A THESIS FOR THE DEGREE OF MASTER OF SCIENCE

Development of an Improved Selective Medium for the Detection of *Shigella* spp.

쉬겔라 분리 검출을 위한 향상된 기능의 선택배지 개발

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석사학위논문

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이 논문을 석사학위 논문으로 제출함

2015 년 8 월

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ABSTRACT

Shigella known as a waterborne pathogen causes outbreaks of Shigellosis. The outbreaks have been traced to contaminated water and food. To detect the *Shigella* present in contaminated foods is very important for helping to ensure food hygiene. There are several selective media including MacConkey agar, Xylose-Lysine Deoxycholate agar, Hektoen-Enteric agar approved by The U.S. Food and Drug Administration (FDA) and International Organization for Standardization (ISO) for the isolation of *Shigella* from foods. But conventional culture media for *Shigella* spp. are neither specific nor sensitive. Therefore, I developed an improved selective medium (HEX, HE-Xylose medium) for the detection of *Shigella* spp. from foods. HEX contains lactose, sucrose, D-xylose, and salicin as a differentiation marker. The concentration of bile salts No.3 was reduced to a level of 0.3% which completely inhibited tested gram positive bacteria. All *Shigella* spp. tested (*S. flexneri*, *S. dysenteriae*, *S. boydii*, and *S. sonnei*) produced green colonies on HEX, while *Hafnia alvei*, found to be false positive for *Shigella* on HE agar, appeared as differentiable orange colonies

on HEX. HEX supported a higher recovery of heat- or acid-injured *Shigella* than conventional medium. A total of 300 uninoculated and inoculated food samples were used to evaluate the specificity and sensitivity of HEX. The specificity was 13.50, 38.50, and 83.50 (%) on MacConkey agar, HE agar, and HEX, respectively. The sensitivity was 76.00 and 84.00 (%) for *S. flexneri* and 80.00 and 92.00 (%) for *S. sonnei* on HE agar and HEX, respectively. HEX had superior specificity and sensitivity compared to HE agar. Therefore HEX can be an appropriate selective and differential medium for detection of *Shigella* spp. from foods.

***Keywords: Shigella* spp.; Selective and differential media; Food safety; Detection of foodborne pathogens**

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I. INTRODUCTION

Shigella is gram-negative pathogenic bacterium belonging to the family Enterobacteriaceae responsible for illness outbreaks of shigellosis worldwide. *Shigella* is divided into four species on the basis of serogroup : *S. dysenteriae* (serogroup A), *S. flexneri* (serogroup B), *S. boydii* (serogroup C), and *S. sonnei* (serogroup D). All serogroups of *Shigella* are pathogenic but each one shows a different epidemiology (Warren et al., 2006). The major symptoms of shigellosis include occasional bloody diarrhea, fever, and stomach cramps (CDC, 2014a). Shigellosis, bacillary dysentery, is known to occur worldwide. Public health authorities in the United States reported that shigellosis is the third most common foodborne bacterial infection and about 14,000 cases of shigellosis occur every year in the USA (CDC, 2003 and CDC, 2014a). Because many milder cases are not reported, the Centers for Disease Control and Prevention (CDC) states that the actual number of infections may be greater (CDC, 2014a). In developing countries with relatively poor public health, *Shigella* is especially considered as a critical foodborne pathogens (CDC, 2003). Also, due to increasing numbers of overseas travelers, the numbers of patients contacting shigellosis are increasing (CDC, 2014c). *Shigella* is often reported to be isolated from a variety of foods such as

potato salad, meat, raw oysters, fish, and vegetables (Warren et al., 2006). Thus, it is very important to detect *Shigella* in contaminated foods to ensure food safety.

The Bacteriological Analytical Manual of the U.S. Food and Drug Administration (FDA) recommends MacConkey agar for the isolation of *Shigella* from foods as the conventional culture method (Wallace and Jacobson, 2013). Also, the International Organization for Standardization (ISO) suggests streaking on a combination of three media, including MacConkey agar, Xylose-Lysine Deoxycholate agar (XLD), and Hektoen-Enteric agar (HE agar) after enrichment (ISO, 2004). However, MacConkey agar has very low selectivity for *Shigella* and that problem has been reported for a long time (de Boer, 1998; In et al., 2011; Taylor and Schenlhart, 1971; Uyttendaele et al., 2001; Warren et al., 2005, 2006). In this regard, although XLD is of intermediate selectivity for this pathogen, it usually forms very small or difficult to recognize colonies due to competitive flora also being the same color as the medium (Altwegg et al., 1996). HE agar was developed which has high selectivity but is too stringent for some strains of *Shigella*, due to the high level of selective agents. In addition, it is still difficult to isolate only *Shigella* spp. from HE agar. The necessity to develop a *Shigella* selective medium has been continuously raised but no such medium has yet appeared.

In this study, I selected HE agar as our basal medium because it has the highest selectivity among *Shigella* selective media. Also, HE agar is known to aid recovery of *Shigella* spp. by using a higher concentration of carbohydrates and lower concentrations of toxic indicators such as acid fuchsin and bromothymol blue (Mary Jo Zimbro et al., 2009). Thus the purpose of this study was to develop a newly improved selective medium for *Shigella* spp. (HE-Xylose medium, HEX) and to evaluate and compare the performance of HEX and conventional medium from food samples using stock cultures.

II. MATERIALS AND METHODS

2.1. Stock cultures

Test bacteria were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA), the National Culture Collection for Pathogens (NCCP) (Osong, South Korea), and the bacterial culture collection of Seoul National University (SNCC; Seoul, Korea) for this study. Stock cultures were stored frozen at -80°C.

