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Development of an improved selective medium for the detection of *Shigella* spp.

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ABSTRACT

We developed an improved selective medium (HEX medium, modified Hektoen Enteric agar medium) for the detection of *Shigella* spp. from foods. Conventional culture media for *Shigella* spp. are neither specific nor sensitive. HEX medium 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 (*Shigella flexneri*, *Shigella dysenteriae*, *Shigella boydii*, and *Shigella sonnei*) produced green colonies on HEX medium, while *Hafnia alvei*, found to be false positive for *Shigella* on HE agar, appeared as differentiable orange colonies on HEX medium. A total of 300 uninoculated and inoculated food samples were used to evaluate the specificity and sensitivity of HEX medium. The specificity was 13.50, 38.50, and 83.50 (%) on MacConkey agar, HE agar, and HEX medium, 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 medium, respectively. HEX medium had superior specificity and sensitivity compared to HE agar. Therefore HEX medium can be an appropriate selective and differential medium for detection of *Shigella* spp. from foods.

1. 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: *Shigella dysenteriae* (serogroup A), *Shigella flexneri* (serogroup B), *Shigella boydii* (serogroup C), and *Shigella sonnei* (serogroup D). All serogroups of *Shigella* are pathogenic but each one shows a different epidemiology (Warren, Parish, & Schneider, 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 & Jacobson, 2013, chap. 6). 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, Ha, & Oh, 2011, In, Ha, Kim, & Oh, 2011; Taylor & Schenlhart, 1971; Uyttendaele, Bagamboula, De Smet, Wilder, & Debevere, 2001; Warren, Parich, & Schneider, 2005,

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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, Buser, & von Graevenitz, 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. on HE agar. The necessity to develop a *Shigella* selective medium has been continuously raised but no such medium has yet appeared.

In this study, we 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, Power, Miller, Wilson, & Johnson, 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.

2. 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. Isolation and identification of background mesophilic bacteria of food samples and confirmation of carbohydrate fermentation of Shigella

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 °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 °C for 24 h. After incubation, greenish colonies suspected of being false-positive for *Shigella* were identified using the Vitek 2 system (bioMérieux). Characteristics of carbohydrate fermentation patterns of 40 strains of *Shigella* spp, were determined using the API CH50 system (bioMérieux).

2.3. Growth of gram positive bacteria and comparison of background mesophilic bacterial populations on food samples on medium containing reduced concentrations of selective agent

The growth of gram positive bacteria was tested on modified medium with reduced levels of Oxoid Bile Salts No.3 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 2) were incubated in 5 ml of tryptic soy broth (TSB; Difco, Sparks, MD, USA) at 37 °C for 24 h. After incubation, one loopful of each culture was streaked for isolation onto each prepared medium and incubated at 37 °C for 24 h and growth was observed. 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 °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. The plates were incubated at 37 °C for 24 h. After incubation, the numbers of colonies were counted.

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

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) and 0.1 g of acid fuchsin (Sigma) 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 °C, and poured into 9-cm-diameter petri dishes.

2.5. Comparison of HE agar with HEX

A total of 40 strains of *Shigella* (15 *S. flexneri*, 8 *S. dysenteriae*, 10 *S. boydii* and 7 *S. sonnei*) and 72 strains of *Hafnia alvei* were incubated in 5 ml of tryptic soy broth (TSB; Difco) 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.

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

A total of 300 food samples (200 uninoculated food samples for evaluating specificity, 50 food samples inoculated with *S. flexneri* and 50 food samples inoculated with *S. sonnei* for evaluating sensitivity) 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, South 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 with 0.5 mg/L novobiocin (MB cell, Seoul, Korea) for 2 min. The enrichment broth was prepared according to manufacturer's directions. After enrichment at 37 °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 °C for 24 h. After incubation, the number of false positive samples was counted. 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 °C for 24 h. Twenty-five g or 25 ml of each food were inoculated with the

Table 1
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 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 °C for 18 h, one loopful of each sample was streaked for isolation onto HE agar and HEX and incubated at 37 °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. 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. The sensitivity was calculated as the proportion of *Shigella* positive samples correctly found to be positive (Park, Chang, Ryu, & Kang, 2014).

2.7. 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.

Table 2
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.

3. Results

3.1. Isolation and identification of background mesophilic bacteria of food samples and characteristics of carbohydrate-fermenting Shigella

Background mesophilic bacteria that grew on HE agar were isolated from various food samples and identified. Many microorganisms such as *H. 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 (data not shown). Of 92 uninoculated food samples, 108 false-positive colonies were isolated and *H. alvei* accounted for around 70% of the total. The isolated *H. alvei* strains were used in the following experiments. Carbohydrate-fermenting abilities of 40 strains of *Shigella* were evaluated using the API CH 50 system. As shown in Table 1, none of the tested *Shigella* strains could ferment lactose, sucrose and salicin during 24 and 48 h of incubation.

3.2. 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 medium containing different concentrations of bile salts No.3 (Table 2). 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 medium with reduced concentrations of selective agents were enumerated (Table 3). Overall, there were no significant differences in total bacterial counts on medium containing bile salts No.3 from 0.9% (original medium) to 0.3%.

Table 3
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 ($P > 0.05$).

3.3. 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 4). 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).

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

Tables 5 and 6 show results of specificity and sensitivity of HEX media 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 50 inoculated food samples, the sensitivity of HE agar, and HEX, was 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*.

