coli* and *E. coli* O157:H7 is needed.

This report describes the development of *E. coli* O157:H7 (EOH) medium, comparison with SMAC for enumerating *E. coli* O157:H7 from inoculated food samples, and

TABLE 1. *Cultures used in the study and their sources*

Cultures	Sources
E. coli (11775)	ATCC[a]
E. coli (5922)	ATCC
E. coli (E-2)	FMCC[b]
E. coli (E-4)	FMCC
E. coli (E-6)	FMCC
E. coli (E-9)	FMCC
E. coli O157:H7 (Eh-7-1)	FMCC
E. coli O157:H7 (Eh-7-2)	FMCC
E. coli O157:H7 (Eh-7-3)	FMCC
E. coli O157:H7 (Eh-7-4)	FMCC
E. coli O157:H7 (Eh-7-5)	FMCC
E. coli O157:H7 (Eh-7-6)	FMCC
E. coli O157:H7 (Eh-7-7)	FMCC
E. coli O157:H7 (Eh-7-8)	FMCC
E. coli O157:H7 (Eh-7-9)	FMCC
E. coli O157:H7 (Eh-7-10)	FMCC

[a] American Type Culture Collection.
[b] Food Microbiology Culture Collection at Kansas State University.

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TABLE 2. *Dyes examined and their sources*

Dyes	Sources
Bismarck brown	Matheson Coleman & Bell
Brilliant yellow	Sigma
Congo red	Sigma
Erichrome black T	Fisher Scientific Co.
Evans blue	Sigma
Janus green B	Sigma
Trypan blue	Sigma
Resiren violet TR	Mobay Chemical Co.
Thiazol yellow G	Sigma
Brilliant cresyl blue	Sigma
Crystal violet	Fisher Scientific Co.
Methylene blue	Allied Chemical Co.
Neutral red	Sigma
Phenolsafranin	Sigma
Safranin O	Matheson Coleman & Bell Co.
Brilliant green	Sigma
Fast green FCF	Sigma
Malachite green	National Aniline Division
Metanil yellow	Sigma
Methyl green	Sigma
Pararosaniline	Sigma
Acridine orange	Sigma
Acridine yellow	Sigma
Acriflavine	Sigma
Brom cresol purple	Fisher Scientific Co.
Bromochlorophenol blue	Sigma
Bromophenol blue	Fisher Scientific Co.
Cresol red	Fisher Scientific Co.
Eosin B	Sigma
Phenol red	Fisher Scientific Co.
Rose bengal	Sigma
Indigo carmine	Sigma
Orcein	Sigma
Astrazon orange G200	Mobay Chemical Co.
Giemsa stain	National Aniline Division

also evaluation of the recovery rates of heat- and cold-injured *E. coli* O157:H7on both media.

MATERIALS AND METHODS

Cultures tested. Table 1 shows the list of cultures used and sources. Each culture was inoculated to brain heart infusion (BHI; Difco Laboratories) broth and incubated at 37°C for 24 h. The cultures were maintained on BHI agar slants until use.

Development of new medium. Thirty-five dyes were used in the screening test. The names and sources of these dyes are listed in Table 2. Stock solutions of 1% dye in suitable solvents were made. Dyes from the stock solution were mixed randomly and added to the basal agar medium (SMAC medium excluding neutral red and crystal violet) to obtain 1:1,000, 1:10,000, and 1:100,000 final dye concentration to find the best combination of dyes. After inoculation of *E. coli* and *E. coli* O157:H7 and incubation at 37°C for 24 h, the differences in colonies were visually evaluated. After selection of potential dyes that could differentiate between *E. coli* and *E. coli* O157:H7, the concentration of each dye was evaluated to find the most effective concentration in the agar medium.

TABLE 3. *The characteristics of cultures tested on EOH medium*

Tested cultures	Growth[a]	Color[b]	Clear zone[c]
E. coli (11775)	+++	Yellow	+
E. coli (15922)	+	Yellow	+
E. coli (E-2)	++	Yellow	+
E. coli (E-4)	+	Yellow	+
E. coli (E-6)	+++	Yellow	+
E. coli (E-9)	+++	Yellow	+
E. coli O157:H7 (Eh-7-1)	++	Red	−
E. coli O157:H7 (Eh-7-2)	++	Red	−
E. coli O157:H7 (Eh-7-3)	+++	Red	−
E. coli O157:H7 (Eh-7-4)	+++	Red	−
E. coli O157:H7 (Eh-7-5)	+++	Red	−
E. coli O157:H7 (Eh-7-6)	++	Red	−
E. coli O157:H7 (Eh-7-7)	+	Red	−
E. coli O157:H7 (Eh-7-8)	++	Red	−
E. coli O157:H7 (Eh-7-9)	+++	Red	−
E. coli O157:H7 (Eh-7-10)	+++	Red	−

[a] +++, Excellent growth; ++, good growth; +, growth.
[b] Color was evaluated after 24 h incubation at 37°C.
[c] Clear zone was evaluated after 24 h incubation at 37°C; +, presence; −, absence.

