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Evaluation of a Chromogenic Medium Supplemented with Glucose for Detecting *Enterobacter sakazakii*

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A commercial chromogenic agar medium (DFI) was supplemented with glucose (mDFI) to enhance the specificity of *Enterobacter sakazakii* (*E. sakazakii*) detection. *Escherichia vulneris* (*E. vulneris*), a putative false-positive strain on the DFI medium, produces α-glucosidase. The enzyme α-glucosidase hydrolyzes a substrate, 5-bromo-4-chloro-3-indolyl-α,D-glucopyranoside (XαGlc), producing green colonies. *E. sakazakii* strains produced green colonies on both DFI and mDFI agar, whereas *E. vulneris* produced green colonies on DFI agar but small white colonies on mDFI agar. *E. sakazakii* and *E. vulneris* were also readily differentiated by colony color when the mixed culture of the two strains was plated on mDFI agar and incubated for 24 h at 37°C. The results indicate that the selectivity of the commercial chromogenic agar medium could be improved by a simple supplementation with glucose.

Keywords: Chromogenic agar, *Enterobacter sakazakii*, *Escherichia vulneris*, glucose

Enterobacter sakazakii (*E. sakazakii*) has been implicated in severe forms of neonatal infections such as meningitis and sepsis [2–5, 11, 13, 17, 19]. Although the natural habitat of *E. sakazakii* is unknown, dehydrated powdered infant formula (DIF) has been epidemiologically identified as the source of *E. sakazakii* infections [1, 8, 24]. The current U.S. Food and Drug Administration method for detection of *E. sakazakii* includes a pre-enrichment procedure in buffered peptone water, enrichment in Enterobacteriaceae enrichment broth, plating on violet red bile glucose agar, and picking of five grown colonies onto tryptone soy agar plates, which are incubated at 25°C for 48–72 h. Yellow pigmented colonies, typical for *E. sakazakii*, on the TSA plates are confirmed using the API 20E systems [15]. This approach is laborious and requires up to 7 days to obtain a confirmed positive result [7]. Hence, there is a strong need for simplified methods for isolation and identification of *E. sakazakii*. Muytjens *et al.* [16] described enzymatic profiles of *E. sakazakii* and related species with specific reference to the α-glucosidase reaction. Farmer *et al.* [6] found 53 of 57 strains of *E. sakazakii* were positive for α-glucosidase activity whereas other members of the Enterobacteriaceae lack the enzyme. Therefore, detection of α-glucosidase activity would be a powerful tool in developing a differential medium. Oh and Kang [19] developed a fluorogenic agar medium using 4-methylumbelliferyl-α-D-glucoside (MUG) as a substrate for α-glucosidase, and the fluorogenic medium requires UV radiation to generate visible fluorescence from the individual colonies. It has been reported that each *E. sakazakii* showed a different intensity of fluorescence at the rate of the different usability of α-glucosidase activity [22]. Iversen *et al.* [9] developed a chromogenic agar (Druggan-Forsythe-Iversen; DFI) using the characteristic of the enzyme α-glucosidase expressed in the majority of *E. sakazakii*. Indolyl substrate 5-bromo-4-chloro-3-indolyl-α,D-glucopyranoside (XαGlc) was used as a chromogenic substrate, enabling to differentiate *Enterobacter sakazakii* with entirely blue-green colonies from other members of the Enterobacteriaceae. The enzyme α-glucosidase hyrolyzes XαGlc, liberating the aglycone (the nonsugar compound remaining after replacement of the glycosyl group from a glycoside by a hydrogen atom) 5-bromo-4-chloro-indolyl, which can be recognized as green color on media. However, 16 of 18 *Escherichia vulneris* strains, 2 of 3 *Pantoea* spp. strains, and 1 of 8 *Citrobacter koseri* strains gave false-positive results on DFI agar, implying that those strains also have α-glucosidase [8]. Therefore, screening the α-glucosidase activity of *E.*

