Comparison of Chromogenic Selective Media for the Detection of Cronobacter spp. (Enterobacter sakazakii)

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The four types of chromogenic selective media that are commercially available in Japan were compared for establishing a Japanese standard method for detecting Cronobacter spp. based on ISO/TS 22964:2006. When assessed using 9 standard Cronobacter spp. strains and 29 non-Cronobacter strains, Enterobacter sakazakii isolation agar, Chromocult Enterobacter sakazakii agar, CHROMagar E. sakazakii, and XM-sakazakii agar demonstrated excellent inclusivity and exclusivity. Using the ISO/TS 22964:2006 method, the recovered numbers of 38 Cronobacter spp. strains, including 29 C. sakazakii isolates obtained from each medium, were equivalent, indicating that there was no significant difference (p > 0.05) among the four types of chromogenic selective media. Thus, we demonstrated that these four chromogenic selective media are suitable alternatives when using the standard method for detecting Cronobacter spp. in Japan, based on the ISO/TS 22964:2006.

Key words : Cronobacter spp. / Enterobacter sakazakii / Chromogenic medium / Detection / ISO/TS 22964:2006.

Cronobacter have been suggested as a new genus of Enterobacter sakazakii (Iversen et al., 2008; Nazarowec-White and Farber, 1997a) comprising seven species (C. condimenti, C. dublinensis, C. malonicus, C. muytjensii, C. sakazakii, C. tunicins, and C. universalis), and three subspecies of C. dublinensis (C. dublinensis subsp. dublilnensis, C. dublinensis subsp. lactaridi, and C. dublinensis subsp. lausannensis) (Iversen et al., 2008; Joseph et al., 2012). Additionally, it has been reported that Cronobacter spp. can cause meningitis, sepsis, and necrotic enteritis in infants (Hawkins et al., 1991; Urmeyi and Franklin, 1961). Cronobacter spp. have a tolerance for heat and desiccation (Nazarowec-White and Farber, 1997b) and are associated with infections in infants through the consumption of contaminated infant formula (Biering et al., 1999; Simmons et al., 1989; van Acker et al., 2001). Indeed, infant formula is considered as a major source of Cronobacter infection, although Cronobacter spp. have also been isolated from other food products, including powdered milk, dry vegetables, and grain powder (Chen et al., 2016; Huang et al., 2015).

Considering these factors, the control of Cronobacter spp. is important to maintain the quality of infant formula and thus prevent their contamination (Food and Agriculture Organization, 1994; Muytjens et al., 1988). For detecting Cronobacter spp., the ISO/TS 22964:2006 method (International Organization for Standardization, 2006) has been recommended along with the procedures outlined in the U.S. Food and Drug Administration Bacteriological Analytical Manual (2012). Chromogenic selective agar media, several of which have been developed, are recommended for use in these methods.
TABLE 1. Formulation of various chromogenic selective media for the detection of Cronobacter spp.*

| Ingredient                                      | ESIA | CESA | CAEA | XMSA |
|------------------------------------------------|------|------|------|------|
| Peptone                                         | 7.0  | 6.0  | 7.0  | 15.0 |
| Yeast extract                                   | 3.0  | 3.0  | 3.0  | 5.0  |
| Sodium chloride                                 | 5.0  | 5.0  | 5.0  | 5.0  |
| Sodium deoxycholate                             | 0.6  | 0.6  | 0.6  |      |
| Bile salts mixture                              | 1.5  |      |      |      |
| Sodium lauryl sulfate                           |      |      |      | 0.15 |
| Tryptophan                                      |      | 1.0  |      |      |
| Disodium hydrogen phosphate                     |      |      |      | 2.0  |
| Sodium pyruvate                                 |      |      | 1.0  |      |
| Potassium nitrate                               |      |      | 1.0  |      |
| 5-bromo-4-chloro-3-indolyl-α-D-glucopyranoside  | 0.15 | 0.1  | 0.15 |      |
| Chromogenic mixture                             |      |      |      | 0.2  |
| Crystal violet                                  | 0.002| 0.002|      |      |
| Agar                                            | 12.0 | 12.0 | 15.0 | 15.0 |
| pH                                              | 7.0±0.2| 7.0±0.2| 7.0±0.2| 7.0±0.2 |

*ESIA; Enterobacter sakazakii isolation agar, CESA; Chromocult Enterobacter sakazakii agar, CAEA; Chromoagar E. sakazakii, XMSA; XM-sakazakii agar. Culture conditions for all selective media were at 44°C for 24 h.