4. 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 & Jacobson, 2013, chap. 6). For conventional plating, there are many selective media used to detect *Shigella* spp. such as MacConkey agar, Tergitol-7 agar (T7-agar), XLD, SSA and HE agar. But the limitations of currently used selective media have nevertheless been raised in many studies (Taylor & 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, we developed an improved selective medium for *Shigella* spp.

As a first step, we screened the background microorganisms from foods to search for false-positive bacteria growing on HE agar. *H. alvei*, *Pseudomonas* spp., *Acinetobacter* spp. *E. hermanii*, *K. oxytoca*, *M. morganii*, and *Serratia* spp. are similar in appearance to colonies of *Shigella* (data not shown). Several studies also reported similar results (Altwegg et al., 1996; In, Ha, et al., 2011, In, Ha, Kim, et al, 2011; Taylor & 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, we tried to selectively distinguish *Shigella* spp. from *H. alvei* on new medium.

We 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, D-mannose. We found that most *Shigella* could not ferment D-xylose and confirmed that *H. alvei* was

Table 4
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
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

Table 4 (*continued*)

Strains	Strain[a]	Colony colors on	
		HE agar	HEX
H. alvei	SNCC 33	green	orange
H. alvei	SNCC 34	green	orange
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.

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*. The *H. alvei* produced acid by using D-xylose as a carbon source. The area *H. alvei* is present had lowered pH and orange colors by indicators such as bromothymol blue and acid fuchsin. We observed that colonies of *H. alvei* appeared orange on HEX medium and thus could be easily differentiated from green colonies of *Shigella* spp. Also, we expected to distinguish other bacteria which show up as false-positives on HE agar such as *E. hermanii*, *K. oxytoca*, and some *Serratia* spp. which according to Farmer et al. (1985) can also ferment D-xylose.

Table 5
Specificity of HEX compared with those of MacConkey agar and HE agar in microbiological analysis of 200 uninoculated 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 6
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.

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. Bile salts can dissolve membrane structure such as membrane lipids and proteins (Begley, Gahan, & Hill, 2005). Commonly, it is known that gram negative bacteria inherently more resistant to bile salts than gram positive bacteria (Mary Jo Zimbro et al., 2009). But these selective agents discourage growth of injured *Shigella* strains resulting from exposure to severe environments like a food matrix (Tollison & Johnson, 1985, 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

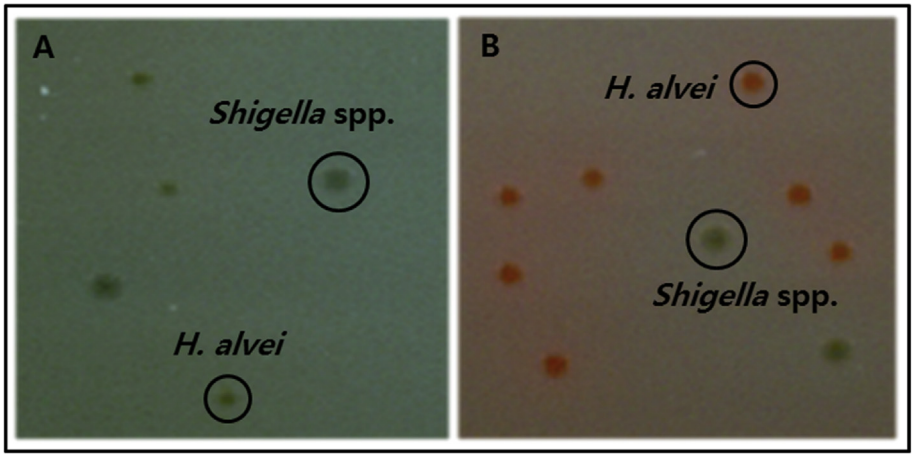

Fig. 1. Colonies produced by *Shigella* spp. and *H. alvei* on HE agar (A) and HEX (B). The two species could not be differentiated on HE agar (A). *Shigella* spp. produced green colonies while *H. alvei* produced orange colonies on HEX (B).

recovering heat stressed *S. flexneri* should be formulated to contain less than 0.85% bile salts. Based on these previous studies, we reduced the concentration of bile salt No.3 from 0.9% to 0.3% which completely inhibited tested gram positive bacteria without affecting background bacteria. Consequently, HEX shows higher sensitivity for *S. flexneri* and *S. sonnei* than HE agar (Table 6).

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 and *H. alvei* comprised the majority of presumptive *Shigella* colonies (green) on HE agar (data not shown). In addition to *H. alvei*, other false positive bacteria were isolated from foods using HEX, and were identified using Vitek 2 system. They included *M. morganii*, *Pseudomonas putida*, *Acinetobacter group*, *Aeromonas salmonicida*, *Shewanella putrefaciens*, *Providencia rustigianii*, and *Vibrio parahaemolyticus* (data not shown). In, Ha, et al. (2011), In, Ha, Kim, et al (2011) explained that false positive results attributed to *M. morganii* may be minimized by using 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 (83.5%) was much superior to that of HE agar (38.5%) and MacConkey agar (13.5%). 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, we 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). Thus *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 & 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, we inoculated *Shigella* at 3–4 log CFU/25 g or ml. Our study showed that HEX had 8% higher selectivity than HE agar for S. *flexneri*, and 12% higher selectivity for *S. sonnei* under the same conditions (Table 6). 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 & 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* 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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