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sakazakii may not be sufficient for selectively differentiating *E. sakazakii* from other Enterobacteriaceae, especially in environmental samples in which many microbial floras that encode α-glucosidase present. α-Glucosidases are enzymes involved in breaking down complex carbohydrates such as starch and glycogen. They catalyze the cleavage of individual glucosyl residues from various glycoconjugates, including alpha- or beta-linked polymers of glucose. The metabolism of alternative carbon sources can be inhibited by glucose, which is known as "catabolite repression" or the "glucose effect" [26, 27]. In this study, the catabolite repression of α-glucosidase activity by glucose in *E. sakazakii* and *E. vulneris* was screened, and a chromogenic medium supplemented with glucose was evaluated for detecting *Enterobacter sakazakii.*

Materials and Methods

Bacterial Strains

All bacterial strains were obtained from the culture collection of U.S. Food and Drug Administration, College Park, MD. Cells were cultured on brain heart infusion (BHI: Oxoid, Columbia, MD, U.S.A.) agar slants and maintained at 4°C. All strains used in this study consisted of *E. sakazakii*, *E. vulneris*, *E. cloacae*, and *Salmonella* spp. that had been isolated from dehydrated powdered infant formula and the environment.

Chromogenic Agar Media for *E. sakazakii*

The basic formulation for Oxoid Chromogenic *Enterobacter sakazakii* Agar (Druggan-Forsythe-Iversen: DFI; Oxoid, U.K.) is as follows: tryptone (15 g/l), soya peptone (5 g/l), sodium chloride (5 g/l), ferric ammonium citrate (1 g/l), sodium deoxycholate (1 g/l), 5-bromo-4-chloro-3-indolyl-α,D-glucopyranoside (0.1 g/l), sodium thiosulfate (1 g/l), and agar (15 g/l) were dissolved in distilled water. The complete medium (pH 7.3) was heated in a boiling water bath to dissolve the components before autoclaving at 121°C for 15 min. Modified DFI (mDFI) agar was made by adding 10 g of glucose per liter to the DFI agar formula.

Presumptive Selection of *E. sakazakii* from the Chromogenic Agars

All strains were grown on tryptic soy broth at 37°C. Purity of the culture was confirmed using a biotyping kit (API 20E; bioMerieux, Hazelwood, MO, U.S.A.). Culture was grown in TSB, and a population of viable *E. sakazakii* or *E. vulneris* was obtained by plating 10-fold serial dilutions of broth cultures onto two different chromogenic agar, DFI agar and mDFI agar, respectively, and then incubating the plates at 37°C for 24 h. Mixed culture of *E. sakazakii* and *E. vulneris* was prepared by combining the 10-fold diluted overnight cultures of the two strains and plating as above. Colony colors were observed and viable cells were counted.

Confirmation of Colonies from the Overnight Pre-enrichment Broth

Commercial infant powdered milk samples (10 g each) in duplicate were collected in 500-ml sterile bottles and then artificially inoculated

