Journal of Clinical Microbiology

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J. Clin. Microbiol. **2012, 50(10):3222. DOI: 10.1128/JCM.01228-12.**
Published Ahead of Print 18 July 2012.

JCM
Journals.ASM.org

Development of an Improved Selective and Differential Medium for Isolation of *Salmonella* spp.

Sang-Hyun Park, Sangryeol Ryu, and Dong-Hyun Kang

Department of Food and Animal Biotechnology, Department of Agricultural Biotechnology, Center for Agricultural Biomaterials, and Research Institute for Agriculture and Life Sciences, Seoul National University, Seoul, Republic of Korea

We describe an improved selective, differential, and cost-effective medium, XA medium, which contains D-arabinose, to facilitate the selective isolation of *Salmonella* spp. The sensitivity and the specificity of XA medium were compared to those of xylose lysine desoxycholate agar (XLD) using stock cultures and naturally contaminated food samples. XA medium and XLD were evaluated with a total of 82 *Salmonella* and 69 non-*Salmonella* stock cultures. Of 82 strains of *Salmonella* spp. tested, 76 produced a characteristic black colony on XA medium and XLD. The remaining 6 strains belonged to *Salmonella enterica* serovars Berta ($n = 1$), Paratyphi A ($n = 1$), Gallinarum ($n = 2$), and Pullorum ($n = 2$). The sensitivities of XA medium and XLD were identical (92.7%). *Citrobacter freundii* ($n = 21$) and *Proteus mirabilis* ($n = 21$) stock cultures produced black colonies on XLD, whereas only 4 strains of *P. mirabilis* appeared as black colonies on XA medium. In the second phase of the study, a total of 180 food samples were cultured onto XA medium and XLD after selective enrichment. The sensitivities of XA medium and XLD were equal (100%), and a total of 6 *Salmonella* strains were isolated from the 180 food samples. The specificity of XA medium (92.0%) was superior to that of XLD (73.0%), with a total of 14 and 47 false-positive results found on XA medium and XLD, respectively. On the basis of its good specificity, XA medium is useful for the isolation of *Salmonella* spp. from food samples.

Salmonella has been associated with many food-borne diseases across the world (19). It is the cause of an estimated 1.4 million illnesses annually in the United States (25). Various foods, such as chicken, beef, and pork, have been implicated in outbreaks caused by *Salmonella* spp. (8, 27, 33). Thus, effective methods for the isolation of *Salmonella* spp. from various foods are important to ensure food quality and safety. The choice of a suitable sampling procedure combined with a sensitive culture method is important for the successful detection of *Salmonella* (4).

The use of selective and differential plating media is a simple method for the isolation of *Salmonella* spp. A wide variety of selective and differential media has been developed for this purpose, including xylose lysine desoxycholate agar (XLD), Hektoen enteric (HE) agar, and bismuth sulfite (BS) agar (6). XLD and HE agar are the most popular media for isolating *Salmonella* spp., and their differentiation abilities rely on characteristics of *Salmonella*, such as hydrogen sulfide production and the nonfermentation of lactose (28). However, these characteristics are shared with other microorganisms, such as *Proteus* and *Citrobacter* (11, 32). Thus, numerous false-positive results are observed on these media which require further confirmation testing, a time-consuming and labor-intensive activity (13). BS agar is the medium of choice for the isolation of *Salmonella enterica* serovar Typhi, and it is used for the isolation of atypical salmonellae, such as those which ferment lactose (7). However, BS agar has several disadvantages, such as low sensitivity and long incubation time for development of the characteristic colony morphology (18).

Several chromogenic media have been developed to increase the specificity of conventional selective and differential media for the detection of *Salmonella* spp. (14, 21, 26, 23, 31). These media incorporate chromogenic substrates which are metabolized by *Salmonella* spp. (29). Although chromogenic media have higher specificities than conventional media, some of them have a low sensitivity (10, 13, 30), which results in more false negatives observed on these media. Also, chromogenic media are relatively expensive, making them less appropriate for routine laboratory use (24).

Recognizing the limits of currently used selective and differential media, it is desirable to improve the specificity and the sensitivity of the medium while maintaining cost-effectiveness. Particularly, it is desirable to differentiate *Salmonella* spp. from *Proteus* spp., as well as from *Citrobacter* spp. Hydrogen sulfide production depends on several factors, such as the sulfide production rate of the microorganisms, the oxygen concentration in the colony, pH, and the iron concentration in the medium (28). Acid production by microorganisms in consequence of carbohydrate fermentation could inhibit hydrogen sulfide production (3).

