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Modified sorbitol MacConkey agar for the rapid isolation of *Escherichia coli* O157 : H7

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Abstract : Unlike most *Escherichia coli* strains, *E coli* O157 : H7 didn't ferment sorbitol within 24h of incubation and showed a negative reaction for β-glucuronidase. We developed a new medium for the rapid isolation of *E coli* O157 : H7 using sorbitol MacConkey agar with cefixime, potassium tellurite and 4-methylumbelliferyl-β-D-glucuronide (MUG) as a primary plating medium. The addition of 20μg/ml of vancomycin in enrichment broth for *E coli* O157 : H7 inhibited lots of Gram positive bacteria. Three strains (10.3%) of 29 non-O157 *E coli* strains and 3 strains (8.3%) of 36 *Salmonella* spp were inhibited at the 0.05μg/ml of cefixime and 23 strains (79.3%) of 29 non-O157 *E coli* strains and 12 strains (33.3%) of 36 *Salmonella* spp were inhibited at the 2.0μg/ml of potassium tellurite. But none of the *E coli* O157 : H7 was affected at these concentration. The addition of MUG at 100μg/ml level to sorbitol MacConkey agar with cefixime and potassium tellurite (CTM-SMAC) aided in the rapid isolation of *E coli* O157 : H7 from samples by checking sorbitol-negative and β-glucuronidase negative phenotypes simultaneously. In conclusion, inoculation of a positive in the O157 screening test from enrichment broth on CTM-SMAC appeared to be a rapid, cost-effective and sensitive method for the isolation of *E coli* O157 : H7.

Key words : *E coli* O157 : H7, vancomycin, cefixime, potassium tellurite, 4-methylumbelliferyl-β-D-glucuronide.

Introduction

E coli O157 : H7 has been recognized as a significant human pathogen. Studies have revealed that *E coli* O157 : H7 is the third or fourth most common enteric pathogen recovered from human stool[1,2]. Hemorrhagic colitis caused by *E coli* O157 : H7 can be fatal to young children and im-

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munocompromised persons, primarily due to complications such as hemolytic uremic syndrome and thrombotic thrombocytopenic purpura[3].

Although 80.3% of *E coli* strains are sorbitol fermenters and approximately 97% are β-glucuronidase positive[4], *E coli* O157 : H7 is sorbitol-negative, β-glucuronidase negative[5,6]. This characteristic has been utilized by both clinicians and food microbiologists to differentiate *E coli* O157 : H7 from other *E coli*[7,8]. Sorbitol MacConkey agar without antimicrobial agents was used as a differential medium for the isolation of *E coli* O157 : H7 from samples[7], but because *E coli* O157 : H7 was usually present in low numbers and accompanied by competitive microflora including other strains of *E coli* and lots of non-sorbitol-fermenting organisms also occurred mostly in large numbers in samples[9], it was common to use a selective medium. A selective medium with cefixime and potassium tellurite was commonly used to isolate *E coli* O157 : H7[10], but it was also needed to check β-glucuronidase activity in the additional step[11].

We developed a new medium for the rapid isolation of *E coli* O157 : H7 using sorbitol MacConkey agar with cefixime, potassium tellurite and 4-methylumbelliferyl-β-D-glucuronide as a primary plating medium.

Materials and Methods

Bacterial strains : Minimum inhibitory concentration of 32 *E coli* O157 : H7 strains was tested ; 3 of these were isolated from bovine feces in Korea and 16 bovine feces isolates and 12 human isolates were kindly provided by Cornell University and *E coli* Reference Center, USA, respectively. *E coli* ATCC 35150 was used as a positive control. Twenty nine non-O157 *E coli* and 36 *Salmonella* standard strains were received from Statens Serum Institute, Denmark and WHO collaborating Center, France, respectively. Sorbitol fermenting *E coli* O157 (H serotyping was not determined), *Aeronomas salmonicida* (FPC 365, Japan), *Klebsiella pneumoniae* (NVRQS 2948, Korea), *Edwardsiella tarda* (FPC 231, Japan), *Citrobacter amalonaticus* (Fairfax hospital, USA), 3 strains of *Staphylococcus aureus* (ATCC 6538, NCTC 7428, NCTC 5663), *Streptococcus pyogenes* (ATCC 21059), *Streptococcus agalactiae* (ATCC 13812), *Streptococcus uberis* (NCTC 2190), *Listeria monocytogenes* (ATCC 19114) were used in this test.