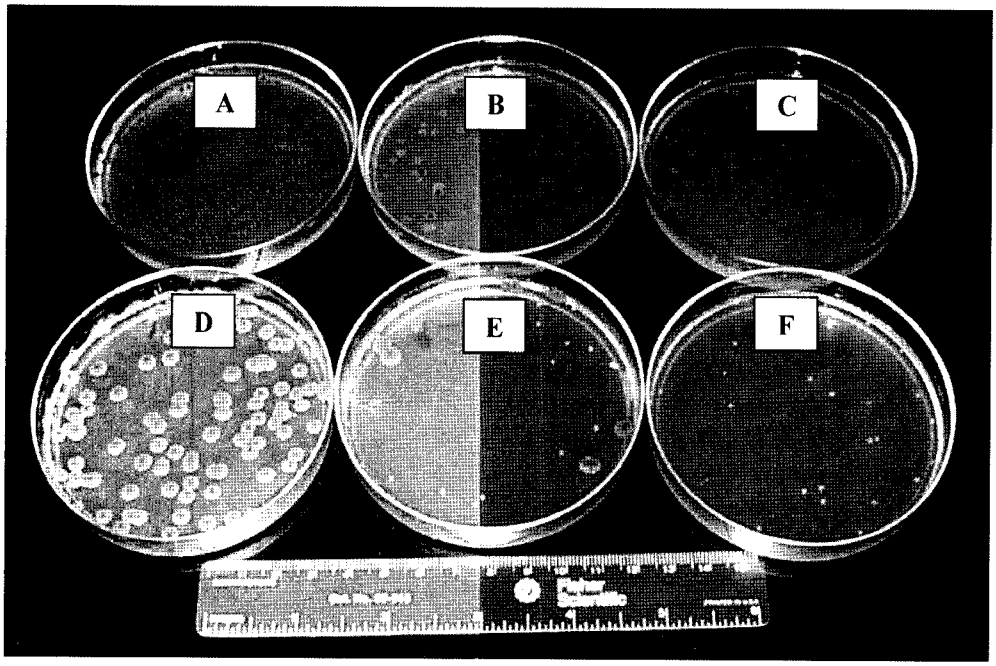

Fig. 1. Colony color and morphological characteristics of *E. sakazakii*, *E. vulneris*, and mixed cocktail grown on DFI agar and mDFI agar.

A. *Enterobacter sakazakii* grown on DFI agar showed green colonies as true positive. **B.** Mixed culture of *Enterobacter sakazakii* and *Escherichia vulneris* grown on DFI agar showed green colonies. **C.** *Escherichia vulneris* grown on DFI agar showed green colonies as false positive. **D.** *Enterobacter sakazakii* grown on mDFI agar showed green colonies with mucoid formation as true positive. **E.** Mixed culture of *Enterobacter sakazakii* and *Escherichia vulneris* grown on mDFI agar showed two different colonies; one was green colonies with mucoid formation in *E. sakazakii* and the other was white colonies without mucoid formation in *E. vulneris*. **F.** *Escherichia vulneris* grown on mDFI agar showed white colonies as true negative.

with 100 µl of serially diluted *E. sakazakii* (strain 2.76) at 6 CFU per g of formula. The spiked powdered formula samples were dissolved in 90 ml of sterile water and incubated for 24 h at 37°C. A loopful of the overnight pre-enrichment broth was streak-plated on DFI and mDFI agars and incubated for 24 h at 37°C. Five presumptive colonies with blue-green color on DFI and mDFI agars were randomly selected and confirmed by API 20E (bioMerieux, St. Louis, MO, U.S.A.), Rapid ID 32 E (bioMerieux), and real-time PCR assay as described in a previous study [23].