Formulation of each chromogenic media was referred from manufacturer information.
subsp. *lausannensis* JCM 16469 obtained from ESIA, CESA, and XMSA were approximately 1 log lower than those obtained from TSA. The range of recovered *Cronobacter* spp. numbers (log CFU/ml) was 7.4 to 9.2; the mean log CFU/ml ± SD for ESIA, CESA, CAEA, XMSA, and TSA were 8.8 ± 0.5, 8.7 ± 0.4, 8.8 ± 0.4, 8.7 ± 0.5, and 8.9 ± 0.1, respectively. Thus, no significant difference in recovered numbers (*p* > 0.05) was observed among the four types of chromogenic selective media.

The results of the exclusivity study are shown in Table 3. Twenty-five strains grew on nonselective TSA at 44°C, whereas five strains failed to grow under these conditions. Of the 29 strains, 16, 22, 17, and 20 grew weakly on ESIA, CESA, CAEA, and XMSA, respectively. ESIA and CAEA allowed non-*Cronobacter* strains to form pale blue-gray colored colonies. All non-*Cronobacter* strains that grew formed white colored colonies on CESA. Of the 21 strains that grew on XMSA, 11, nine, and one formed magenta, pale yellow, and light brown colored colonies, respectively. All non-*Cronobacter* strains that grew were different in appearance from typical *Cronobacter* colonies on all chromogenic media. Therefore, it appears that *Cronobacter* spp. can easily be distinguished from competitive non-*Cronobacter* spp. using these four types of chromogenic media with no significant differences.

**TABLE 2. Growth of *Cronobacter* spp. on various chromogenic selective media**

| Strains of *Cronobacter* spp. | Mean Log CFU/ml ± SD (colony color) |
|-------------------------------|--------------------------------------|
|                              | ESIA       | CESA       | CAEA       | XMSA       | TSA        |
| C. dublinensis subsp. dublinensis JCM 16457 | 8.6±0.3 (blue-green) | 8.5±0.1 (blue-green) | 8.9±0.2 (blue-green) | 8.9±0.2 (navyblue) | 8.9±0.1 |
| C. dublinensis subsp. lactaridii JCM 16468 | 8.8±0.2 (blue-green) | 8.8±0.1 (blue-green) | 8.3±0.7 (blue-green) | 8.6±0.2 (navyblue) | 9.0±0.1 |
| C. dublinensis subsp. lausannensis JCM 16469 | 7.6±0.1 (blue-green) | 7.9±0.8 (blue-green) | 8.4±0.1 (blue-green) | 7.4±0.3 (navyblue) | 8.7±0.1 |
| C. malonaticus DSM 18702 | 9.0±0.1 (blue-green) | 8.9±0.1 (blue-green) | 9.0±0.0 (blue-green) | 8.9±0.0 (navyblue) | 9.0±0.1 |
| C. muelensis ATCC 51329 | 8.8±0.2 (blue-green) | 8.7±0.2 (blue-green) | 8.8±0.2 (blue-green) | 8.8±0.2 (navyblue) | 8.9±0.2 |
| C. sakazakii ATCC 12868 | 9.0±0.1 (blue-green) | 8.5±0.1 (blue-green) | 8.6±0.3 (blue-green) | 8.9±0.1 (navyblue) | 8.9±0.1 |
| C. sakazakii ATCC 293004 | 9.2±0.0 (blue-green) | 9.1±0.3 (blue-green) | 9.0±0.1 (blue-green) | 9.0±0.2 (navyblue) | 9.0±0.1 |
| C. sakazakii ATCC 29644 | 8.9±0.2 (blue-green) | 8.8±0.2 (blue-green) | 8.9±0.1 (blue-green) | 8.8±0.2 (navyblue) | 9.0±0.2 |
| C. turicensis DSM 18703 | 9.1±0.1 (blue-green) | 9.0±0.2 (blue-green) | 9.0±0.3 (blue-green) | 9.0±0.2 (navyblue) | 9.0±0.1 |
| Overall | 8.6±0.5 | 8.7±0.4 | 8.8±0.4 | 8.7±0.5 | 8.9±0.1 |

*p* value (ANOVA) *p* > 0.05 shows no significant difference between the two media.