2.2. Screening of the false-positive bacteria on HE agar from food samples

A variety of 92 food samples, including ground beef, pork, chicken, onions, celery, lettuce, cabbage, spinach, parsley, cucumbers, potatoes, bell peppers, broccoli, cheese, shrimp, short-necked clams, manila clams, and sea cucumbers, were purchased from local retail markets (Seoul, Korea). Each sample (25 g) was homogenized for 2 min with a stomacher (EASY MIX, AES Chemunex, Rennes, France) in sterile stomacher bags (Labplas Inc., Sainte-Julie, Quebec, Canada) containing 225 ml of 0.2% peptone water and incubated at 37^{o}C for 18 h. Samples were 10-fold serially diluted in peptone

water, and 0.1 ml of sample was spread-plated onto HE agar (Oxoid, UK) and incubated at 37^{o}C for 24 h. After incubation, greenish colonies suspected of being false-positive for *Shigella* were identified using the Vitek 2 system (bioMérieux).

2.3. Carbohydrates fermentation test

Characteristics of carbohydrate fermentation patterns of 40 strains of *Shigella* spp, (15 strains of *S. flexneri*, 8 strains of *S. dysenteriae*, 10 strains of *S. boydii*, 7 strains of *S. sonnei*) were determined using the API 50 CH system (bioMérieux SA, Marcy 1'Etoile, France). The test was used according to manufacturer's instructions.

2.4. Growth of test bacteria on medium containing reduced concentration of selective agent

2.4.1. Growth of gram positive bacteria

The growth of gram positive bacteria was tested on modified medium with reduced levels of bile Salts No.3 (Oxoid, UK) from HE agar. The levels of bile salts No.3 tested in modified medium were 9 (normal amount of bile

salts No.3 in HE agar), 5, 3, 2 and 1 g/liter, respectively. A total of 26 strains of gram positive bacteria (Table 3) were incubated in 5 ml of tryptic soy broth (TSB; Difco, Sparks, MD, USA) at 37^{o}C for 24 h. After incubation, one loopful of each culture was streaked for isolation onto each prepared medium with reduced concentrations of bile salts No.3 and incubated at 37^{o}C for 24 h and growth was observed.

2.4.2. Comparison of background mesophilic bacterial populations on food samples

To study the effect of reduced concentrations of selective agent on background mesophilic bacteria of foods, food samples (25 g), including ground beef, pork and chicken, were placed in stomacher bags containing 225 ml of peptone water and incubated at 37^{o}C for 18 h. Samples were 10-fold serially diluted in peptone water after homogenizing with a stomacher for 2 min and 0.1 ml of diluents were spread plated onto each prepared medium with reduced concentrations of bile salt No.3. The plates were incubated at 37^{o}C for 24 h. After incubation, the numbers of colonies were counted.

2.5. Formulation and preparation of new selective medium (HEX)

HEX (HE-Xylose medium) used HE agar as a basal medium. The ingredients of HEX are as follows : 12.0 g of proteose peptone (Merck, Darmstadt, Germany), 3.0 g of yeast extract (Difco), 3.0 g of bile salts No.3 (Oxoid), 12.0 g of lactose (Difco), 12.0 g of sucrose (Difco), 12.0 g of D-xylose (Sigma, USA), 2.0 g of salicin (Sigma, USA), 5.0 g of sodium chloride (Daejung Chemicals & Metals co. Ltd, Gyonggido, Korea), 5.0 g of sodium thiosulfate (Duchefa Biochemie, Haarlem, The Netherlands), 1.5 g of ferric ammonium citrate (Acros Organics, NJ, USA), 14.0 g of agar (Difco), 65.0 mg bromothymol blue (Sigma, USA) and 0.1 g of acid fuchsin (Sigma, USA) per liter. These ingredients were added to 1 L of distilled water and the preparation was heated with agitation just until the medium boiled. The medium was cooled to 50^{o}C, and poured into 9-cm-diameter petri dishes.

2.6. Comparison of HEX with HE agar

A total of 40 strains of *Shigella* (15 *S. flexneri*, 8 *S. dysenteriae*, 10 *S. boydii*, and 7 *S. sonnei*) and 72 strains of *H. alvei* were incubated in 5 ml of Tryptic Soy Broth (TSB; Difco, Becton Dickinson, Sparks, MD, USA) at 37°C for 24 h. After incubation, one loopful of each culture was streaked for

isolation onto HE agar and HEX and incubated at 37°C for 24 h. The color of colonies was compared and recorded. Colonies suspected of being *Shigella* spp. were defined as green colonies on both media.

2.7. Recovery of injured Shigella on HE agar and HEX

2.7.1. Bacterial cultures and cell suspension

Three strains each of *S. flexneri* (NCCP 10852, NCCP 11251, and NCCP 14744), *S. dysenteriae* (NCCP 10097, NCCP 10101, and NCCP 14746), *S. boydii* (NCCP 10098, NCCP 10342, and NCCP 11190), and *S. sonnei* (NCCP 11180, NCCP 11204, and NCCP 11220) were provided by the National Culture Collection for Pathogens (NCCP) (Osong, South Korea). Each strain of *Shigella* was cultured in Tryptic Soy Broth (TSB; Difco, Becton Dickinson, Sparks, MD, USA) at 37^{o}C for 24 h, collected by centrifugation at 4000×g for 20 min at 4^{o}C, and washed three times with 0.2% Peptone Water (PW; Bacto, Becton, Dickinson, Sparks, MD, USA). The final pellets were resuspended in sterile PW, corresponding to approximately 10^{7}-10^{8} CFU/ml.

2.7.2. Recovery of heat-injured Shigella spp.

To investigate the recovery of heat-injured *Shigella* spp., 1 ml of suspension was added to test tubes containing 5 ml of PW that had been preheated at 60^{o}C and held for 1 min in water bath. After heating, test tube was removed immediately from water bath and serially 10-fold diluted in PW, and 0.1 ml aliquots of diluted samples was spread plate onto Tryptic Soy Agar (TSA; Difco, Becton Dickinson, Sparks, MD, USA), HE agar, and HEX, respectively. All plates were incubated at 37^{o}C for 24 h.