This study yielded XA medium, which contains D-arabinose as a differential agent and neutral red as a pH indicator to differentiate *Salmonella* from *Citrobacter freundii*, *Proteus mirabilis*, and other enteric bacteria. The effectiveness of XA medium was compared to that of XLD using stock cultures and naturally contaminated food samples.

MATERIALS AND METHODS

Stock cultures. *S. enterica* serovar Typhimurium (ATCC 19585, ATCC 43971, ATCC 700408), *S. enterica* serovar Enteritidis ATCC 13078, *C. freundii* ATCC 8090, *Escherichia coli* (ATCC 8739, ATCC 11775, ATCC 25922), *E. coli* O157:H7 (ATCC 35150, ATCC 43889, ATCC 43890), *Hafnia alvei* (ATCC 13337, ATCC 29926, ATCC 29927), *Yersinia enterocolitica* (ATCC 9610, ATCC 23715, ATCC 55075), *Cronobacter sakazakii* (ATCC 12868, ATCC 29004), *Klebsiella pneumonia* ATCC 13883, *Shigella*

Received 9 May 2012 Returned for modification 1 June 2012
Accepted 16 July 2012
Published ahead of print 18 July 2012
Address correspondence to Dong-Hyun Kang, kang7820@snu.ac.kr.

doi:10.1128/JCM.01228-12

flexneri (ATCC 12022, ATCC 29903), and *Pseudomonas aeruginosa* (ATCC 15692, ATCC 27853) were obtained from the American Type Culture Collection (ATCC; Manassas, VA). *S. enterica* serovar Agona NCCP 12231, *S. enterica* serovar Berta NCCP 10270, *S. enterica* serovar California NCCP 10319, *S. enterica* serovar Chester NCCP 10317, *S. enterica* serovar Derby NCCP 12238, *S. enterica* serovar Dublin NCCP 10860, *S. enterica* serovar Florida NCCP 10361, *S. enterica* serovar Gallinarum NCCP 10323, *S. enterica* serovar Heidelberg NCCP 10322, *S. enterica* serovar Illinois NCCP 10437, *S. enterica* serovar Infantis NCCP 12233, *S. enterica* serovar London NCCP 10831, *S. enterica* serovar Maarssen NCCP 10951, *S. enterica* serovar Montevideo NCCP 12211, *S. enterica* serovar Newington NCCP 10440, *S. enterica* serovar Newport (NCCP 10325, NCCP 12235), *S. enterica* serovar Oranienburg NCCP 10441, *S. enterica* serovar Panama NCCP 10333, *S. enterica* serovar Pullorum NCCP 10335, *S. enterica* serovar SaintPaul NCCP 10329, *S. enterica* serovar Senftenberg NCCP 12240, *S. enterica* serovar Schottmuelleri NCCP 10324, *S. enterica* serovar Schwarzergrund NCCP 12212, *S. enterica* serovar Sloterdijk NCCP 10331, *S. enterica* serovar Stanley NCCP 10332, *S. enterica* serovar Tennessee NCCP 10861, *S. enterica* serovar Thompson NCCP 11011, and *S. enterica* serovar Weltevreden NCCP 12239 were obtained from the National Culture Collection for Pathogens (NCCP; Osong, South Korea). *S. enterica* serovar Choleraesuis (KCCM 13076, KCCM 14028), *S. enterica* subsp. *arizonae* (KCCM 41035, KCCM 41575), *S. enterica* subsp. *salamae* (KCCM 41651, KCCM 41762), *S. enterica* subsp. *indica* KCCM 41759, *S. enterica* subsp. *houtenae* KCCM 41760, and *S. enterica* subsp. *diarizonae* KCCM 41761 were obtained from the Korean Culture Center of Microorganisms (KCCM; Seoul, South Korea). *S.* Gallinarum KVCC 1457, *S.* Heidelberg KVCC 0506, *S.* Pullorum KVCC 2509, *S.* Senftenberg KVCC 0590, *S.* Tennessee KVCC 0592, and *S. enterica* serovar Virchow KVCC 0595 were obtained from the Korea Veterinary Culture Collection (KVCC; Anyang, South Korea). The remaining strains were isolates obtained from the bacterial culture collection of the Food Hygiene Laboratory at Seoul National University (SNCC; Seoul, South Korea). They were stored frozen at −80°C.