*ESIA: Enterobacter sakazakii isolation agar, CESA: Chromocult Enterobacter sakazakii agar, CAEA: Chromoagar *E. sakazakii*, XM-SAK: XMSA agar, TSA: Tryptic soy agar. All strains were cultured at 44°C for 24 h.*

*Strains were from ATCC (American Type Culture Collection), DSM (Leibniz Institute DSMZ-German Collection of microorganisms and Cell Cultures, Germany), and JCM (Japan Collection of Microorganisms).*

*For each strain, mean ± SD of the log CFU/ml on ESIA, CESA, CAEA, and XMSA is shown.*

*For each strain, mean ± SD of the log CFU/ml on ESIA, CESA, CAEA, and XMSA is shown.*
false-positive results.

The growth of Cronobacter spp. was assessed using 38 strains including 29 C. sakazakii isolates from foods, to determine whether the four types of chromogenic media are amenable to the ISO/TS 22964:2006 method. In brief, 100 µl of Cronobacter spp. strains were cultured in TSB at 37°C for 18 h and then used to inoculate 10 ml of modified lauryl sulfate tryptose broth with vancomycin (mLST with vancomycin) in accordance with the ISO/TS 22964:2006 method. After incubation at 44°C for 24 h, each culture was then subjected to 10-fold serial dilution in BPW. The following procedures were conducted according to the description above. When these chromogenic media were used in the ISO/TS

| Name of organism          | ESIA | CESA | CAEA | XMSA | TSA |
|--------------------------|------|------|------|------|-----|
| Cedecea neteri ATCC 33855 |      |      |      |      |     |
| Citrobacter koseri JCM 1658 |      | W    | M    |      |     |
| Enterobacter cloacae NBRC 3320 | ±PBG | W    | M    |      |     |
| E. cloacae IAM 12349      | ±PBG | W    | M    |      |     |
| E. gergoviae ATCC 33028   |      |      |      |      |     |
| Escherichia coli JCM 5491 | ±PBG | W    | M    |      |     |
| E. coli O157 ATCC 35150   | ±PBG | W    | M    |      |     |
| E. coli O157 ATCC 43889   | ±PBG | W    | M    |      |     |
| Hafnia alvei NBRC 3731    |      |      |      |      |     |
| Klebsiella oxytoca ATCC 8724 | ±PBG | W    | M    |      |     |
| K. pneumoniae ATCC 13883  |      |      |      |      |     |
| Klyuyera ascorbata JCM 1681 |      | W    | M    |      |     |
| Leclercia adecarboxylata JCM 1667 | ±PBG | W    | M    |      |     |
| Pantoea agglomerans JCM 1236 |      |      |      |      |     |
| P. agglomerans NBRC 12686 |      |      |      |      |     |
| Plesiomonas shigelloides ATCC 14029 |      | W    | M    |      |     |
| Proteus mirabilis NBRC 13300 | ±PBG | W    | M    |      |     |
| P. mirabilis ATCC 29906   | ±PBG | W    | M    |      |     |
| Providencia rettgeri NBRC 13501 |      | W    | M    |      |     |
| Rahnella aquatilis JCM 1683 |      |      |      |      |     |
| Racultella planticola NBRC 14939 | ±PBG | W    | M    |      |     |
| Salmonella Enteritidis NBRC 3313 | ±PBG | W    | M    |      |     |
| S. Typhimurium ATCC 7823  | ±PBG | W    | M    |      |     |
| S. Vellore ATCC 15611     | ±PBG | W    | M    |      |     |
| Serratia marcescens ATCC 13880 | ±PBG | W    | M    |      |     |
| S. liquefaciens NBRC 12979 |      | W    | M    |      |     |
| S. rubidaea JCM 1240      | ±PBG | W    | M    |      |     |
| S. rubidaea NBRC 12973    | ±PBG | W    | M    |      |     |
| Yersinia intermedia JCM 7579 | ±PBG | W    | M    |      |     |

*ESIA; Enterobacter sakazakii isolation agar, CESA; Chromocult Enterobacter sakazakii agar, CAEA; Chromoagar E. sakazakii, XMSA; XM-sakazakii agar, TSA; Tryptic soy agar. All strains were cultured at 44°C for 24 h.

*Strains were from ATCC (American Type Culture Collection), IAM (IAM Culture Collection, Center for Cellular and Molecular Research, Institute of Molecular and Cellular Biosciences, University of Tokyo, Japan), JCM (Japan Collection of Microorganisms) and NBRC (NITE Biological Resource Center, Japan).