2.7.3. Recovery of acid-injured Shigella spp.

To evaluate the recovery of acid-injured *Shigella* spp., 1 ml of suspension was added to test tubes containing 5 ml of PW that was adjusted to pH 2.38 with 0.5% lactic acid (w/w) and held for 3 min. After treatment, cell suspension was diluted serially 10-fold with PW, and 0.1 ml aliquots was spread plate onto TSA, HE agar, and HEX, respectively. All plates were incubated at 37^{o}C for 24 h.

2.8. Assessment of the performance of HEX using contaminated food samples

2.8.1. Evaluation of the specificity of HEX

A total of 200 naturally contaminated food samples for evaluating specificity were tested. These food samples consisting of beef, pork, chicken, bacon, ham, sausage, milk, cheese, parsley, onions, spinach, celery, lettuce, cabbage, tomatoes, potatoes, bean sprouts, chili, bell peppers, broccoli, radish, eggplant, cucumbers, green pumpkins, mushrooms, sweet potatoes, carrots, fresh salad, potato salad, sandwiches, shrimp, manila clams, cuttlefish, oysters, shellfish, snails, and mussels were purchased from local retail markets (Seoul, Korea). To evaluate the specificity, 25 g or 25 ml subsamples of each food was homogenized with a stomacher (EASY MIX, AES Chemunex, Rennes, France) in sterile stomacher bags (Labplas Inc., Sainte-Julie, Quebec, Canada) containing 225 ml of *Shigella* broth supplemented novobiocin with 0.5 mg/liter (MB cell, Seoul, Korea) for 2 min. The enrichment broth was prepared according to manufacturer's directions. After enrichment at 37^{o}C for 18 h, one loopful of each sample was streaked for isolation onto MacConkey agar (Difco), HE agar, and HEX and incubated at 37^{o}C for 24 h. After incubation, the number of false positive

samples was counted. Colonies suspected of being *Shigella* were defined as colorless on MacConkey agar and greenish on HE agar and HEX. The specificity was evaluated by calculating the proportion of *Shigella* negative samples correctly found to be negative.

2.8.2. Evaluation of the sensitivity of HEX

A total of 100 inoculated food samples were tested for evaluating sensitivity. These food samples were selected among the same food samples for the test of specificity. All foods that were used in the inoculated food sample test were previously screened to ensure they were *Shigella* free. To prepare inoculated food samples, each strain of *S. flexneri* (ATCC 25929, NCCP 10852, and NCCP 14744) and *S. sonnei* (ATCC 9290, ATCC 29930, and NCCP 10935) was grown in 5 ml of TSB at 37^{o}C for 24 h. Twenty-five g or 25 ml of each food were inoculated with the 3 different *S. flexneri* and *S. sonnei* strains at an inoculum level of 3-4 log CFU/25 g or ml. Inoculated samples were introduced into a sterile stomacher bags containing 225 ml *Shigella* broth and homogenized with a stomacher for 2 min. After enrichment at 37^{o}C for 18 h, one loopful of each sample was streaked for isolation onto HE agar and HEX and incubated at 37^{o}C for 24 h. After incubation, 5 colonies suspected of being *Shigella* on these media were

selected for identification. Bacterial colonies were identified using the Vitek 2 system (bioMérieux). The sensitivity was calculated as the proportion of *Shigella* positive samples correctly found to be positive (Park et al., 2014).

2.9. Statistical analysis

All experiments were repeated three times and data were converted to log CFU/ml. Data were analyzed by ANOVA using the Statistical Analysis System (SAS Institute, Cary, NC, USA). Significant means ($P < 0.05$) were separated using Duncan's multiple range test.

III. RESULTS

3.1. Screening of the false-positive bacteria on HE agar from food samples

Background mesophilic bacteria that grew on HE agar were isolated from various food samples and identified. Many microorganisms such as *Hafnia alvei*, *Pseudomonas* spp., *Acinetobacter* spp., *Escherichia hermanii*, *Klebsiella oxytoca*, *Morganella morganii*, *Serratia* spp. and others except *Shigella* grew on HE agar and were suspected of being false-positive (Table 1). Of 92 uninoculated food samples, 108 false-positive colonies were isolated and 72 strains were identified as *H. alvei*. The isolated *H. alvei* strains were used in the following experiments.

Table 1. False-positive bacteria on HE agar

Bacteria	Number
Acinetobacter group	4
Aeromonas group	3
Buttiauxella agrastis	1
Enterobacter group	4
Escherichia hermannii	1
Escherichia fergusonii	2
Escherichia vulneris	1
Hafnia alvei	72
Klebiella oxytoca	4
Morganella morganii	1
Providenoia rettgeri	1
Psedomonas aeruginosa	2
Psedomonas fluorescens	2
Psedomonas putida	2
Raoultella ornithinolytica	1
Serratia liquefaciens group	2
Yoknella regensburgei	2
Total	108

3.2. Characteristics of carbohydrate-fermenting Shigella spp.

Carbohydrate-fermenting abilities of 40 strains of *Shigella* were evaluated using the API 50 CH system. As shown in Table 2, none of the tested *Shigella* strains could ferment lactose, sucrose, salicin, and D-Xylose during 24 and 48 h of incubation.

Table 2. Fermentation of carbohydrates by *Shigella* spp.

Carbohydrate	Number of strains[a]			
	S. flexneri (n=15)	*S. dysenteriae* (n=8)	*S. boydii* (n=10)	*S. sonnei* (n=7)
Lactose	0[b]	0	0	0
Sucrose	0	0	0	0
Salicin	0	0	0	0
D-Xylose	0	0	0	0

[a] n, number of strains examined.
[b] Number of strains that fermented each carbohydrates.

