Research Note

Evaluation of a Revised U.S. Food and Drug Administration Method for the Detection of Cronobacter in Powdered Infant Formula: A Collaborative Study

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ABSTRACT

A revised U.S. Food and Drug Administration (FDA) method for the isolation and detection of Cronobacter from powdered infant formula was recently developed, which combines real-time PCR, chromogenic agars, and RAPID ID 32E biochemical tests. This method provides an expedient analysis within 24 to 48 h. A collaborative validation study involving four different laboratories was conducted to compare the revised FDA method with the reference FDA method using casein- and soy-based powdered infant formula inoculated with different Cronobacter strains. Valid results from 216 test portions and controls from collaborating laboratories were obtained and showed that the revised FDA method performed significantly better than the reference FDA method. Newly revised PCR protocols and VITEK 2 were also evaluated to be integrated into the complete detection procedure.

Cronobacter is a gram-negative pathogen that causes rare but severe foodborne diseases (4). Cronobacter is a newly defined genus which was formerly Enterobacter sakazakii (5). In recent years, this bacterium has been associated with illnesses in neonates, infants, and children (3 months to 4 years). Contaminated powdered infant formula (PIF) has been linked to recent outbreaks in the United States (4).

In 2002, the U.S. Food and Drug Administration (FDA) released a protocol for the isolation and enumeration of E. sakazakii from dehydrated PIF (8). According to the protocol, infant formula samples are first enriched in Enterobacteriaceae enrichment (EE) broth, followed by plating on violet red bile glucose (VRBG) agar and then Trypticase soy agar (TSA). Typical colonies are confirmed with API 20E. This method is laborious and can take 6 to 7 days to complete. Recently, a revised FDA method (1, 3) was developed for rapid detection and isolation of Cronobacter in PIF. This method utilizes a real-time PCR-based assay that targets the dnaG gene in the macromolecular synthesis operon, which is specific to Cronobacter (7). Additionally, enriched samples in buffered peptone water were streaked onto different chromogenic agars to isolate Cronobacter species.

This work extends beyond the precollaborative study that compared the revised FDA method with the reference FDA method (1). Two types of PIF samples, casein based and soy based, were assessed with two different strains of Cronobacter. Results obtained from the 216 test portions and controls showed that the revised FDA method is either significantly better (\(P < 0.05\)) than or equivalent to the reference FDA method for the detection of Cronobacter strains in PIF. The objective of this work was to complete the validation of the revised FDA method for the detection of Cronobacter in PIF in a collaborative study that involved four laboratories.

MATERIALS AND METHODS

Sample preparation. The samples were prepared by Silliker Inc. (Chicago, IL). Briefly, two types of PIF were selected for this study: cow’s milk (casein) based and soy milk based. Presently, these two products account for at least 90% of the market share. PIF samples were screened for the presence of Cronobacter prior to inoculation by the current FDA method. Bulk samples of the milk-based formula were inoculated with Cronobacter sakazakii strain E604, and the soy-based formula was inoculated with Cronobacter malonaticus strain E902. Strain E604 was originally isolated from a patient in a children’s hospital in Canada. Strain E902 was originally isolated from human blood in the United States. Infant formula samples were artificially seeded with two different levels of cells. Cronobacter strains were grown in 100 ml of brain heart infusion broth for 18 to 24 h at 35 ± 1°C. The
cultures were then centrifuged (10,000 × g), suspended in 10 ml of 10% nonfat dry milk, and then lyophilized. The lyophilized cultures were ground to a fine powder with a mortar and pestle and mixed with approximately 500 g of appropriate PIF (soy and milk based) to make a seed inoculum. Each seed inoculum was stored at 25°C for up to 7 days to enable the microorganism to adapt to the product. On each day during the 7-day period, the seed inoculum cell count was determined by plating 100 μl of serial dilutions onto R&F E. sakazakii chromogenic agar (R&F Laboratories, Downers Grove, IL). The plates were incubated at 35 ± 1°C for 24 ± 2 h prior to enumeration.

A total of four sets of samples were prepared. Two sets of samples were prepared for each of the milk- and soy-based formulas. One set was analyzed by the revised FDA method (1) and the other by the reference FDA method (8). Infant formula samples inoculated were achieved to target levels of approximately 0.2 to 2 CFU/100 g of sample for the low level and 5 to 10 CFU/100 g of sample for the high level when measured by the most-probable-number (MPN) method using an MPN calculator (http://www.lcflltd.com/customer/LCFMPNCalculator.exe) on the day of initiation of analysis. Samples with either the low or high level of cells were expected to yield fractional positive results (25 to 75% of the total number of samples).

Inoculated samples were held at room temperature for at least 2 weeks prior to analysis to allow populations to stabilize. These samples were then centrifuged and then distributed to three FDA laboratories located in Atlanta, GA, New York, NY, and Alameda, CA. Each laboratory analyzed six test portions representing the high contamination level, six test portions representing the low contamination level, and six uninoculated controls. Another set of samples was sent to the FDA laboratory in College Park, MD, for MPN analysis.