Preparation of the basal medium. Xylose lysine desoxycholate agar (XLD; Difco, Sparks, MD) was used as a base for the development of XA medium. The ingredients of the basal medium were as follows: 3.75 g D-xylose (Fluka, Paris, France), 5.0 g of L-lysine (Junsei Chemical Co. Ltd., Tokyo, Japan), 7.5 g of lactose (Difco), 7.5 g of sucrose (Difco), 5.0 g of sodium chloride (Samchun Chemical Co. Ltd., Pyeongtaeksi, South Korea), 3.0 g of yeast extract (Difco), 2.5 g of sodium desoxycholate (Difco), 6.8 g of sodium thiosulfate (Junsei Chemical), 0.8 g of ferric ammonium citrate (Acros Organics, NJ), 15.0 g of Bacto agar (Difco), and 0.03 g of neutral red (Samchun Chemical) per liter. Various quantities of D-arabinose (7.5, 9.0, or 10.5 g/liter) (Fluka) were added to the basal medium to optimize the concentration of this component. The ingredients were added to distilled water, and the preparation was heated with agitation just until the medium boils. The medium was cooled to 50°C and poured into 9-cm-diameter petri dishes.

Evaluation of the effect of D-arabinose concentration. Test bacteria (82 strains of *Salmonella*, 21 strains of *C. freundii*, and 21 strains of *P. mirabilis*) were incubated in 10 ml of tryptic soy broth (TSB; Difco) at 37°C for 18 h. After incubation, one loopful (ca. 10 μl) of each culture was streaked onto XLD and each of the basal media (containing 7.5, 9.0, or 10.5 g/liter D-arabinose) to obtain single, isolated colonies and incubated at 37°C for 48 h. The colors of colonies were compared and recorded after 24, 36, and 48 h. The experiments were repeated twice with each tested strain. Colonies suspected of being *Salmonella* spp. were defined as black colonies on both XLD and the basal media.

The specificity testing of XA medium with other Gram-negative bacteria. XA medium was prepared with the previously determined optimum D-arabinose concentration. Twenty-seven strains of Gram-negative bacteria (see Table 2) were incubated in 10 ml of TSB at 37°C for 18 h. After incubation, one loopful of each culture was streaked onto XLD and XA medium. The plates were incubated at 37°C for 48 h, and the colors of colonies were compared and recorded. The experiments were repeated twice with each tested strain.

Assessment of the performance of XA medium using naturally contaminated food samples. The conventional culture method described by the U.S. Food and Drug Administration (FDA) was used for the microbiological analysis of naturally contaminated foods (1). A total of 180 samples consisting of chicken ($n = 80$), ground beef ($n = 50$), and ground pork ($n = 50$) were purchased from local retail markets (Seoul, South Korea). Each sample (25 g) 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 lactose broth (Difco) for 2 min and incubated at 37°C for 22 h. One hundred microliters of preenriched culture was transferred to 10 ml of Rappaport-Vassiliadis (RV) medium (Difco) and incubated at 42°C for 24 h. After selective enrichment, one loopful of each RV culture was streaked onto XLD and XA medium and incubated at 37°C for 24 h. After incubation, a maximum of 5 colonies suspected of being *Salmonella* on XLD and XA medium were selected for identification. Bacterial colonies were identified using the API 20E system (bioMérieux SA) and the *Salmonella* latex agglutination kit (Oxoid Ltd., Cambridge, United Kingdom). The specificity was evaluated by calculating the proportion of *Salmonella*-negative samples correctly found to be negative (i.e., those that did not appear as *Salmonella*-like colonies).

RESULTS

A total of 124 strains (82 strains of *Salmonella*, 21 strains of *C. freundii*, and 21 strains of *P. mirabilis*) were streaked onto each basal medium (containing 7.5, 9.0, or 10.5 g/liter D-arabinose) (Table 1). At a D-arabinose concentration of 7.5 to 10.5 g/liter, most *Salmonella* strains produced typical black colonies after 24 h of incubation, except *S.* Paratyphi A, *S.* Berta, *S.* Gallinarum, and *S.* Pullorum. *S.* Paratyphi A, *S.* Gallinarum, and *S.* Pullorum produced colorless colonies, and *S.* Berta appeared as pink colonies on each basal medium. *Salmonella* strains belonging to *S. enterica* serovars California, Chester, Maarssen, and Montevideo produced pink colonies with black centers on each basal medium after 24 h of incubation. Also, 3 strains of *S.* Enteritidis, *S.* Florida, and *S.* London appeared as pink colonies with black centers at a D-arabinose concentration of ≥9.0 g/liter. After 36 h of incubation, these strains appeared as black colonies with no pink halo. *S.* Typhi ($n = 1$) produced colonies with small black centers on both XLD and XA medium.