Media. The SMAC medium was purchased from Difco. The ingredients of EOH medium are as follows: 15.5 g peptone, 3 g proteose peptone, 10 g D-sorbitol, 1.5 g bile salts, 5 g sodium chloride, 15 g agar, 0.036 g phenol red, and 0.03 g indigo carmine final dye concentration per liter.

Cell suspension. The *E. coli* O157:H7 was transferred to 9.0 ml BHI broth and incubated at 37°C for 24 h. After incubation, 1 ml of culture broth was transferred to sterilized 500 ml polycarbonate centrifuge bottles (Nalgene, Rochester, N.Y.) containing 100 ml of BHI broth, and the bottles were incubated at 37°C for 24 h. Cells were harvested by centrifugation (model JA-22A, International Equipment Co., Needhan Heights, Mass.) at 12,400 × *g* for 25 min at 4°C. After centrifugation, the pellet was resuspended in sterile Butterfield's phosphate buffer. The resuspended cells were centrifuged and resuspended again to be used as culture suspension. The number of *E. coli* O157:H7 was adjusted with diluent (0.1% sterile peptone water).

Recovery from inoculated foods. Three samples each of milk and ground beef, pork, and turkey were purchased from a local commercial source. One hundred grams or milliliters of each sample was placed aseptically into individual stomacher bags. The samples were inoculated with prepared 5-ml *E. coli* O157:H7 culture suspensions containing about 7.0, 8.0, and 9.0 log CFU to construct different initial numbers (ca. 5.0, 6.0, and 7.0 log CFU/ml or g). After inoculation, the meat samples were mixed well with gloved hands to achieve homogeneous distribution for 2 min. After mixing, the samples were incubated at 37°C for 24 h. Before and after incubation, 25 g or ml of each sample was transferred aseptically to a stomacher bag (Spiral Biotech, Inc., Bethesda, Md.) and mixed with 225 ml 0.1% sterile peptone water. This 1:10 dilution of the sample was stomached for 2 min with the stomacher (Seward Medical, London, UK) for 2 min. After a 10-fold serial dilution, each dilution was spiral spread on EOH and SMAC agar plates in duplicate with Spiral Plater (Spiral Biotech, Inc.) and incubated aerobically at 37°C for 24 h. The recovered popu-

