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Method for the isolation and detection of *Enterobacter sakazakii* (*Cronobacter*) from powdered infant formula

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ABSTRACT

In the United States, there are approximately 76 million foodborne cases annually. Although the number of food-related infections caused by *Enterobacter sakazakii* is relatively low, the United States Food and Drug Administration in 2002 became concerned about the incidence of *E. sakazakii* infections related to powdered infant formula (PIF). At that time, a method to isolate this pathogen from PIF was developed and implemented in several cases. This protocol requires multiple steps and up to 7 days to complete. Recently, a new method was developed that incorporates a real-time PCR-based assay and chromogenic agars to improve isolating and detecting this pathogen in PIF. The updated protocol has undergone and successfully concluded an AOAC pre-collaborative study and is in the process of further validation for the inclusion into the FDA's *Bacteriological Analytical Manual*. This manuscript describes the performance evaluation of the new method.

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1. Introduction

Enterobacter sakazakii has been implicated as a cause of bacterial infections in infants since 1961 (Bowen and Braden, 2006). Although foodborne infections caused by *E. sakazakii* are not common, this pathogen has a 40–80% mortality rate in infected infants. In the United States, the number of cases reported each year has been between one and six. In 2001, an infant died as a result of ingestion of PIF contaminated with *E. sakazakii* (U.S. Center for Diseases Control and Prevention, 2002). Almost a year later, the product was recalled by the manufacturer (http://www.fda.gov/oc/po/firmrecalls/meadjohnson03_02.html) and the US Food and Drug Administration provided guidelines for health care providers involved in preparation of PIF. Prior to establishing a strong causative link between *E. sakazakii* infections and ingestion of PIF as a primary transmission vehicle, microbiological analysis of PIF showed that this pathogen was present in a high number of samples, i.e., 20 out of 141 (14%) (Muytjens et al., 1988). In a later survey conducted in 2003, the rate of *E. sakazakii* in PIF dropped noticeably to 2.4% (Iversen and Forsythe, 2004). The importance of this pathogen and its relationship to PIF was once again highlighted by an international meeting sponsored by the World Health Organization and the Food and Agricultural Organization in 2004 (http://www.who.int/foodsafety/publications/micro/summary.pdf). Here it was noted that infants, particularly premature neonates, are at greater risk of infection than older children and adults. As recently as November 2008, two cases were identified in New Mexico in which one infant died due to complications from *E. sakazakii* infection (http://www.health.state.nm.us/).

Since PIF was considered a likely source of *E. sakazakii* infections, the US FDA devised a method for isolating this pathogen from PIF (http://www.cfsan.fda.gov/~comm/mmesakaz.html). This protocol requires 5–7 days for complete analysis and has multiple growth conditions in broth and selective agar media. Briefly, PIF samples are pre-enriched in water overnight, further enriched in *Enterobacteriaceae* Enrichment (EE) broth overnight, then plated on Violet Red Bile Glucose (VRBG) agar for overnight incubation, and finally plated onto Tripticase Soy Agar (TSA) for another 48 to 72 h incubation. Typical colonies, those producing yellow pigment, are confirmed by biochemical analysis using API 20E (bioMérieux Inc., Durham, NC) biochemical test (http://www.cfsan.fda.gov/~comm/microbio.html). The major limitations of this method are the length of time for a complete analysis, complexities in various growth conditions, and the lack of discrete specificity in identifying *E. sakazakii* particularly in discriminating between this pathogen and other *Enterobacter* species.

Recently, FDA has devised a new method for the detection of *E. sakazakii* in PIF. This method now incorporates two important components. A real-time PCR-based assay with bi-functional applications, utilized as a screening tool and also for culture confirmation. Buffered peptone water is used as the enrichment media, followed by PCR for screening, and two different chromogenic agars for obtaining isolated *E. sakazakii* colonies. From the latter, PCR and RAPID ID 32E (bioMérieux) biochemical tests are then used to confirm the culture from chromogenic agars. The assurance that false negative and positive results will be minimized is a built-in attribute of the entire method. This protocol is currently undergoing a collaborative validation study for

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final adoption into the FDA's *Bacteriological Analytical Manual* (http://www.cfsan.fda.gov/~ebam/bam-toc.html).

It should be noted that, recently, a new genus, *Cronobacter*, was proposed to redefine *E. sakazakii* and previously identified biotypes of *E. sakazakii* were redefined as different species in the genus of *Cronobacter* (Iversen et al., 2008). Since the putative new genus was designated after this work was completed, the nomenclature *E. sakazakii* applies and, as such, was used throughout this manuscript.