C. freundii produced no false-positive results on any of the basal media within 48 h of incubation. Four strains of *P. mirabilis* produced pink colonies with small black centers on the basal medium containing 7.5 g/liter of D-arabinose after 24 h of incubation, and among these, two strains produced pink colonies with small black centers on the basal medium containing 9.0 g/liter of D-arabinose. However, all *P. mirabilis* strains failed to produce black colonies at a D-arabinose concentration of 10.5 g/liter. The final XA medium formulation contained 7.5 g/liter of D-arabinose. The sensitivities (the ratio of true positives over the number of true positives and false negatives) of XA medium and XLD were identical (92.7%). Twenty-seven strains of other Gram-negative bacteria (Table 2) did not produce false-positive results on either XLD or XA medium.

Figure 1 shows that colonies of *S.* Typhimurium, *C. freundii*, and *P. mirabilis* formed on XLD and XA medium. *S.* Typhimurium (Fig. 1A) produced typical black colonies on XLD after 24 h of incubation. However, *C. freundii* (Fig. 1B) produced black colonies or black colonies surrounded by yellow halos, and *P. mirabilis* (Fig. 1C) appeared as black colonies on XLD, similar to those

TABLE 1 Colony color reactions of *Salmonella* spp., *C. freundii*, and *P. mirabilis* on XLD and XA medium

Strain	No. of strains tested	No. of strains that showed black colonies[a] on:			
		XLD	XA medium with D-arabinose concn (g/liter) of:		
			7.5	9.0	10.5
Salmonella enterica subsp. *enterica* serovars					
S. Typhimurium	17	17	17	17	17
S. Enteritidis	15	15	15	15	15
S. Paratyphi A	1	0	0	0	0
S. Paratyphi B	1	1	1	1	1
S. Paratyphi C	2	2	2	2	2
S. Agona	1	1	1	1	1
S. Berta	1	0	0	0	0
S. California	1	1	1	1	1
S. Chester	1	1	1	1	1
S. Choleraesuis	2	2	2	2	2
S. Derby	1	1	1	1	1
S. Dublin	2	2	2	2	2
S. Florida	1	1	1	1	1
S. Gallinarum	2	0	0	0	0
S. Heidelberg	2	2	2	2	2
S. Illinois	1	1	1	1	1
S. Infantis	1	1	1	1	1
S. London	1	1	1	1	1
S. Maarssen	1	1	1	1	1
S. Montevideo	1	1	1	1	1
S. Newington	1	1	1	1	1
S. Newport	2	2	2	2	2
S. Oranienburg	1	1	1	1	1
S. Panama	1	1	1	1	1
S. Pullorum	2	0	0	0	0
S. SaintPaul	1	1	1	1	1
S. Senftenberg	2	2	2	2	2
S. Schottmuelleri	1	1	1	1	1
S. Schwarzergrund	1	1	1	1	1
S. Sloterdijk	1	1	1	1	1
S. Stanley	1	1	1	1	1
S. Tennessee	2	2	2	2	2
S. Thompson	1	1	1	1	1
S. Typhi	1	1	1	1	1
S. Virchow	1	1	1	1	1
S. Weltevreden	1	1	1	1	1
Other *Salmonella enterica* subsp.					
S. enterica subsp. *arizonae*	2	2	2	2	2
S. enterica subsp. *salamae*	2	2	2	2	2
S. enterica subsp. *indica*	1	1	1	1	1
S. enterica subsp. *houtenae*	1	1	1	1	1
S. enterica subsp. *diarizonae*	1	1	1	1	1
Enterobacteriaceae					
C. freundii	21	21	0	0	0
P. mirabilis	21	21	4	2	0

[a] Expected *Salmonella* spp. color reactions: XLD, black; XA medium, black.

TABLE 2 Colony colors of Gram-negative bacteria on XLD and XA medium

Strain	No. of strains tested	No. of strains that showed black colonies[a] on:	
		XLD	XA medium
Escherichia coli	7	0	0
E. coli O157:H7	3	0	0
Hafnia alvei	5	0	0
Yersinia enterocolitica	3	0	0
Cronobacter sakazakii	3	0	0
Klebsiella pneumoniae	2	0	0
Shigella flexneri	2	0	0
Pseudomonas aeruginosa	2	0	0

[a] Expected *Salmonella* spp. color reactions: XLD, black; XA medium, black.

of *Salmonella*. *S.* Typhimurium (Fig. 1D) appeared as black colonies on XA medium, whereas *C. freundii* and *P. mirabilis* produced pink colonies (Fig. 1E and F).