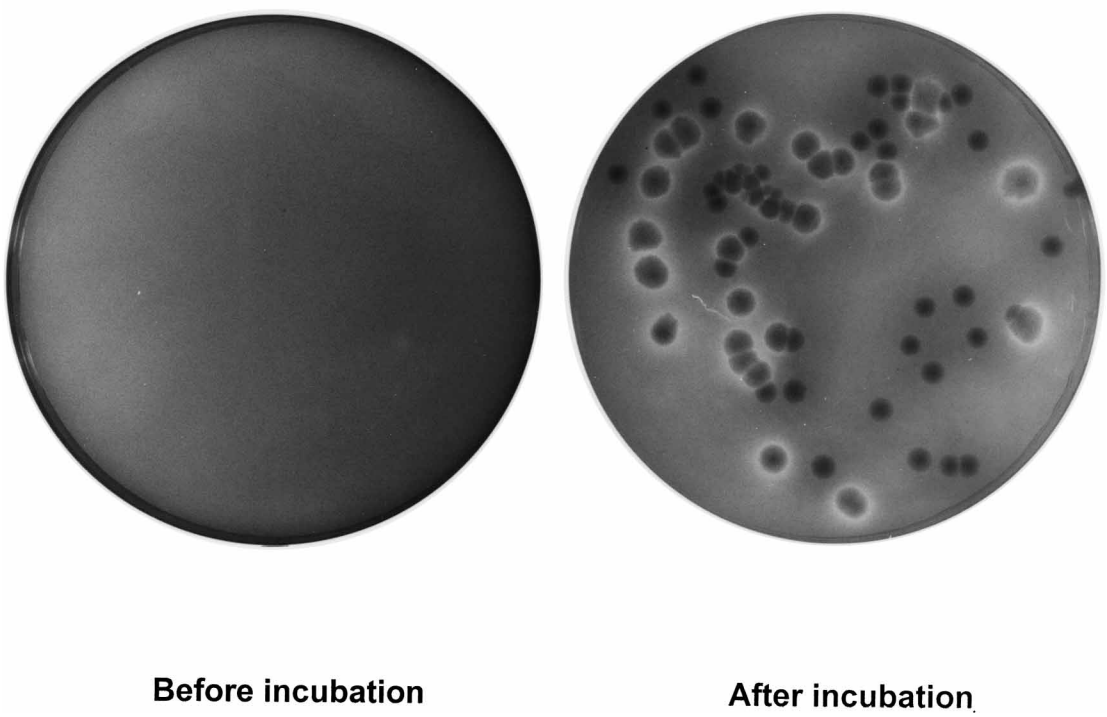

FIGURE 1. *Color changes of* E. coli *O157:H7 and* E. coli *in EOH medium. Yellow color with clear zone was developed by* E. coli, *whereas red color without clear zone was developed by* E. coli *O157:H7.*

lations were enumerated using a bacterial colony counter (Spiral Biotech, Inc.) or a manual method. This experiment was performed three times.

Recovery of heat- and cold-injured *E. coli* O157:H7. Fifty microliters of *E. coli* O157:H7 culture suspension (diluted to ca. 7.0 log CFU/ml) was added separately into three test tubes with 5 ml of Butterfield's phosphate buffer/tube that had been preheated and maintained at 50, 55, or 60°C. After inoculation, each tube was sealed tightly, immersed completely in a shaking water bath, and heated at 50, 55, or 60°C for 120, 10, or 1.25 min, respectively. After heating, tubes were cooled immediately in slush ice and spiral-plated onto EOH and SMAC media. Plates were incubated at 37°C for 24 h.

Fifty microliters of *E. coli* O157:H7 culture suspension (7.0 log CFU/ml) was spiked into two test tubes that also contained 5 ml of buffer solution. Tubes were frozen at −20°C for 2 or 4 h and then thawed rapidly. All tubes were enumerated for CFU/ml by preparing serial dilutions in 0.1% sterile peptone water, plating each dilution in duplicate on EOH and SMAC, and incubated at 37°C for 24 h.

Statistical analysis. These experiments were repeated three times, and the means of values are listed in the tables. Data for each treatment and bacterial isolate were converted to $\log_{10}$ CFU/g or ml and analyzed statistically by *t* test (factor = media) using the SAS general linear models procedure *(14).*

TABLE 4. *The recovery rate of* E. coli *O157:H7 from different food samples with EOH and SMAC media*

	SMAC		EOH	
Samples	Initial[a]	Final[b]	Initial	Final
Milk	5.41 ± 0.04	9.12 ± 0.14	5.32 ± 0.03	9.16 ± 0.41
Ground beef	5.38 ± 0.21	8.13 ± 0.33	5.41 ± 0.45	8.14 ± 0.23
Pork	5.28 ± 0.17	8.12 ± 0.42	5.32 ± 0.27	8.21 ± 0.34
Turkey	5.39 ± 0.25	7.87 ± 0.38	5.36 ± 0.36	7.79 ± 0.25
Milk	6.81 ± 0.02	9.32 ± 0.42	6.83 ± 0.04	9.46 ± 0.49
Ground beef	6.32 ± 0.17	8.23 ± 0.36	6.21 ± 0.14	8.42 ± 0.27
Pork	6.59 ± 0.06	8.95 ± 0.41	6.64 ± 0.24	8.36 ± 0.35
Turkey	6.67 ± 0.12	8.68 ± 0.38	6.45 ± 0.21	8.77 ± 0.22
Milk	7.21 ± 0.05	9.32 ± 0.36	7.35 ± 0.12	9.46 ± 0.12
Ground Beef	7.22 ± 0.10	8.23 ± 0.42	7.51 ± 0.16	8.30 ± 0.28
Pork	7.31 ± 0.09	8.72 ± 0.25	7.27 ± 0.26	8.78 ± 0.37
Turkey	7.27 ± 0.11	8.66 ± 0.17	7.48 ± 0.23	8.87 ± 0.33

[a] Mean values of three replicated data sets ± standard deviation before incubation, log CFU/g or ml.
[b] Mean values of three replicated data sets ± standard deviation after 24 h incubation, log CFU/g or ml.

TABLE 5. *The recovery rates of heat-injured* E. coli *O157:H7 with EOH and SMAC media*

	SMAC		EOH	
Treatment	Initial[a]	Final[b]	Initial	Final
50°C for 120 min	5.49 ± 0.04	3.11 ± 0.42	5.43 ± 0.11	3.05 ± 0.22
55°C for 10 min	5.51 ± 0.21	2.24 ± 0.61	5.32 ± 0.35	2.29 ± 0.36
60°C for 1.2 min	5.50 ± 0.17	1.96 ± 0.42	5.41 ± 0.37	1.86 ± 0.26

[a] Mean values of three replicated data sets ± standard deviation before treatment, log CFU/ml.
[b] Mean values of three replicated data sets ± standard deviation after treatment, log CFU/ml.