2. Materials and methods

2.1. Bacterial strains

All strains, 51 strains of *E. sakazakii* (Table 1) and 42 strains of non-*E. sakazakii* (Table 2) were provided by Center for Food Safety and Applied Nutrition (CFSAN) at the U.S. FDA. These bacterial strains were cultured in Brain Heart Infusion Broth (BHI; Becton Dickinson,

Table 1
Inclusivity of the new FDA method for detection of *E. sakazakii*.

Supplying labs	Original ID	Organism	Source	Country origin	DFI[a]	R&F[b]	RAPID ID 32E	Real-time PCR
UCD[c]/UZH[d]/NRC[e]	E265	*E. sakazakii*	Milk powder	Malaysia	Positive	Positive	*E. sakazakii*	Positive
ILSI[f]	F6-036	*E. sakazakii*	Environment (milk powder)	Malaysia	Positive	Positive	*E. sakazakii*	Positive
ILSI	F6-038	*E. sakazakii*	Environment (milk powder)	Holland	Positive	Positive	*E. sakazakii*	Positive
ILSI	F6-040	*E. sakazakii*	Environment (milk powder)	Holland	Positive	Positive	*E. sakazakii*	Positive
UCD/UZH/NRC	E464	*E. sakazakii*	Environment (milk powder)	Zimbabwe	Positive	Positive	*E. sakazakii*	Positive
ATCC[g]; NCTC[h]	ATCC 29544; NCTC 11467	*E. sakazakii*	Human (throat)	Unknown	Positive	Positive	*E. sakazakii*	Positive
FDA[i]	607	*E. sakazakii*	Unknown	Unknown	Positive	Positive	*E. sakazakii*	Positive
UCD/UZH/NRC	E515	*E. sakazakii*	Water	Switzerland	Positive	Positive	*E. sakazakii*	Positive
ATCC	ATCC 12868	*E. sakazakii*	Unknown	Unknown	Positive	Positive	*E. sakazakii*	Positive
ATCC	ATCC 51329	*E. sakazakii*	Unknown	Unknown	Positive	Positive	*E. sakazakii*	Positive
HCSC[j]; FDA	SK90	*E. sakazakii*	Clinical (children's hospital)	Canada	Positive	Positive	*E. sakazakii*	Positive
UCD/UZH/NRC	E632	*E. sakazakii*	Food	USA	Positive	Positive	*E. sakazakii*	Positive
HCSC	HPB 2848	*E. sakazakii*	Clinical	Canada	Positive	Positive	*E. sakazakii*	Positive
HCSC	HPB 2873	*E. sakazakii*	Clinical	Canada	Positive	Positive	*E. sakazakii*	Positive
HCSC	HPB 2874	*E. sakazakii*	Clinical	Canada	Positive	Positive	*E. sakazakii*	Positive
UCD/UZH/NRC	H. Muytjens (Prague 72 26248)	*E. sakazakii*	Unknown	Czech Republic	Positive	Positive	*E. sakazakii*	Positive
UCD/UZH/NRC	H. Muytjens 52	*E. sakazakii*	Milk powder	Australia	Positive	Positive	*E. sakazakii*	Positive
UCD/UZH/NRC	H. Muytjens 58	*E. sakazakii*	Milk powder	Belgium	Positive	Positive	*E. sakazakii*	Positive
UCD/UZH/NRC	H. Muytjens 15	*E. sakazakii*	Milk powder	Denmark	Positive	Positive	*E. sakazakii*	Positive
UCD/UZH/NRC	H. Muytjens 8	*E. sakazakii*	Milk powder	France	Positive	Positive	Non-E.sak	Positive
UCD/UZH/NRC	H. Muytjens 35	*E. sakazakii*	Milk powder	Russia	Positive	Positive	*E. sakazakii*	Positive
UCD/UZH/NRC	H. Muytjens 26	*E. sakazakii*	Milk powder	Russia	Positive	Positive	*E. sakazakii*	Positive
UCD/UZH/NRC	H. Muytjens (Nijmegen 15)	*E. sakazakii*	Neonate	Holland	Positive	Positive	*E. sakazakii*	Positive
UCD/UZH/NRC	H. Muytjens (Nijmegen 21)	*E. sakazakii*	Neonate	Holland	Positive	Positive	*E. sakazakii*	Positive
CDC[k]	CDC 5960-70	*E. sakazakii*	Human (blood)	USA	Positive	Positive	*E. sakazakii*	Positive
CDC	CDC 3523-75	*E. sakazakii*	Human (bone marrow)	USA	Positive	Positive	*E. sakazakii*	Positive
NCTC	NCTC 9238	*E. sakazakii*	Human (abdominal pus)	UK	Positive	Positive	*E. sakazakii*	Positive