Minimum inhibitory concentration (MIC) : Solutions of the novobiocin (Sigma, St Louis, MO, USA), vancomycin (Sigma) were prepared as described by Anhalt and Washington[12]. Cefixime (Lyphocin ; Lyphomed, Deerfield, USA) was resolved with absolute ethanol (Merck, Darmstadt, Germany) and potassium tellurite solution (Difco, Detroit, MI, USA) was used in commercial products. Depend on the antimicrobial agents, the various dilutions were dispensed in sterile tissue-culture quality, 96-well U-bottomed microtitration plates. Bacterial strains were tested for antimicrobial susceptibility by the modified microbroth dilution methods[13] to the following procedures.

A range of dilutions of antimicrobial agents were prepared in 100μl with tryptic soy broth (TSB) containing 1% glucose and 0.0018% phenol red as a pH indicator. Isolates to be tested were grown in TSB at 37℃ overnight aerobically and these cultures diluted 1 in 100 in 0.85% sterile saline ; 100μl of inoculum were added to and mixed with each dilution of antimicrobial agents to give an inoculum of 5×10^5 organisms/ml. The plates were incubated at 37℃ overnight aerobically. Growth was determined by comparison with the growth control well, the lowest dilution of antimicrobial agents that inhibited growth (no turbidity or color change) was recorded as the MIC.

Media : To make enrichment broth for *E coli* O157 : H7, we used the modified TSB (tryptic soy broth 30g/l, bile salts No. 3 1.5g/l, K_2HPO_4 1.5g/l) containing cefixime (0.05 μg/ml) and vancomycin (20μg/ml) (CV-mTSB).

The primary plating medium was sorbitol MacConkey agar (Difco) containing 0.05μg/ml of cefixime, 2.0μg/ml of potassium tellurite and 100μg/ml of MUG (Biolife, Milano, Italy) (CTM-SMAC).

Isolation procedure of *E coli* O157 : H7 : Fifty ground beef and 50 bovine feces were analyzed by O157 screening methods[14] and the screening positive samples were examined for *E coli* O157 : H7 with CTM-SMAC as shown in Figure 1. Each sample was enriched according to Jung *et al*[14]. After screening of O157 by enzyme immunoassay, the positive

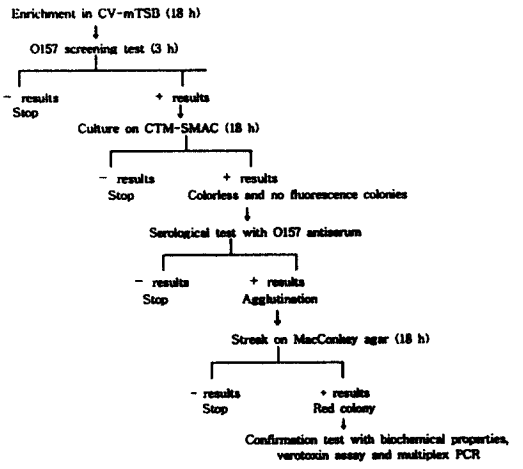

Fig 1. Flowchart on sequence of analyses for *E coli* O157 : H7.

cultures were serially diluted from 10^{-3} to 10^{-5} in 0.85% sterile saline and 0.1ml from dilutions were spread on CTM-SMAC. The plates were incubated at 37℃ overnight aerobically. From the sorbitol negative (colorless) colonies, MUG negative (no fluorescence under UV light) colonies were picked up and taken the slide agglutination with O157 antiserum. Those cultures which were sorbitol negative, MUG negative and O157 positive cultures were streaked on MacConkey agar and then incubated at 37℃ overnight aerobically. To confirm *E coli* O157 : H7 the red colonies on MacConkey agar were tested with biochemical properties, verotoxin assay and multiplex PCR[15] to detect *slt* I, *slt* II, *eae* A, *uid* A genes.