3.3. Growth of gram positive bacteria and comparison of the number of background mesophilic bacteria in food samples on medium containing reduced concentrations of selective agent

A total of 26 gram positive bacteria were tested for their ability to grow on media containing different concentrations of bile salt No.3 (Table 3). None of the *Listeria* strains were able to grow on medium containing any tested level (0.9 - 0.1%) of bile salts No.3. However, *Staphylococcus aureus* ATCC 13565, *Lactobacillus reuteri* ATCC 23272, and *Bifidobacterium adolescentis* ATCC 15703 grew on medium containing 0.2% bile salts No.3. Medium containing 0.3% bile salts No.3 completely inhibited the growth of all tested gram positive bacteria.

Also, background mesophilic bacteria from food samples plated on media with reduced concentrations of selective agents were enumerated (Table 4). Overall, there were no significant differences in total bacterial counts on media containing bile salts No.3 from 0.9% (original medium) to 0.3%.

Table 3. Growth of gram positive bacteria on medium with reduced concentrations of bile salts No.3

Tested bacteria	Source[a]	Growth[b] on medium with varying concentrations of bile salts No.3 (%)				
		0.9	0.5	0.3	0.2	0.1
Bacillus cereus ATCC 13061	ATCC	-	-	-	-	-
Staphylococcus aureus ATCC 6538	ATCC	-	-	-	-	-
Staphylococcus aureus ATCC 13565	ATCC	-	-	-	+	+
Staphylococcus aureus ATCC 29213	ATCC	-	-	-	-	-
Listeria innocua SNCC 1	SNCC	-	-	-	-	-
Listeria innocua SNCC 2	SNCC	-	-	-	-	-
Listeria innocua ATCC 51742	ATCC	-	-	-	-	-
Listeria innocua ATCC 33090	ATCC	-	-	-	-	-
Listeria monocytogenes ATCC 19111	ATCC	-	-	-	-	-
Listeria monocytogenes ATCC 19115	ATCC	-	-	-	-	-
Listeria monocytogenes ATCC 19116	ATCC	-	-	-	-	-
Listeria monocytogenes ATCC 19118	ATCC	-	-	-	-	-
Listeria monocytogenes ATCC 15313	ATCC	-	-	-	-	-
Listeria grayi NCCP 10879	NCCP	-	-	-	-	-
Listeria welshimeri NCCP 10965	NCCP	-	-	-	-	-
Streptococcus mutans 1	SNCC	-	-	-	-	-
Streptococcus mutans 2	SNCC	-	-	-	-	-
Pediococcus pentosaceus	SNCC	-	-	-	-	-
Aerococcus viridans	SNCC	-	-	-	-	-
Lactobacillus brevis ATCC 14869	ATCC	-	-	-	-	-
Lactobacillus plantarum	ATCC	-	-	-	-	-
Lactobacillus reuteri ATCC 23272	ATCC	-	-	-	+	+
Weissella confuse ATCC 10881	ATCC	-	-	-	-	-
Bifidobacterium adolescentis ATCC 15703	ATCC	-	-	-	+	+
Bifidobacterium animalis ATCC 25527	ATCC	-	-	-	-	+
Bifidobacterium breve ATCC 15700	ATCC	-	-	-	-	+

[a] ATCC, American Type Culture Collection; NCCP, National Culture Collection for Pathogens; SNCC, Seoul National University Culture Collection.
[b] +, growth; -, no growth.

Table 4. Enumeration (log CFU/g) of background mesophilic bacteria of food samples on medium with reduced concentrations of bile salts No.3

Sample	Concentrations of bile salt No.3 (%)			
	0.9	0.7	0.5	0.3
Ground beef	5.23 ± 0.55 a	5.49 ± 0.54 a	5.67 ± 0.09 a	5.66 ± 0.11 a
Pork	4.75 ± 0.65 a	4.94 ± 0.58 a	5.10 ± 0.28 a	5.27 ± 0.25 a
Chicken	6.32 ± 0.76 a	6.65 ± 0.32 a	6.88 ± 0.43 a	6.97 ± 0.37 a

a Means ± standard deviations from three replications. Values followed by the same letters within the row per concentration of bile salts No.3 are not significantly different ($P > 0.05$).

3.4. Comparison of HEX with HE agar

A total of 112 strains (15 *S. flexneri*, 8 *S. dysenteriae*, 10 *S. boydii*, 7 *S. sonnei* and 72 *H. alvei*) were streaked for isolation onto HE agar and HEX (Table 5). On HE agar, all *Shigella* spp. (n=40) and *H. alvei* (n=72) formed green colonies when incubated for 24 h. On HEX, *Shigella* also formed green colonies but no false-positive results occurred with *H. alvei* which formed orange colonies on this medium.

Fig. 1 shows colonies of *Shigella* spp. and *H. alvei* formed on HE agar and HEX. *Shigella* spp. produced typical green colonies on HE agar (Fig. 1A) and HEX (Fig. 1B) after 24 h of incubation. However, *H. alvei* produced green colonies on HE agar (Fig. 1A) and orange colonies on HEX (Fig. 1B).