*The symbols indicate growth; +, good; ±, poor; −, inhibited.

*Characteristics indicate colony appearance: PBG, pale blue gray; W, white; M, magenta; PY, pale yellow; LB, light brown.
| Strains of Cronobacter spp. tested | Mean Log CFU/ml ± SD |
|-----------------------------------|----------------------|
|                                   | ESIA | CESA | CAEA | XMSA | TSA |
| C. dublinensis subsp. dublinensis JCM 16467<sup>7</sup> | 6.9±0.0 | 6.8±0.1 | 6.9±0.0 | 6.8±0.2 | 6.7±0.1 |
| C. dublinensis subsp. lactarid JCM 16468<sup>2</sup> | 7.0±0.1 | 6.9±0.0 | 7.0±0.0 | 6.9±0.0 | 6.9±0.1 |
| C. dublinensis subsp. laussannensis JCM 16469<sup>7</sup> | 5.9±0.3 | 5.8±0.1 | 5.5±0.4 | 4.9±0.5 | 5.8±0.3 |
| C. malonicatus DSM 18702<sup>7</sup> | 7.4±0.0 | 7.4±0.1 | 7.5±0.1 | 7.4±0.1 | 7.5±0.1 |
| C. muytjens ATCC 51329<sup>T</sup> | 6.7±0.1 | 6.7±0.1 | 6.8±0.0 | 6.9±0.2 | 6.9±0.1 |
| C. sakazakii ATCC 12868 | 7.7±0.1 | 7.8±0.0 | 7.8±0.1 | 7.9±0.1 | 7.9±0.0 |
| C. sakazakii ATCC 29004 | 7.9±0.1 | 7.9±0.1 | 7.8±0.1 | 7.8±0.1 | 7.7±0.2 |
| C. sakazakii ATCC 29544<sup>T</sup> | 7.4±0.1 | 7.6±0.1 | 7.8±0.4 | 7.2±0.3 | 7.3±0.1 |
| C. sakazakii NFH 0905006 | 7.7±0.1 | 7.6±0.0 | 7.8±0.1 | 7.3±0.2 | 7.7±0.1 |
| C. sakazakii NFH 0905206 | 7.4±0.0 | 7.3±0.0 | 7.3±0.1 | 7.3±0.2 | 7.5±0.1 |
| C. sakazakii NFH 0905406 | 7.7±0.0 | 7.7±0.1 | 7.7±0.1 | 7.5±0.0 | 7.7±0.1 |
| C. sakazakii NFH 0906206 | 7.7±0.0 | 7.7±0.1 | 7.7±0.1 | 7.5±0.2 | 7.5±0.1 |
| C. sakazakii NFH 0906311 | 7.4±0.1 | 7.5±0.1 | 7.4±0.2 | 7.5±0.1 | 7.5±0.0 |
| C. sakazakii NFH 0906605 | 7.9±0.1 | 7.9±0.1 | 7.9±0.1 | 8.0±0.1 | 7.6±0.5 |
| C. sakazakii NFH 0907106 | 7.4±0.1 | 7.4±0.1 | 7.6±0.0 | 7.5±0.0 | 7.4±0.2 |
| C. sakazakii NFH 0907111 | 7.6±0.1 | 7.8±0.2 | 7.7±0.3 | 7.6±0.1 | 7.7±0.1 |
| C. sakazakii NFH 0907406 | 7.7±0.1 | 7.7±0.0 | 7.7±0.1 | 7.4±0.3 | 7.8±0.1 |
| C. sakazakii NFH 0907411 | 7.2±0.0 | 7.2±0.2 | 7.3±0.1 | 7.3±0.1 | 7.3±0.0 |
| C. sakazakii NFH 0907412 | 7.0±0.1 | 7.2±0.1 | 7.2±0.2 | 7.2±0.1 | 7.3±0.0 |
| C. sakazakii NFH 0907706 | 7.4±0.1 | 7.6±0.1 | 7.5±0.0 | 7.5±0.2 | 7.6±0.1 |
| C. sakazakii NFH 0907806 | 7.2±0.2 | 7.2±0.1 | 7.4±0.1 | 7.5±0.0 | 7.8±0.1 |
| C. sakazakii NFH 0908102 | 6.0±0.6 | 6.4±0.1 | 6.3±0.1 | 6.5±0.1 | 6.3±0.1 |
| C. sakazakii NFH 0908106 | 7.6±0.1 | 7.7±0.1 | 7.8±0.0 | 7.6±0.1 | 7.7±0.1 |
| C. sakazakii NFH 0908307 | 8.0±0.1 | 7.7±0.1 | 8.0±0.1 | 8.0±0.1 | 7.9±0.1 |
| C. sakazakii NFH 0908808 | 7.0±0.1 | 7.1±0.0 | 7.0±0.0 | 7.2±0.1 | 7.1±0.1 |
| C. sakazakii NFH 0909407 | 7.2±0.0 | 7.2±0.1 | 7.0±0.3 | 7.3±0.0 | 7.1±0.1 |
| C. sakazakii NFH 0909611 | 7.7±0.1 | 7.8±0.0 | 7.8±0.0 | 7.9±0.0 | 7.7±0.1 |
| C. sakazakii NFH 0909612 | 7.6±0.1 | 7.6±0.1 | 7.6±0.1 | 7.3±0.1 | 7.6±0.1 |
| C. sakazakii NFH 1002408 | 7.6±0.1 | 7.8±0.1 | 7.7±0.1 | 7.7±0.1 | 7.6±0.0 |
| C. sakazakii NFH 1004106 | 7.0±0.1 | 7.0±0.1 | 7.0±0.1 | 7.1±0.2 | 7.0±0.1 |
| C. sakazakii NFH 1006207 | 7.1±0.4 | 6.6±0.1 | 6.7±0.0 | 6.5±0.1 | 6.8±0.0 |
| C. sakazakii NFH 1006306 | 7.7±0.1 | 7.7±0.1 | 7.6±0.1 | 7.6±0.1 | 7.8±0.1 |
| C. sakazakii NFH 1006702 | 6.7±0.1 | 6.7±0.0 | 6.6±0.1 | 6.6±0.1 | 6.6±0.1 |
| C. sakazakii NFH 1006707 | 7.4±0.0 | 7.6±0.0 | 7.5±0.1 | 7.4±0.2 | 7.5±0.2 |
| C. sakazakii NFH 1006806 | 7.9±0.0 | 7.8±0.0 | 7.8±0.1 | 7.7±0.1 | 7.8±0.1 |
| C. sakazakii NFH 1006807 | 7.2±0.1 | 7.3±0.0 | 7.1±0.1 | 7.3±0.0 | 7.3±0.1 |
| C. sakazakii NFH 1007006 | 7.4±0.0 | 7.6±0.1 | 7.3±0.1 | 7.4±0.1 | 7.4±0.1 |
| C. turicensis DSM 18703<sup>T</sup> | 7.0±0.0 | 7.0±0.1 | 7.0±0.2 | 7.1±0.1 | 7.0±0.1 |
| Overall | 7.3±0.5 | 7.4±0.5 | 7.3±0.5 | 7.3±0.6 | 7.4±0.5 |