NCTC	NCTC 9529	*E. sakazakii*	Water	UK	Positive	Positive	*E. sakazakii*	Positive
ATCC	ATCC BAA893	*E. sakazakii*	Unknown	USA	Positive	Positive	*E. sakazakii*	Positive
ATCC	ATCC BAA894	*E. sakazakii*	Unknown	USA	Positive	Positive	*E. sakazakii*	Positive
CDC	CDC 996-77	*E. sakazakii*	Human (spinal fluid)	USA	Positive	Positive	*E. sakazakii*	Positive
CDC	CDC 1058-77	*E. sakazakii*	Human (breast abscess)	USA	Positive	Positive	*E. sakazakii*	Positive
CDC	CDC 407-77	*E. sakazakii*	Human (sputum)	USA	Positive	Positive	*E. sakazakii*	Positive
CDC	CDC 3128-77	*E. sakazakii*	Human (sputum)	USA	Positive	Positive	*E. sakazakii*	Positive
CDC	CDC 9369-75	*E. sakazakii*	Unknown	USA	Positive	Positive	*E. sakazakii*	Positive
UZH	3032	*E. sakazakii*	Neonate (meningitis)	Switzerland	Positive	Positive	*E. sakazakii*	Positive
HCSC; ILSI	SK81; F6-023	*E. sakazakii*	Human	Canada	Positive	Positive	*E. sakazakii*	Positive
ILSI; RAD[l]	F6-029	*E. sakazakii*	Neonate	Holland	Positive	Positive	*E. sakazakii*	Positive
ILSI	01-10-2001; F6-034	*E. sakazakii*	Clinical	USA	Positive	Positive	*E. sakazakii*	Positive
ILSI	8397; F6-043	*E. sakazakii*	Clinical	USA	Positive	Positive	*E. sakazakii*	Positive
CDC; ILSI	CDC 289-81; F6-049	*E. sakazakii*	Clinical	USA	Positive	Positive	*E. sakazakii*	Positive
CDC; ILSI	CDC 1716-77; F6-052	*E. sakazakii*	Human (blood)	USA	Positive	Positive	*E. sakazakii*	Positive
ILSI; RAD	F6-032; H. Muytjens 7	*E. sakazakii*	Milk powder	Uruguay	Positive	Positive	*E. sakazakii*	Positive
UCD	CFS112	*E. sakazakii*	Milk powder	Ireland	Positive	Positive	*E. sakazakii*	Positive
UCD	CFS349N	*E. sakazakii*	Milk powder	New Zealand	Positive	Positive	*E. sakazakii*	Positive
UCD	CFS352N	*E. sakazakii*	Milk powder	New Zealand	Positive	Positive	*E. sakazakii*	Positive
UCD	CFS237	*E. sakazakii*	Milk powder	Ireland	Positive	Positive	*E. sakazakii*	Positive
CDC	CDC 9363-75	*E. sakazakii*	Stool	USA	Positive	Positive	Non-E.sak	Positive
CDC	CDC 4963-71	*E. sakazakii*	Stool	USA	Positive	Positive	*E. sakazakii*	Positive
CDC	CDC 1895-73	*E. sakazakii*	Human (faeces)	USA	Positive	Positive	*E. sakazakii*	Positive
RF[m]	Es626	*E. sakazakii*	Rice flour	USA	Positive	Positive	*E. sakazakii*	Positive

[a] Positive of DFI shows green colony as *E. sakazakii*.
[b] Positive of R&F shows blue–green–black colony as *E. sakazakii*.
[c] UCD S. Fanning, Centre for Food Safety, University College Dublin, Belfield, Dublin 4, Ireland.
[d] UZH R. Stephan, Institute for Food Safety, University of Zurich, Winterthurerstrasse 270, CH-8057, Switzerland.
[e] NRC Nestlé Research Centre, Vers-Chez-les-Blanc, Lausanne, CH-1000, Switzerland.
[f] ILSI R. Ivy, Food Safety lab, Cornell University, 412 Stocking Hall, Ithaca, NY, USA.
[g] ATCC American Type Culture Collection, Manassas, VA, USA.
[h] NCTC National Collection of Type Cultures, London, UK.
[i] FDA R. Buchanan, FDA-CFSAN, College Park, MD, USA.
[j] HCSC F. Pagotto, Health Products and Food branch, Health Canada, Ottawa, Canada.
[k] CDC Center for Disease Control, Atlanta, GA, USA.
[l] RAD Department of Medical Microbiology, University of Nijmegen, Radboud, Netherlands.
[m] RF L. Restaino, R&F Laboratories, Downers Grove, IL, USA.