Table 5. Colony colors of stock cultures on HE agar and HEX

Strains	Strain[a]	Colony colors on	
		HE agar	HEX
Shigella spp.			
Shigella flexneri	NCCP 10107	green	green
S. flexneri	NCCP 10108	green	green
S. flexneri	NCCP 10111	green	green
S. flexneri	NCCP 10114	green	green
S. flexneri	NCCP 10115	green	green
S. flexneri	NCCP 10116	green	green
S. flexneri	NCCP 10117	green	green
S. flexneri	NCCP 10612	green	green
S. flexneri	NCCP 10852	green	green
S. flexneri	NCCP 10853	green	green
S. flexneri	NCCP 10855	green	green
S. flexneri	NCCP 11203	green	green
S. flexneri	NCCP 11251	green	green
S. flexneri	NCCP 14744	green	green
S. flexneri	NCCP 15744	green	green
Shigella dysenteriae	NCCP 10097	green	green
S. dysenteriae	NCCP 10101	green	green
S. dysenteriae	NCCP 10103	green	green
S. dysenteriae	NCCP 10104	green	green
S. dysenteriae	NCCP 10105	green	green
S. dysenteriae	NCCP 10341	green	green
S. dysenteriae	NCCP 10344	green	green
S. dysenteriae	NCCP 14746	green	green
Shigella boydii	NCCP 10098	green	green
S. boydii	NCCP 10245	green	green
S. boydii	NCCP 10342	green	green
S. boydii	NCCP 10426	green	green
S. boydii	NCCP 10554	green	green
S. boydii	NCCP 10614	green	green
S. boydii	NCCP 10616	green	green
S. boydii	NCCP 10854	green	green
S. boydii	NCCP 11190	green	green
S. boydii	NCCP 14745	green	green
Shigella sonnei	NCCP 10875	green	green
S. sonnei	NCCP 10935	green	green
S. sonnei	NCCP 11180	green	green

(Continued)

Table 5 (Continued)

S. sonnei	NCCP 11204	green	green
S. sonnei	NCCP 11220	green	green
S. sonnei	NCCP 11221	green	green
S. sonnei	NCCP 14743	green	green
Hafnia alvei	SNCC 1	green	orange
H. alvei	SNCC 2	green	orange
H. alvei	SNCC 3	green	orange
H. alvei	SNCC 4	green	orange
H. alvei	SNCC 5	green	orange
H. alvei	SNCC 6	green	orange
H. alvei	SNCC 7	green	orange
H. alvei	SNCC 8	green	orange
H. alvei	SNCC 9	green	orange
H. alvei	SNCC 10	green	orange
H. alvei	SNCC 11	green	orange
H. alvei	SNCC 12	green	orange
H. alvei	SNCC 13	green	orange
H. alvei	SNCC 14	green	orange
H. alvei	SNCC 15	green	orange
H. alvei	SNCC 16	green	orange
H. alvei	SNCC 17	green	orange
H. alvei	SNCC 18	green	orange
H. alvei	SNCC 19	green	orange
H. alvei	SNCC 20	green	orange
H. alvei	SNCC 21	green	orange
H. alvei	SNCC 22	green	orange
H. alvei	SNCC 23	green	orange
H. alvei	SNCC 24	green	orange
H. alvei	SNCC 25	green	orange
H. alvei	SNCC 26	green	orange
H. alvei	SNCC 27	green	orange
H. alvei	SNCC 28	green	orange
H. alvei	SNCC 29	green	orange
H. alvei	SNCC 30	green	orange
H. alvei	SNCC 31	green	orange
H. alvei	SNCC 32	green	orange
H. alvei	SNCC 33	green	orange
H. alvei	SNCC 34	green	orange

(Continued)

Table 5 (Continued)

H. alvei	SNCC 35	green	orange
H. alvei	SNCC 36	green	orange
H. alvei	SNCC 37	green	orange
H. alvei	SNCC 38	green	orange
H. alvei	SNCC 39	green	orange
H. alvei	SNCC 40	green	orange
H. alvei	SNCC 41	green	orange
H. alvei	SNCC 42	green	orange
H. alvei	SNCC 43	green	orange
H. alvei	SNCC 44	green	orange
H. alvei	SNCC 45	green	orange
H. alvei	SNCC 46	green	orange
H. alvei	SNCC 47	green	orange
H. alvei	SNCC 48	green	orange
H. alvei	SNCC 49	green	orange
H. alvei	SNCC 50	green	orange
H. alvei	SNCC 51	green	orange
H. alvei	SNCC 52	green	orange
H. alvei	SNCC 53	green	orange
H. alvei	SNCC 54	green	orange
H. alvei	SNCC 55	green	orange
H. alvei	SNCC 56	green	orange
H. alvei	SNCC 57	green	orange
H. alvei	SNCC 58	green	orange
H. alvei	SNCC 59	green	orange
H. alvei	SNCC 60	green	orange
H. alvei	SNCC 61	green	orange
H. alvei	SNCC 62	green	orange
H. alvei	SNCC 63	green	orange
H. alvei	SNCC 64	green	orange
H. alvei	SNCC 65	green	orange
H. alvei	SNCC 66	green	orange
H. alvei	SNCC 67	green	orange
H. alvei	SNCC 68	green	orange
H. alvei	SNCC 69	green	orange
H. alvei	SNCC 70	green	orange
H. alvei	SNCC 71	green	orange
H. alvei	SNCC 72	green	orange

[a] NCCP, National Culture Collection for Pathogens; SNCC, Seoul National University Culture Collection.

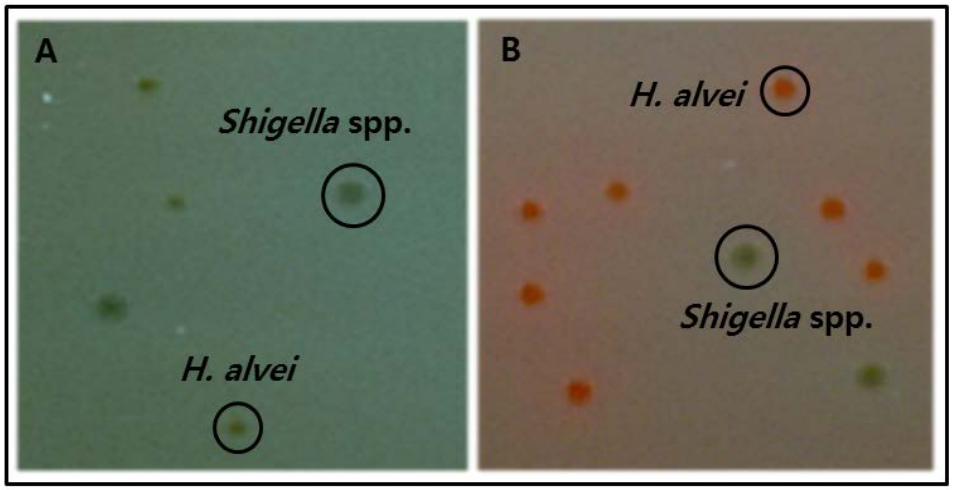

Fig. 1. Colonies produced by *Shigella* spp. and *H. alvei* on HE agar (A) and HEX (B). The two species could not be differentiated on HE agar (A). *Shigella* spp. produced green colonies while *H. alvei* produced orange colonies on HEX (B).