Results

All the bacterial strains were examined in the MIC study. The MICs of 4 antimicrobial agents were shown in Table 1 to 4. Most of the Gram positive bacteria were highly susceptible in vancomycin and novobiocin (Table 1, 2). All *E coli* O157 : H7 strains weren't inhibited in 0.05μg/ml of cefixime but 3 strains (10.3%) of 29 non-O157 *E coli* and 3 strains (8.3%) of 36 *Salmonella* spp were inhibited at the concentration (Table 3). At 2.0μg/ml of potassium tellurite, all *E coli* O157 : H7 strains were not inhibited but 23

Table 1. Number of strains inhibited by each concentration of novobiocin

Bacterial strains (n)	No. of strains with indicated MIC (μg/ml)										
	〉320	320	160	80	40	20	10	5	2.5	1.26	≤0.63
E coli O157 : H7 (23)		2	7	14							
E coli O157 : Hund* (1)			1								
non-O157 *E coli* (29)		3	15	10		1					
Salmonella spp (36)	23	11	2								
Aeromonas salmonicida (1)			1								
Klebsiella pneumoniae (1)			1								
Edwardsiella tarda (1)					1						
Citrobacter amalonaticus (1)		1									
Staphylococcus aureus (3)										3	
Streptococcus pyogenes (1)											1
Streptococcus agalactiae (1)											1
Streptococcus uberis (1)											1
Listeria monocytogenes (1)											1

* H serotyping was not determined.

Table 2. Number of strains inhibited by each concentration of vancomycin

Bacterial strains (n)	No. of strains with indicated MIC (μg/ml)										
	〉640	640	320	160	80	40	20	10	5	2.5	≤1.26
E coli O157 : H7 (23)					21	2					
E coli O157 : Hund* (1)					1						
non-O157 *E coli* (29)			1	10	14	4					
Salmonella spp (36)	9	20	5	1	1						
Aeromonas salmonicida (1)			1								
Klebsiella pneumoniae (1)	1										
Edwardsiella tarda (1)		1									
Citrobacter amalonaticus (1)			1								
Staphylococcus aureus (3)											3
Streptococcus pyogenes (1)											1
Streptococcus agalactiae (1)											1
Streptococcus uberis (1)											1
Listeria monocytogenes (1)											1

* H serotyping was not determined.

Table 3. Number of strains inhibited by each concentration of cefixime

Bacterial strains (n)	No. of strains with indicated MIC (μg/ml)										
	〉0.8	0.8	0.4	0.2	0.1	0.05	0.025	0.013	0.006	0.003	≤0.002
E coli O157 : H7 (32)			6	26							
E coli O157 : Hund* (1)				1							
non-O157 *E coli* (29)		7	9	8	2	3					
Salmonella spp (36)	1		4	11	17	2	1				
Aeromonas salmonicida (1)		1									
Klebsiella pneumoniae (1)					1						
Edwardsiella tarda (1)					1						
Citrobacter amalonaticus (1)					1						

* H serotyping was not determined.

strains (79.3%) of 29 non-O157 *E coli* and 12 strains (33.3 %) of 36 *Salmonella* spp were inhibited (Table 4).

One hundred samples were analyzed during this study (Table 5). Of these, 9 samples with positive results by O157 screening test[14] were subjected to cultural analyses by using CTM-SMAC. *E coli* O157 : H7 was not recovered from the samples but isolated from the bovine feces to which *E coli* O157 : H7 had been added artificially (Fig 2).

Table 4. Number of strains inhibited by each concentration of potassium tellurite

Bacterial strains (n)	No. of strains with indicated MIC (μg/ml)										
	〉16	16	8.0	4.0	2.0	1.0	0.5	0.25	0.13	0.06	≤0.03
E coli O157 : H7 (32)	7	11	8	6							
E coli O157 : Hund* (1)			1								
non-O157 *E coli* (29)			1	5	12	2	2	6	1		
Salmonella spp (36)	1	2	6	15	11				1		
Aeromonas salmonicida (1)	1										
Klebsiella pneumoniae (1)	1										
Edwardsiella tarda (1)		1									
Citrobacter amalonaticus (1)						1					

* H serotyping was not determined.

Table 5. Results from analyses of ground beef and bovine feces for the *E coli* O157 : H7

Sample types	No. tested	No. of positive samples for	
		O157 screening test	O157 : H7 cultural test
Ground beef	50	2	0
Bovine feces	50	7	0