Table 3 represents results of the bacteriological analysis of naturally contaminated food samples. A total of 6 *Salmonella* spp. were isolated on both XLD and XA medium from the same food samples (sensitivity, 100%) (data not shown). The specificity of XA medium (92.0%) was superior to that of XLD (73.0%), with a total of 14 and 47 false-positive results found on XA medium and XLD, respectively. They consisted of *P. mirabilis* on XA medium and *P. mirabilis* and *C. freundii* on XLD. No false-positive results with *C. freundii* were observed on XA medium. All strains of *P. mirabilis* that produced false-positive results on XA medium also produced false-positive results on XLD.

DISCUSSION

Consumption of contaminated foods of animal origin is responsible for the great majority of salmonellosis cases (31). Plating on XLD, HE agar, and BS agar after preenrichment followed by selective enrichment in RV medium or tetrathionate (TT) broth has been recommended for the isolation of *Salmonella* from foods by the U.S. FDA (1). However, colonies of *C. freundii* and *Proteus* spp., such as *P. mirabilis*, may resemble these of *Salmonella* due to hydrogen sulfide production on HE agar (2). Although XLD has a high sensitivity and specificity, *Proteus* and *Citrobacter* produce colonies indistinguishable from those of *Salmonella* on this medium (6, 28, 32).

Acid formation in consequence of carbohydrate fermentation may affect hydrogen sulfide production (3, 28). Veron and Gasser (34) reported that under the acid conditions of carbohydrate metabolism, H_2S-positive *Enterobacteriaceae* were unable to produce the black precipitate of iron sulfide. In the present study, the carbohydrate-fermenting abilities of 15 strains each of *C. freundii* and *P. mirabilis* were examined using the API CH50 system (bioMérieux SA, Marcy 1′Etoile, France) for screening additional carbohydrate sources (data not shown). *C. freundii* and *P. mirabilis* strains commonly fermented D-arabinose, ribose, galactose, glucose, *N*-acetylglucosamine, and trehalose. Among these carbohydrates, we selected D-arabinose to add to XA medium in addition to D-xylose, lactose, and sucrose, which are contained in XLD, because most major *Salmonella* spp., including *S.* Typhimurium, did not ferment D-arabinose (data not shown).

The final XA medium formulation contained 7.5 g/liter of D-

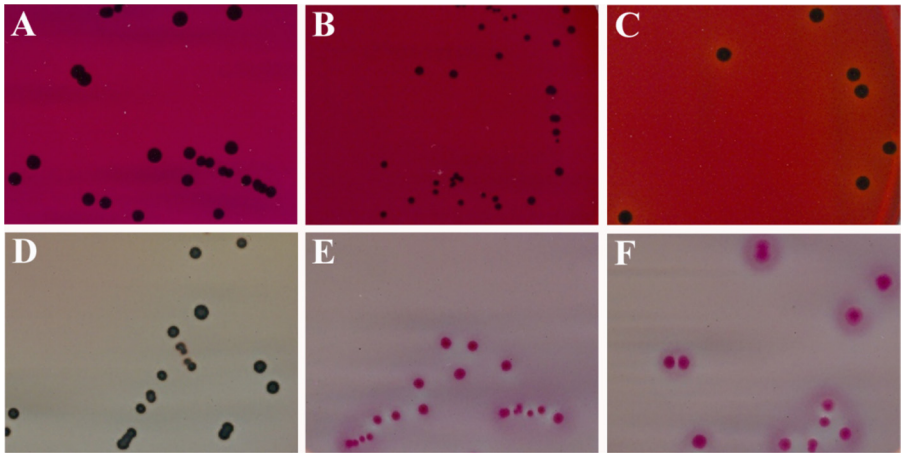

FIG 1 Colonies produced by *S.* Typhimurium, *C. freundii*, and *P. mirabilis* on XLD (A, B, and C) and XA medium (D, E and F). Colonies of *S.* Typhimurium (A), *C. freundii* (B), and *P. mirabilis* (C) appeared as black colonies on XLD. On XA medium, *S.* Typhimurium (D) produced black colonies, whereas *C. freundii* (E) and *P. mirabilis* (F) produced pink colonies.

arabinose in spite of some false-positive results with *P. mirabilis* strains, because some *Salmonella* strains which fermented D-arabinose produced pink colonies with black centers after 24 h of incubation. It is assumed that these *Salmonella* strains produced an initial acidification of the medium due to fermentation of D-arabinose. Then, these strains apparently produced an alkaline reversion in XA medium because of decarboxylation of lysine (7, 12). D-Arabinose may delay alkaline reversion by these strains, but blackening of the colonies of these strains was obvious after 36 h. This suggests that XA medium should be examined within 36 h after inoculation.