3.5. Recovery of heat-injured Shigella spp.

Table 6 shows recovery abilities of injured *Shigella* spp. after heat treatment at 60^{o}C for 1 min on HE agar and HEX. Tryptic Soy Agar (TSA) was used for control medium. Compared to TSA (8.14 log CFU/ml), there were no significant ($P > 0.05$) differences in recovery of heat-injured *S. flexneri* on HEX (7.46 log CFU/ml). However, *S. flexneri* were represented significantly ($P < 0.05$) low recovery abilities on HE agar (6.61 log CFU/ml). Heat-injured *S. dysenteriae* and *S. sonnei* on HEX did not lead to complete recovery compared to TSA, but showed significantly ($P < 0.05$) higher recovery than HE agar. Heat-injured *S. boydii* represented a similar level of recovery on HE agar and HEX. Overall, HEX medium supported a higher recovery of heat-injured *Shigella* spp. than HE agar.

Table 6. The recovery of heat (60°C for 1 min) injured *Shigella* spp. on HE agar and HEX

Strains	Medium[b]		
	TSA	HE agar	HEX
S. flexneri	8.14 ± 0.11 A[a]	6.61 ± 0.69 B	7.46 ± 0.08 A
S. dysenteriae	7.05 ± 0.19 A	4.78 ± 0.13 C	5.64 ± 0.30 B
S. boydii	7.00 ± 0.15 A	4.90 ± 0.65 B	5.22 ± 0.56 B
S. sonnei	6.69 ± 0.53 A	4.52 ± 0.24 C	5.56 ± 0.27 B

[a] log CFU/ml. Means with different letters within the row are significantly different ($P < 0.05$).

[b] TSA, Tryptic Soy Agar; HE agar, Hektoen-Enteric agar; HEX, HE-Xylose medium.

3.6. Recovery of acid-injured Shigella spp.

Table 7 represents recovery of acid-injured *Shigella* spp. after treatment with 0.5% lactic acid for 3 min on HE agar and HEX. There was a significant ($P < 0.05$) difference in the number of acid-injured all tested *Shigella* spp. among three media. After acid treatment, the level of recovery for *S. flexneri* showed 7.83, 7.02, and 7.52 log CFU/ml on TSA, HE agar, and HEX, respectively. The acid-injured *S. dysenteriae* were recovered by 7.16, 5.71, and 6.45 log CFU/ml on TSA, HE agar, and HEX, respectively. *S. boydii* were recovered by 7.76, 5.04, and 5.95 on TSA, HE agar, and HEX, respectively after acid treatment. Following acid treatment, injured *S. sonnei* were recovered by 7.57, 5.26, and 6.35 on TSA, HE agar, and HEX, respectively. For all tested *Shigella* spp., TSA resulted in the highest recovery. And HEX supported greater recovery of acid-injured *Shigella* spp. than HE agar.

Table 7. The recovery of acid (0.5% lactic acid for 3 min) injured *Shigella* spp. on HE agar and HEX

Strains	Medium[b]		
	TSA	HE agar	HEX
S. flexneri	7.83 ± 0.19 A[a]	7.02 ± 0.14 C	7.52 ± 0.03 B
S. dysenteriae	7.16 ± 0.21 A	5.71 ± 0.34 C	6.45 ± 0.30 B
S. boydii	7.76 ± 0.08 A	5.04 ± 0.43 C	5.95 ± 0.59 B
S. sonnei	7.57 ± 0.22 A	5.26 ± 0.29 C	6.35 ± 0.10 B

[a] log CFU/ml. Means with different letters within the row are significantly different ($P < 0.05$).

[b] TSA, Tryptic Soy Agar; HE agar, Hektoen-Enteric agar; HEX, HE-Xylose medium.

3.7. Assessment of the performance of HEX using uninoculated and inoculated food samples

Table 8 and Table 9 show results of specificity and sensitivity of HEX medium using uninoculated and inoculated food samples, respectively. Out of 200 uninoculated food samples, no *Shigella* strains were isolated on any of the tested media so the number of negative samples was equal to the number of total food samples. The specificity of HEX (83.50%) was the highest followed by HE agar (38.50%) and MacConkey agar (13.50%). Among 200 food samples, totals of 173, 123 and 33 false-positive results were observed on MacConkey agar, HE agar, and HEX, respectively. Based on 100 inoculated food samples, the sensitivity of HE agar and HEX, were 76.0% and 84.0%, respectively, for *S. flexneri*, and 80.0% and 92.0%, respectively, for *S. sonnei*. The sensitivity of HEX was shown to be superior to that of HE agar for *S. flexneri* and *S. sonnei*.

Table 8. Specificity of HEX compared with those of MacConkey agar and HE agar in microbiological analysis of 200 uninouculated food samples

Medium	No. of results		Specificity (%)[a]
	True negative	False positive	
MacConkey agar	27	173	13.50
HE agar	77	123	38.50
HEX	167	33	83.50

[a] (No. of true-negative results on this medium/ no. of negative samples)×100.