MPN values for the high- and low-level inoculums were estimated for both the reference and the revised FDA methods. For the determination of the high level of inoculation, 18 replicates of 100-g test portions and three replicates each of 10-g and 1-g test portions were analyzed following each method’s specified test procedure (8). Because of the extremely low levels targeted, a slightly different design was used for the determination of the low level of inoculum. Three replicates of 300-g, 18 replicates of 100-g, and three replicates of 33-g test portions were evaluated following each method’s specified test procedure. The 100-g test portions were the same as those used for the method comparison component of the study.

Sample analysis and confirmation. Only 100-g samples were used for the comparison study. Presumptive-positive results that were culturally confirmed were considered positive for both methods. All other results were considered negative for both methods. Duplicate test portions were tested with a different primary enrichment for the revised and reference methods. Therefore, method equivalence was determined by comparing the results of the duplicate revised and reference methods by the Mantel-Haenszel chi-square formula for unpaired samples (6). A chi-square value of less than 3.84, in most cases, indicates no significant difference between the two methods, at the 5% probability level. The methods were compared on a per-level and per-sample type basis.

Reference FDA method. The reference FDA method was performed as described previously (8).

Revised FDA method. The revised FDA method was performed according to the precollaborative study by Chen et al. (1) with four modifications. First, only 24-h enrichment was used because in the precollaborative validation study, a 6-h enrichment procedure was shown to be insufficient. Second, in addition to being plated onto chromogenic agars after centrifugation, concentrated enrichment mixtures were also streaked onto chromogenic agars in duplicates. Third, all extracted DNA was tested by a revised PCR assay with internal control in addition to the original PCR assay. Fourth, newly revised PCR protocols (2) and the use of VITEK 2 were evaluated for integration into a complete detection procedure.

Modification of the PCR assay for use with ABI 7500 Fast thermal cycler. The PCR protocols developed for SmartCycler Real-time PCR system (Cepheid, Sunnyvale, CA) (1, 2) were modified to be used with an ABI 7500 Fast thermal cycler (Life Technologies Inc, Foster City, CA). All PCR volumes were 25 μl. In addition, the original PCR protocol was modified with the addition of an internal control (2). Each reaction mixture contained 1 μl of 10 μM dnaG forward and reverse primers, 1.5 μl of 5 μM dnaG Taqman probe labeled with FAM (6-carboxyfluorescein), 2 μl of genomic DNA, 12.5 μl of IQ Supermix (Bio-Rad Labs, Hercules, CA), 0.5 μl of Platinum Taq DNA polymerase (5 U/μl; Invitrogen, Inc., Carlsbad, CA), 1.2 μl of InC DNA at 0.09 pg/μl (equivalent to 2.4 × 105 plasmid copies per μl), 0.375 μl of 10 μM InC forward and reverse primers, 0.75 μl of 5 μM InC probe labeled with Cy5, 1.5 μl of 50 mM MgCl2, and 2.3 μl of water. For the PCR protocol without internal control, each reaction mixture contained 1 μl of 10 μM dnaG forward and reverse primers, 1.5 μl of 5 μM dnaG Taqman probe labeled with FAM, 2 μl of genomic DNA, 12.5 μl of IQ Supermix (Bio-Rad Labs), 0.1 μl of Platinum Taq DNA polymerase (5 U/μl; Invitrogen, Inc.), and 6.9 μl of water. All reactions were performed in the ABI 7500 Fast thermal cycler (Life Technologies Inc.) with an initial denaturation of 3 min at 95°C, followed by 40 cycles of denaturation of 15 s at 95°C, annealing of 40 s at 52°C, and extension of 15 s at 72°C. The threshold values were determined manually such that all the negative controls were negative.

RESULTS AND DISCUSSION

This study compared 144 portions of two types of PIF inoculated with two Cronobacter strains at two different levels of cells with 72 uninoculated PIF portions. For each lot of infant formula, 18 high-level inoculation samples, 18 low-level inoculation samples, and 18 uninoculated controls were analyzed. Confirmed results from the revised method and the reference method were compared.

All uninoculated controls were negative by both methods. Chi-square analysis demonstrated that the revised FDA method was statistically equivalent to the reference method for both sets of samples. However, the revised method still detected a higher number of positives than the reference method in three sets of samples (Table 1). One sample inoculated at low level was positive by PCR, but the results on the chromogenic agars were uninterpretable; the plates were overgrown with background flora, and no typical Cronobacter colonies were isolated. Single colonies of background flora were picked and tested negative for Cronobacter by PCR. In addition, the PCR protocol with the internal control generated two false-negative results; this error was corrected by the PCR without the internal control. This demonstrated that the use of both PCR protocols with and without internal control increases the possibility of
detecting extremely low levels of *Cronobacter* in PIF samples.