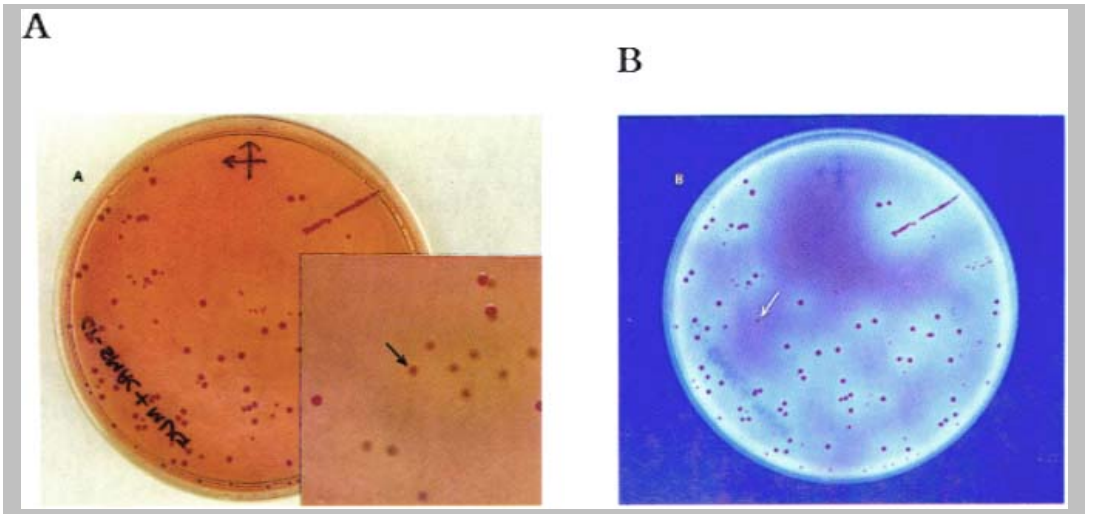

Fig 2. Sorbitol-negative and MUG-negative phenotypes of *E coli* O157 : H7 on the CTM-SMAC. *E coli* O157 : H7 was inoculated artificially in the bovine feces. This sample was enriched for 18h and then diluted with 0.85% sterile saline for spreading on CTM-SMAC. After overnight incubation, *E coli* O157 : H7 was shown as colorless colonies with dark-brown pigment (left arrow) and the inset showed magnification of CTM-SMAC (A). These types of colonies were tested under the 365nm UV light to check the MUG activity (right arrow). The colony indicating right arrow had sorbitol-negative and MUG-negative phenotypes and grew in 0.05μg/ml cefixime and 2.0μg/ml potassium tellurite (B).

Discussion

The goal of this study was to determine the effect of antimicrobial agents and MUG for the isolation of *E coli* O157 : H7 in samples. Most of Gram positive bacteria were highly sensitive in novobiocin or vancomycin (Table 1, 2). We suggested that addition of these kinds of antimicrobial agents into enrichment broth for *E coli* O157 : H7 could suppress the growing of Gram positive bacteria that were existed as microflora or contaminants in samples.

Novobiocin had been used to improve the *E coli* O157 : H7 isolation efficiency in some commercialized media. According to our results, novobiocin was not selective antimicrobial agent for the isolation of the *E coli* O157 : H7, but effective in inhibition Gram positive bacteria like vancomycin (Table 1).

All *E coli* O157 : H7 strains weren't inhibited in 0.05μg/ml of cefixime but 10.3% of non-O157 *E coli* strains and 8.3 % *Salmonella* spp were inhibited at the concentration (Table 3). At 2.0μg/ml of potassium tellurite, 32 *E coli* O157 : H7 strains were not inhibited but 23 strains (79.3%) of 29 non-O157 *E coli* strains and 12 strains (33.3%) of 36 *Salmonella* spp were inhibited (Table 4).

Most of the primary selective media of *E coli* O157 : H7 were based on the sorbitol-fermenting activity but at least 47 enteric bacteria strains formed colorless colonies on the sorbitol MacConkey agar, for example up to 20% non-O157 *E coli*, *Citrobacter amalonaticus*, *Enterobacter agglomerans*, *Morganella morganii*, *Proteus mirabilis* and some of the *Salmonella*, *Serratia*, *Shigella*, *Yersinia* spp[9]. Because of lots of sorbitol non-fermenting strains, it has been a laborious to isolate *E coli* O157 : H7 from the samples. Recently a new selective plating medium was developed, which was based on sorbitol MacConkey agar, but was modified by the inclusion of cefixime and tellurite[16~18]. The inclusion of potassium tellurite[16] and use of 5-bromo-4-chloro-3-indoxyl-β-D-glucuronide[19] in sorbitol MacConkey agar were recommended to increase the rate of isolation of verotoxin producing *E coli* O157 from samples.