Table 9. Sensitivity of HEX compared with HE agar for *S. flexneri* and *S. sonnei* from 100 inoculated food samples

Species	Medium	No. of results		Sensitivity (%)[a]
		True positive	False negative	
S. flexneri	HE agar	38	12	76.00
	HEX	42	8	84.00
S. sonnei	HE agar	40	10	80.00
	HEX	46	4	92.00

[a] [No. of true positives/(no. of true positives + no. of false negatives)]×100.

IV. DISCUSSION

The selective detection of *Shigella* in foods is difficult due to their affinity to *Escherichia coli* and the lack of distinctive biochemical activity (Wallace and Jacobson, 2013). For conventional plating, there are many selective media used to detect *Shigella* spp. such as MacConkey agar, Tergitol-7 agar (T7-agar), XLD, *Salmonella-Shigella* Agar (SSA), and HE agar. But the limitations of currently used selective media have nevertheless been raised in many studies (Taylor and Schenlhart, 1971; Uyttendaele et al., 2001; Warren et al., 2006). Based on inherent problems of existing media and the steady occurrence of outbreaks reported earlier, I developed an improved selective medium for *Shigella* spp.

As a first step, I screened the background microorganisms from foods to search for false positive bacteria growing on HE agar. *Hafnia alvei*, *Pseudomonas* spp., *Acinetobacter* spp. *E. hermanii*, *Klebsiella oxytoca*, *Morganella morganii*, and *Serratia* spp. are similar in appearance to colonies of *Shigella* on HE agar (Table 1). Several studies also reported similar results (Altwegg et al., 1996; In et al., 2011; Taylor and Schenlhart, 1971; Uyttendaele et al., 2001). In the present study, *H. alvei* accounted for the highest proportion of 108 false-positive colonies, so it is necessary to

differentiate *Shigella* from *H. alvei*. Therefore, I tried to selectively distinguish *Shigella* spp. from *H. alvei* on new medium.

I compared carbohydrates fermentation between *Shigella* spp. and *H. alvei.* Carbohydrates such as lactose, sucrose, and salicin which HE agar contains, are not used by *Shigella* spp. HE agar includes these carbohydrates to differentiate fermenters (coliforms) from non-fermenters such as *Shigella* by including pH indicators. Farmer et al. (1985) reported that *H. alvei* can ferment several carbohydrates such as D-mannitol, L-arabinose, L-rhamnose, maltose, D-xylose, trehalose, and D-mannose. I found that most *Shigella* could not ferment D-xylose and confirmed that *H. alvei* was able to. D-xylose is an inexpensive, easily available carbohydrate and widely used as a medium component. In this study, D-xylose was added to HE agar for the differentiation of *Shigella* spp. from *H. alvei*. By difference in characteristics of carbohydrate utilizing ability, I observed that colonies of *H. alvei* appeared orange on HEX medium and thus could be easily differentiated from green colonies of *Shigella* spp. Also, I expected to distinguish other bacteria which show up as false positives on HE agar such as *E. hermanii*, *Klebsiella oxytoca*, and some *Serratia* spp. which according to Farmer et al. (1985) can also ferment D-xylose.

Most selective media for the Enterobacteriaceae contain selective agents such as bile salts, and sodium deoxycholate in order to inhibit gram positive

bacteria and background bacteria. But these selective agents discourage growth of injured *Shigella* strains resulting from exposure to severe environments like a food matrix (Tollison and Johnson, 1985 and Uyttendaele et al., 2001). Smith and Buchanan (1992) recommended using MacConkey agar, XLD, and SSA instead of HE agar due to high levels of selective agents in the latter. Tollison and Johnson (1985) suggested that media for recovering heat stressed *S. flexneri* should be formulated to contain less than 0.85% bile salts. Based on these previous studies, I reduced the concentration of bile salt No.3 from 0.9% to 0.3% which completely inhibited tested gram positive bacteria without affecting background gram negative bacteria. Consequently, HEX supported a higher recovery of heat- or acid-injured *Shigella* than conventional medium (Table 6 and Table 7). In addition, HEX shows higher sensitivity for *S. flexneri* and *S. sonnei* than HE agar (Table 9).

Studies on performance of conventional media for isolation of *Shigella* from foods are few. Unacceptably high numbers of false positive colonies (colorless) on MacConkey agar were observed. In contrast, the number of identified bacteria producing false positive green colonies was certainly diminished but *H. alvei* comprised the majority of presumptive *Shigella* colonies (green) on HE agar. In addition to *H. alvei*, other false positive bacteria were isolated from foods using HEX, and were identified using

Vitek 2 system. They included *Morganella morganii, Pseudomonas putida*, *Acinetobacter* group, *Aeromonas salmonicida*, *Shewanella putrefaciens*, *Providencia rustigianii*, and *Vibrio parahaemolyticus* (data not shown). In et al. (2011) reported that for species that are indistinguishable on selective medium, the occurrence of false positives could be minimized by using an enrichment broth selectively. Even though it still yields approximately 16.5% false positives, HEX has superior effectiveness compared to traditional media for isolating *Shigella* spp. The specificity of HEX medium (83.50%) was much superior to that of HE agar (38.50%) and MacConkey agar (13.50%). This high specificity of HEX medium contributes to a reduction in the number of colonies to be subjected to the confirmation test, which can reduce labor, expense, and time.