RESULTS AND DISCUSSION

Morphological Difference of *E. sakazakii* on DFI and mDFI Agars

All of the *E. sakazakii* strains tested produced blue-green colonies as true positive on DFI agar, and mucoid-large-green colonies on mDFI agar (Figs. 1A and 1D). According to the product manual, DFI agar is based on the α-glucosidase reaction, which is detected by incorporating the chromogenic substrate 5-bromo-4-chloro-3-indolyl-α-D-glucopyranoside in the medium. The enzyme α-glucosidase, present in *E. sakazakii*, hydrolyzes the substrate, producing green colonies on the pale yellow medium. *Proteus vulgaris* is also weakly α-glucosidase positive and could grow to give colonies of a similar color to *E. sakazakii*. However, on this medium, *Proteus* spp. grow as grey colonies because they produce hydrogen sulfide in the presence of ferric ions forming ferrous sulfide. Desoxycholate inhibits the growth of most Gram-positive organisms [20]. The result of this study (Table 1) showed that the DFI medium has 100% sensitivity for *E. sakazakii* because of the constitutive expression of α-glucosidase. Five selected presumptive colonies on DFI agar and mDFI agar revealed as *E. sakazakii* when overnight pre-enrichment broth culture of dehydrated powdered infant formula inoculated with *E. vulneris* was streak-plated, incubated for 24 h at 37°C, and confirmed by API 20E, Rapid 32 E, and real-time PCR analysis (data not shown). Real-time PCR assays are characterized by a wide dynamic range of quantification, high sensitivity, and a high degree of precision [10, 12, 21, 23]. Interestingly, all of the *E. sakazakii* strains produced green and mucoid colonies, small to large in size (or diameter), on mDFI agar but not on the original DFI (Table 2) formula. In addition to the color difference, the mucoid colony characteristic of *E. sakazakii* on mDFI could help the readily differentiate the bacteria from other background flora. In a previous study [25], we found the correlation between mucoid formation and carbohydrate composition in the media consisting of 5 g of NaCl, 20 g of tryptone, 15 g of agar, 200 ng/ml ferrioxamine E, and 50 mg α-MUG per liter supplemented with (a) no extra carbohydrate, (b) glucose (10 g per liter), and (c) lactose (10 g per liter), respectively. A total of 58 *E. sakazakii* strains were grown on the three different media. The results were as follows: (a) on unsupplemented media (control), all strains of *E. sakazakii* tested did not produce mucoid; (b) on the media supplemented with glucose, 50 of 58 (86%) *E. sakazakii* produced mucoid; and (c) on the media supplemented with lactose, 22 of 58 (38%) *E. sakazakii* strains produced mucoid [25]. Interestingly, all of the 58 *E. sakazakii* strains showed mucoid on VRBG agar that contains both glucose and lactose (10 g each per liter). Hence, we concluded that a carbohydrate, especially glucose, acts as an inducer in the expression of the mucoid production operon in *E. sakazakii* colonies on the agar medium. Nazarowec-White and Farber [18] observed variations in the colony morphology of *E. sakazakii*, from rubbery to smooth on violet red bile glucose agar. Lehner and Stephan [14] described two morphologically different colony types of *E. sakazakii* type strain ATCC 29544 when streaked on blood agar, brain heart infusion agar, and plate count agar, and incubated at 25°C for 48 h. The rubbery colonies were generated on blood agar and brain heart infusion agar whereas the smooth colonies were exhibited by the organism on plate count agar. However, to the author's knowledge, there has been no report that explains the relationship between the mucoid formation of *E. sakazakii* and glucose supplementation in culture media. Therefore, further studies are necessary to scrutinize the mucoid expression mechanism in *E. sakazakii* as the composition of carbohydrates in a medium varies.

Morphological Difference of *E. vulneris* on DFI Agar and mDFI Agar

All of the *E. vulneris* strains produced green colonies as false positive on DFI agar (Fig. 1C). Five presumptive green colonies displayed by the α-glucosidase activity of a putative organism on the chromogenic agar medium

Table 1. Comparison of colony color, API 20E, Rapid 32 E, and real-time PCR of 5 strains tested in this study.

Strain	Colony color		API 20E	Rapid ID32 E	Real-time PCR
	DFI agar	mDFI agar			
E. sakazakii N6	Green	Mucoid-large-green	*E. sakazakii*	*E. sakazakii*	(+)
E. sakazakii 10	Green	Mucoid-large-green	*E. sakazakii*	*E. sakazakii*	(+)
E. sakazakii N1	Green	Green	*E. sakazakii*	*E. sakazakii*	(+)
E. vulneris 1	Green	White	*E. vulneris*	*E. vulneris*	(-)
E. vulneris 2	Green	White	*E. vulneris*	*E. vulneris*	(-)

Table 2. Comparison of *Enterobacter sakazakii*'s and Enterobacteriaceae's morphology grown on DFI and DFI with glucose.

		DFI		DFI with glucose	
		color	mucoid	color	mucoid
Enterobacter sakazakii	FSM 299	Green	No	Green	Small
	FSM 318	Green	No	Green	Large
	FSM 294	Green	No	Green	Large
	FSM 295	Green	No	Green	Small
	FSM 287	Green	No	Green	Small
	FSM 302	Green	No	Green	Large
	FSM 273	Green	No	Green	Small
	FSM 261	Green	No	Green	Small
	FSM 298	Green	No	Green	Small
	FSM 265	Green	No	Green	Large
	FSM 290	Green	No	Green	Small
	FSM 275	Green	No	Green	Large
	FSM 293	Green	No	Green	Large
	FSM 303	Green	No	Green	Large
	2.76	Green	No	Green	Large
	2.70	Green	No	Green	Large
	2.71	Green	No	Green	Large
	2.74	Green	No	Green	Small
	2.69	Green	No	Green	Large
	2.68	Green	No	Green	Large
	2.47	Green	No	Green	Large
	5	Green	No	Green	Small
	3	Green	No	Green	Large
	2	Green	No	Green	Large
	2.84	Green	No	Green	Small
	N6	Green	No	Green	Large
	ES 626	(Weak) Green	No	Green	Small
Enterobacteriaceae	Salmonella	Black	No	Black	No
	E. cloacae (ES 614)	Yellow	No	Yellow	No
	E. vulneris	Green	No	white	No