After incubation for the primary isolation of *E coli* O157 : H7, many types of colorless colonies were shown on CTM-SMAC. *E coli* O157 : H7 had the same colony size as other *E coli* and dark-brown pigment on the center of the colorless colony on the CTM-SMAC. Most of the pin-point colorless colonies or no dark-brown pigmented colonies were not *E coli* O157 : H7 (Data were not shown). If dark-brown pigmented colorless colonies were shown on CTM-SMAC, they should be checked MUG activity under the 365nm UV light and then slide agglutination test with O157 antiserum for rapid presumptive diagnosis.

In conclusion, inoculation of a positive in the O157 screening test from enrichment broth on CTM-SMAC appeared to be a rapid, cost-effective and sensitive method for the isolation of *E coli* O157 : H7.

References

1. MacDonald KL, O'Leary MJ, Cohen ML, *et al*. *Escherichia coli* O157 : H7, an emerging gastrointestinal pathogen. *JAM*, 259:3567-3570, 1988.
2. Marshall WF, McLimans CA, Yu PKW, *et al*. Results of a 6-month survey of stool cultures for *Escherichia coli* O157 : H7. *Mayo Clin Proc*, 65:787-792, 1990.
3. Kamali MA. Infection by verocytotoxin-producing *Escherichia coli*. *Clin Microbiol Rev*, 2:15-38, 1989.
4. Kilian M, Bulow P. Rapid diagnosis of Enterobacteriaceae. I. Detection of bacterial glycosidases. *Acta Pathol Microbiol Scand*, Sect, B 84:245-251, 1976.
5. Ewing WH. Edwards and Ewing's Identification of Enterobacteriaceae. Elsevier Science Publishing Co. 4th ed, New York, p 95, 1986.
6. Ratnam S, March SB, Ahmed R, *et al*. Characterization of *Escherichia coli* serotype O157 : H7. *J Clin Microbiol*, 26:2006-2012, 1988.
7. March SB, Ratnam S. Sorbitol-MacConkey Medium for detection of *Escherichia coli* O157 : H7 associated with hemorrhagic colitis. *J Clin Microbiol*, 23:869-872, 1986.
8. Wells JG, Davis BR, Wachsmuth IK, *et al*. Laboratory investigation of hemorrhagic colitis outbreaks associated with a rare *Escherichia coli* serotype. *J Clin*

Microbiol, 18:512-520, 1983.

9. Krieg NR. Bergey's Manual of Systematic Bacteriology, Vol 1. Williams and Wilkins, 1984.
10. Bolton FJ, Crozier L, Williamson JK. Isolation of *Escherichia coli* O157 from raw meat products. *Lett Appl Microbiol*, 23:317-321, 1996.
11. Park JH, Vandel NH, Hixon DL, Rapid immunoassay for detection of *Escherichia coli* O157 directly from stool specimens. *J Clin Microbiol*, 34:988-990, 1996.
12. Anhalt JP, Washington II JA. Preparation and storage of antimicrobial solutions. Manual of Clinical Microbiology. American Society for Microbiology. Washington, D.C. 5th ed; 1199-1200, 1991.
13. Sahm DF, Washington II JA. Antibacterial susceptibility tests: dilution methods. Manual of clinical Microbiology. American Society for Microbiology. Washington, D.C. 5th ed; 1105-1116, 1991.
14. Jung BY, Jung SC, Cho DH, *et al*. Development of an enzyme immunoassay for detection of *Escherichia coli* O157 in meat. *Korean J Vet Res*, 38:745-750, 1998.
15. Jung SC, Jung BY, Yoon JW, *et al*. Development of a multiplex-PCR for the rapid detection of *Escherichia coli* O157 : H7 from raw beef. *Korean J Vet Res*, 38:173-181, 1998.
16. Zidik PM, Chapman PA, Siddons CA. Use of tellurite for the selection of verocytotoxigenic *Escherichia coli* O157. *J Med Microbiol*, 39:155-158, 1993.
17. Bolton FJ, Crozier L, Williamson JK. Optimisation of methods for the isolation of *Escherichia coli* O157 from beefburgers. *PHLS Microbiol Dig*, 12:67-70, 1995.
18. Sanderson MW, Gay JM, Hancock DD, *et al*. Sensitivity of bacteriologic culture for detection of *Escherichia coli* O157 : H7 in bovine feces. *J Clin Microbiol*, 33:2616-2619, 1995.
19. Okrend AJG, Rose BE, Lattuada CP. Use of 5-bromo-4-chloro-3-indoxyl-β-D-glucuronide in MacConkey sorbitol agar to aid in isolation of *Escherichia coli* O157 : H7 from ground beef. *J Food Protect*, 53:941-943, 1990.