Regarding the specificity of HEX medium, no *Shigella* spp. were isolated from uninoculated foods on any of the media tested. Thus, in order to examine the sensitivity of this new medium, I inoculated foods with *Shigella* followed by observing the recovery of *Shigella* spp. According to recent research, *S. sonnei* and *S. flexneri* account for the largest percentage of *Shigella* infections having high incidence rates (CDC, 2014b). Therefore, *S. flexneri* and *S. sonnei* were used for assessment of sensitivity. Isolation of *Shigella* from foods is very fastidious due to many different physical attributes such as composition and natural microbial flora (Doyle and

Beuchat, 2007). Our study also indicated that the bacterial flora of foods were able to overgrow *Shigella* spp. on the test media at low levels of inoculum (around 1-2 log CFU/25 g or ml) (data not shown). Uyttendaele et al. (2001) reported on current enrichment and isolation media but did not achieve reliable detection of *Shigella* in foods at low inoculum levels. The study of Zhang and Lampel (2010) involved inoculated *Shigella* spp. populations of 4.8 log CFU/g of food. Based on the previous studies, I inoculated *Shigella* at 3-4 log CFU/25 g or ml. Our study showed that HEX had 8% higher sensitivity than HE agar for *S. flexneri,* and 12% higher sensitivity for *S. sonnei* under the same conditions (Table 9). In addition, the sensitivity of *S. sonnei* was higher than that of *S. flexneri* on both media. This is similar to the results of Uyttendaele et al. (2001) and Warren et al. (2006) who explained that *S. sonnei* has better resistance to stress and competitive flora than *S. flexneri*.

In light of existing limitations, Chromogenic *Shigella* spp. Plating Medium (CSPM) and rapid alternative technologies, such as immunological methods and molecular methods, which have high specificity and sensitivity have been recently developed (Warren et al., 2006). However, these technologies are costly and the latter also require skilled labor and sophisticated instrumentation. Also, an enrichment method for sufficient increase of *Shigella* for detection is still required, as is the case with the

conventional plating method, and thus requires more time and involves high cost (Lindqvis, 1999; Uyttendaele et al., 2001; Warren et al., 2006). Therefore, an effective and appropriate selective conventional culture medium for isolating *Shigella* that offers reasonable cost, ease of use and familiarity is still needed (Gracias and Mckillip, 2004). In addition, many laboratories rely on the conventional plating method for maintaining quality control and assurance of foods.

In conclusion, HEX medium, consisting of HE agar modified with the addition of D-xylose and with a reduced concentration of bile salts, is very sensitive to grow *Shigella* spp. and also has greater specificity than approved conventional media. Therefore, unlike traditional media, HEX can easily distinguish HE false-positive bacteria from most *Shigella* spp. and may provide effectiveness and practicality for detection and isolation of *Shigella* spp. from foods.

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VI. 국문초록

쉬겔라는 그람음성의 병원균으로서 세균성 설사질환을 야기하는 이질균이다. 쉬겔라는 혈청군에 따라 4 가지 그룹으로 분류되며 주로 비 위생적인 환경에서 오염된 물이나 식품을 통해 식중독을 일으킨다. 미국 FDA 에서는 쉬겔라 검출 배지로 MacConkey agar 를 공인 배지로 지정하고 있고, 국제표준화기구 (ISO)와 우리나라 국립보건원에서는 단독배지 사용시 분리 효율이 낮은 단점 때문에 MacConkey 배지, Xylose lysine desoxycholate 배지 (XLD), *Salmonella-Shigella* 배지, Hektoen-Enteric 배지(HE 배지)를 병용하여 사용하는 것을 권장하고 있다. 그러나 이 배지들은 선택성 혹은 민감도가 낮다는 문제점이 꾸준히 제기되고 있고, 그 중에서 선택성이 가장 높은 배지로 알려져 있는 HE 배지에서도 여전히 쉬겔라 균으로 오인하는 허위양성 (false positive) 결과가 많이 보고되고 있다. 따라서 본 연구에서는 기존배지의 단점을 보완하여 높은 민감도와 선택성을 가지는 새로운 쉬겔라 선택배지를 개발하였다. 기존 HE 배지에서 가장 높은 허위양성결과를 나타내는 균으로서 *Hafnia alvei* 를

찾아내었고, 쉬겔라와 *H. alvei* 의 탄소이용능 차이와 선택제의 함량을 낮춤으로써 쉬겔라 균의 회수를 증가시키는 원리를 이용하였다. 분별마커로서 D-xylose, Lactose, Sucrose, Slaicin 을 첨가하였으며, 선택물질인 bile salt No.3 함량을 기존 0.9% 수준에서 0.3% 수준으로 낮추었다. 총 40 개의 쉬겔라 균과 식품에서 분리해 낸 72 개의 *H. alvei* 를 HE 배지와 새로운 배지에 streaking 하여 24 시간 배양한 결과, 새로운 배지에서 *H. alvei* 는 쉬겔라 균과는 달리 D-xylose 를 이용함으로써 산을 생성하고 주변의 pH 를 낮추어 배지에 함유되어 있는 pH 지시약에 의해 오렌지 색 집락을 형성하였다. 따라서 기존 HE 배지에서 쉬겔라와 *H. alvei* 가 모두 녹색의 집락을 형성해 분별되지 않았던 문제점을 해결함으로써, 효과적으로 쉬겔라를 분리해 낼 수 있었다. 또한 열과 산 처리를 통해 손상된 쉬겔라 균의 회복력을 실험해 본 결과, 기존배지에 비해 새로 개발된 배지에서 모두 유의적으로 높은 회복능을 나타내었다. 다양한 식품 샘플을 이용한 배지의 민감도 검증 실험에서는, 새로 개발된 배지에서의 민감도가 기존배지 보다 더 높게 나타났다. 선택배지의 위양성결과를 나타내는 척도인 특이도 실험에서는 MacConkey 배지에서

13.50%, HE 배지에서 38.50%, 새로 개발된 배지에서는 83.50%를 나타냄으로써, 새로운 배지의 선택성이 기존배지에 비해 월등히 뛰어난 것을 확인하였다. 따라서 본 연구를 통해 개발된 배지는 기존 배지의 단점을 보완하여 그 기능을 향상시킴으로써, 식품으로부터 쉬겔라를 검출해 내는데 HE 배지를 대체하여 유용하게 사용될 수 있을 것이다.

주요어: 쉬겔라 균, 선택분별배지, 식품안전성, 식중독 균 검출

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