p value (ANOVA)<sup>c</sup>

|                                | vs. ESIA | CESA | CAEA | XMSA | TSA |
|--------------------------------|----------|------|------|------|-----|
| vs. ESIA                       | 0.64     | 0.82 | 0.71 |      |     |
| vs. CESA                       | 0.64     | 0.82 | 0.42 |      |     |
| vs. CAEA                       | 0.82     | 0.82 | 0.57 |      |     |
| vs. XMSA                       | 0.71     | 0.42 | 0.57 |      |     |

<sup>a</sup>ESIA; Enterobacter sakazakii isolation agar, CESA; Chromocult Enterobacter sakazakii agar, CAEA; Chromoagar E. sakazakii, XMSA; XM-sakazakii agar, TSA; Tryptic soy agar. All strains were cultured at 44°C for 24 h after being cultured in mLST with vancomycin.

<sup>b</sup>Strains were from ATCC (American Type Culture Collection), DSM (Leibniz Institute DSMZ-German Collection of microorganisms and Cell Cultures, Germany), and JCM (Japan Collection of Microorganisms). NFH (C. sakazakii) strains tested were derived from food samples and identified using the 16S rRNA sequence.

<sup>c</sup>p > 0.05 shows no significant difference between the two media.
In conclusion, we compared the performances of four types of chromogenic selective media that are commercially available in Japan and demonstrated that each is a suitable alternative when using the standard ISO/TS 22964:2006 method in Japan for detecting Cronobacter spp.

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