Several modifications or additions were made to the original revised FDA method. The original PCR protocol was designed for the discontinued ABI 7000 thermal cycler and did not have an internal control (7). Subsequently, the method was developed and optimized for the SmartCycler Real-time PCR system, with and without internal control (2, 3). In this thermal cycler, different PCR conditions can be used in individual reaction tubes; however, the throughput of this system is only 16 per block compared with a standard 96-well plate. The reagent setup is also more labor-intensive with SmartCycler due to its unique reaction tube and thermal block design. Therefore, to increase the throughput and resolve potential false-negative issues, the PCR protocols were adapted for use with the ABI 7500 Fast thermal cycler, which uses 96-well plates. The primer and probe sequences remained the same, but their concentrations and thermal cycler conditions were revised. The thermal cycler conditions were identical for the protocols with and without the internal control. When evaluated, these two protocols had similar PCR efficiencies and limits of detection when tested with pure culture. Further evaluation of the revised protocols for the ABI 7500 system using all other DNA extracts generated in the present and the precollaborative studies showed that the data generated were equivalent to the original PCR protocols optimized for SmartCycler Real-time PCR system (data not shown).

Another addition to the detection and isolation protocol was to include an alternative biochemical analytical tool. Evaluation of VITEK 2 showed that this technology can accurately identify all *Cronobacter* strains and differentiate them from non-*Cronobacter* strains that were tested in the precollaborative study. Therefore, the use of this instrument can serve as an alternative for the confirmation of typical colonies.

In the precollaborative validation study, after 24 h of incubation, *Cronobacter* colonies were not isolated due to the overgrowth on essentially all the spread agar plates using spread plating. Therefore, it was necessary to conduct an additional step implementing a second transfer to obtain single colonies. However, in this study, single colonies were obtained from the additional streaking procedure and a second transfer was not necessary, thereby reducing the total time needed for identification.

Additionally, the reference FDA method performed poorly on two of the four sample sets in the precollaborative validation study (1). For one sample set, 38 of 40 test portions were positive on VRBG agar and 21 were positive on TSA, but only 3 samples were confirmed positive by API 20E. For another sample set, 6 of the 40 test portions were positive on VRBG agar and 1 was positive on TSA and confirmed positive by API 20E. This difference in results could be attributed to the natural bacterial background flora of the samples or the fact that the inoculated competing microorganisms outgrew *Cronobacter* in EE broth and VRBG media, thus indicating that EE broth and VRBG agar offer poor selectivity for *Cronobacter*. However, in the present study, all positive VRBG agars were confirmed to have *Cronobacter* by the reference FDA method (data not shown), which indicated that either the PIF samples obtained contained little natural background flora or the background flora did not interfere with the growth of *Cronobacter* in EE broth and VRBG agar. Therefore, in conditions in which the PIF sample is relatively free of background bacteria, the reference FDA method performed equally well as the revised FDA method. However, as we noted, the revised method clearly outperforms the reference method published in 2002.

In summary, the data reported in the present collaborative study, in combination with the data from the precollaborative study (1), indicate that the revised FDA method is highly sensitive and specific and significantly reduces the time for analysis. It is either significantly better than or equivalent to the reference FDA method for the detection of *Cronobacter* in PIF depending on the species and amount of natural background flora present in that food matrix.

**TABLE 1. Comparison between the revised method and reference method**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Level</th>
<th>MPN</th>
<th>No. of test portions</th>
<th>Revised method</th>
<th>Reference method</th>
<th>$\chi^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Presumptive</td>
<td>Confirmed</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Milk based</td>
<td>High</td>
<td>1.352</td>
<td>18</td>
<td>13</td>
<td>13</td>
<td>10.054</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>0.377</td>
<td>18</td>
<td>7</td>
<td>6</td>
<td>3.296</td>
</tr>
<tr>
<td></td>
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<td>18</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Soy based</td>
<td>High</td>
<td>9.387</td>
<td>18</td>
<td>18</td>
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<td>NA</td>
</tr>
<tr>
<td></td>
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<td>10</td>
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<tr>
<td></td>
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<td>18</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* MPN, most-probable-number estimate of bacterial level in CFU/100 g. MPN was estimated for the revised method using the MPN calculator available at http://www.lcfild.com/customer/LCFMPNCalculate.exe.

$\chi^2 = [(n-b)(a+c)-(b-c)(a+d)]^2/[a+b+c+d]$ [1]$, where a is the number of samples confirmed positive by the revised method, b is the number of samples negative by the revised method, c is the number of samples positive by the reference method, and d is the number of samples negative by the reference method, and n is the sum $a + b + c + d$. A $\chi^2$ value greater than 3.84 indicates a statistically significant difference at the 95% probability level.

NA, statistical analysis not applicable.
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REFERENCES