RESULTS AND DISCUSSION

Figure 1 shows the color changes in EOH medium inoculated with *E. coli* O157:H7 and *E. coli.* Table 3 shows the results of several *E. coli* O157:H7 and generic *E. coli* on EOH medium. All tested *E. coli* O157:H7 produced a red color without a clear zone, whereas all tested generic *E. coli* produced a yellow color with a clear zone. On SMAC media, *E. coli* O157:H7 produced a light gray color, whereas *E. coli* produced a red color (data not shown). The most important step for enumerating *E. coli* O157:H7 is differentiating from normal *E. coli. E. coli* O157:H7 shows characteristics typical of most other *E. coli*; however, it does exhibit some unique distinguishing characteristics. It does not ferment sorbitol within 24 h and does not have β-glucuronidase activity (4-methylumbelliferyl-β-D-glucuronide negative). The EOH and SMAC media contained sorbitol; *E. coli* O157:H7 cannot produce acid from sorbitol, whereas *E. coli* can produce acid around the colony. The acid production by *E. coli* produced yellow and red color in EOH and SMAC media, respectively. In the case of EOH, *E. coli* produced a clear zone around colonies, whereas *E. coli* O157:H7 did not. This phenomenon makes differentiation easy. Indigo carmine was used for the characteristic. In low pH, the blue color of indigo carmine was changed to clear (data not shown). A clear zone was produced by the acid produced by *E. coli* through sorbitol fermentation. The dye is an important factor for differentiating the two microorganisms. Neutral red and crystal violet in SMAC medium and phenol red and indigo carmine in EOH medium are used as the indicators and are pH dependent. The color difference between red without clear zone and yellow with clear zone in the EOH medium is more obvious than the difference between red and light gray in the SMAC medium. Furthermore, the yellow color with a clear zone does not mask the red color without a clear zone produced by *E. coli* O157:H7 in EOH medium. Table 4 showed the result of comparison of SMAC and EOH media for enumerating *E. coli* O157:H7 from an inoculated food system. No statistical difference occurred ($P > 0.05$) between results obtained with the two media. *E. coli* O157:H7 grow well in milk and ground beef, pork, and turkey up to ca. 9.0 log CFU/g or ml. From these data, we can postulate that EOH has the same potential as SMAC for use as a selective medium to enumerate *E. coli* O157:H7 from food samples. In the case of ground beef, the initial numbers of natural microorganisms were around 5.0 to 6.0 log CFU/g. The EOH medium contains bile salt (1.5 g/liter) that has strong antimicrobial activities. The EOH medium successfully eliminated those microorganisms to isolate only *E. coli* O157:H7.

Sublethally injured microorganisms are sensitive to antimicrobial agents. For example, *E. coli* O157:H7 has resistance against bacteriocin such as pediocin or nisin. However, if it is sublethally injured by heat, the pathogen can be susceptible to bacteriocin *(6).* Dye in media also can attack sublethally injured microorganisms. Therefore, to develop selective media, the antimicrobial activities of dyes against sublethally injured microorganisms must be considered. Table 5 shows that the dyes in the EOH medium did not have antimicrobial activity against sublethally heat-injured *E. coli* O157:H7 when it compared to SMAC medium. No statistical differences occurred ($P > 0.05$) between the media for isolation of *E. coli* O157:H7 from the three treatments (50, 55, and 60°C). Table 6 shows the recovery rates of cold-injured *E. coli* O157:H7 with EOH and SMAC media. These results also indicated no significant difference between the two media for enumeration of cold-injured *E. coli* O157:H7. In conclusion, the EOH medium was not statistically different from SMAC for isolating healthy and sublethally injured *E. coli* O157:H7 and had more advantages for differentiating this foodborne pathogen from generic *E. coli.*

TABLE 6. *The recovery rates of cold-injured* E. coli *O157:H7 with EOH and SMAC media*

	SMAC		EOH	
Treatment	Initial[a]	Final[b]	Initial	Final
−20°C for 2 h	5.50 ± 0.04	5.27 ± 0.06	5.53 ± 0.02	5.30 ± 0.04
−20°C for 4 h	5.52 ± 0.16	5.11 ± 0.21	5.56 ± 0.06	5.06 ± 0.11

[a] Mean values of three replicated data sets ± standard deviation before treatment, log CFU/ml.
[b] Mean values of three replicated data sets ± standard deviation after treatment, log CFU/ml.

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