All *C. freundii* stock cultures ($n = 21$) that produced black colonies on XLD appeared as pink colonies on XA medium. Also, no false-positive results due to *C. freundii* were observed on XA medium from naturally contaminated food samples, whereas false-positive results were observed on XLD. XA medium was shown to be more specific than XLD for the differentiation of *Salmonella* from *P. mirabilis*, despite some false-positive results with *P. mirabilis* which were still observed on XA medium. Although *P. mirabilis* fermented D-arabinose, it rarely fermented lactose or sucrose contained in XA medium. It is quite likely that the false-positive results with *P. mirabilis* strains were caused by the fact that the amount of acid formation was not sufficient to inhibit hydrogen sulfide production (22). Some *C. freundii* and *P. mirabilis* strains showed variability in the size of black center of colonies on XLD and XA medium. However, positive or negative reactions were identical for each test.

Incorporating D-arabinose does not affect the sensitivity of the medium. The sensitivities were identical for both XA medium and XLD. *S.* Paratyphi A ($n = 1$), *S.* Berta ($n = 1$), *S.* Gallinarum ($n = 2$), and *S.* Pullorum ($n = 2$) produced H_2S-negative colonies on XA medium and XLD. *S.* Paratyphi A and *S.* Berta are known as hydrogen sulfide negative; thus, their colonies do not appear as black on media that detect hydrogen sulfide formation (7, 17). *S.* Gallinarum and *S.* Pullorum rarely produce hydrogen sulfide, and the reaction occurred slowly (5). Also the hydrogen sulfide-generating ability of *S.* Typhi is weak or negative (17). On XA medium, *S.* Typhi produced black colonies, but the black centers were smaller than those of other *Salmonella* strains.

All strains of *S.* Typhimurium ($n = 17$) and *S.* Enteritidis ($n = 15$) were easily detected on XA medium. The detection of these two *Salmonella* serotypes is important, as these serotypes are the ones most frequently associated with food-borne outbreaks (15). Also, *S.* Typhimurium and *S.* Enteritidis alone represent 73.9% of all clinical isolates (16).

In the present study, XA medium contains neutral red as a dye rather than phenol red contained in XLD. When phenol red was used as a dye in XA medium, yellow-colored colonies due to low pH were easily diffusible on agar (data not shown). Thus, neutral red that produces less diffusible colored colonies on agar was used in formulating XA medium.

Several rapid methods have been developed to detect food-borne pathogens present in foods (20). However, conventional selective and differential media are still important, due to several advantages, including cost-effectiveness, ease of use, and familiarity among users (14). A wide range of chromogenic media have been developed and offered commercially. However, they are more expensive than conventional media, largely due to the additional cost of enzyme substrates (24). High costs of chromogenic media present a major obstacle for their use in routine practice. D-Arabinose incorporated in XA medium is cost-effective; thus, it is adequate for routine laboratory use.

In conclusion, XA medium developed in this study has higher specificity than XLD. Especially, XA medium easily differentiates *Salmonella* from *C. freundii* and *P. mirabilis*, which frequently produce false-positive results on XLD. Due to the good specificity of XA medium, labor and time can be saved due to fewer colonies needing further confirmation tests. XA medium may provide a valuable addition to the array of selective media available for the

TABLE 3 The specificity of XA medium compared with that of XLD on the microbiological analysis of naturally contaminated foods

Medium	No. of true-negative results	No. of false-positive results	% specificity[a]
XLD	127	47	73.0
XA medium	160	14	92.0

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

detection of *Salmonella* spp. from foods. We recommend that XA medium be used in parallel with HE agar and BS agar in the examination of food samples. Further studies are required to evaluate the growth of more *Salmonella* serotypes on XA medium, and it is necessary to assess the specificity of this new medium with food samples other than meat products and stool samples.

ACKNOWLEDGMENTS

This work was supported by grant no. R32-2008-000-10183-0 from the World Class University (WCU) project of the Ministry of Education, Science & Technology (MEST) and the KOSEF through Seoul National University.

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