should be confirmed by biochemical test in the following identification step, owing to the possible false-positive strains such as α-glucosidase positive *E. vulneris*. False positives are more problematic when environmental samples are pre-enriched overnight and then plated on a selective or differential agar media. Iversen *et al.* [9] reported that nearly 90% (16 out 18) of *E. vulneris* strains were identified as false positive on DFI agar, the most among the false positives, followed by *Pantoea* spp. strains and *C. koseri* strains. The false-positive result of *E. vulneris* on DFI agar medium was expected since this species was reported to be 25% positive for α-glucosidase and 50% positive for yellow pigment production [16]. The *E. vulneris* tested in this study revealed small white colonies on mDFI agar (Fig. 1F), and colonies with the same morphology appeared on the medium when overnight pre-enchment broth culture of dehydrated powdered infant formula inoculated with *E. vulneris* was streak-plated, incubated for 24 h at 37°C, and confirmed by API 20E, Rapid 32 E, and real-time PCR analysis (data not shown). It is assumed that glucose suppresses the expression of α-glucosidase in *E. vulneris* by the mechanism known as catabolite repression. Further study would be necessary to determine how the carbohydrate affects the expression of the enzyme and the growth of *E. vulneris*.

Morphological Difference of Mixed Culture of *E. sakazakii* and *E. vulneris* on DFI Agar and mDFI Agar

In Table 1 and Fig. 1B, the mixed culture of *E. sakazakii* and *E. vulneris* strains produced green colonies on DFI agar making it difficult to selectively differentiate the two strains on the medium. Hence, it was impossible to isolate only *E. sakazakii* by relying on the colony color on DFI agar. In a previous studies [9, 19], false positives were observed in all methods developed based on the α-glucosidase activity, owing to the presence of the enzyme in other species of Enterobacteriaceae. The mixed culture of *E. sakazakii* and *E. vulneris* that was spread-plated on mDFI agar medium

exhibited two distinctive morphological characteristics, mucoid-large-green and small-white, respectively (Fig. 1E). After the overnight pre-enrichment broth cultures of dehydrated powdered infant formula inoculated with a mixed culture of the two strains was streak-plated on mDFI agar medium and incubated for 24 h at 37°C, five mucoid-large-green colonies revealed as *E. sakazakii*, and 5 small-white colonies as *E. vulneris*, when confirmed using biochemical and real-time PCR analysis (data not shown). The results clearly show that an organism that is positive for the α-glucosidase activity, as expressed in the majority of *E. vulneris* strains, could be distinguishable on mDFI agar by both colony color (green for *E. sakazakii* and white for non-*E.sakazakii*) and morphology (large and mucoid for *E. sakazakii*, and small and nonmucoid for non-*E. sakazakii*). However, further studies should be required to evaluate the performance of this medium with naturally contaminated food and environmental samples.

In conclusion, this study has shown that the performance of the commercial selective medium for *E. sakazakii,* DFI, could be improved by supplementing with glucose to effectively exclude a false-positive strain from the medium. Further studies are being concluded with the elimination of various other false positives shown on DFI agar (the α-glucosidase-positive *Pantoea* spp. or *Citrobacter koseri* or any other organisms), by supplementing the agar medium with various carbohydrate sources that could act as a metabolite repressor or inducer. More strains of *E. sakazakii* need to be screened for the α-glucosidase activity, whether it is expressed constitutively or regulated by the glucose effect along with mucoid formation.

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