TAP SAKAZAKII CONF LAIT V2:Mise en page 1 14/09/09 17:03 Page 1

RAPID´*sakazakii* – A **Short Protocol Method** for the **Detection of *Cronobacter* spp.** in Powdered Infant Formula Milk

Christophe Quiring[1], Maryse Rannou[2], Danièle Sohier[2] , Nathalie Bernard[1] and Nicolas Duquennoy[1]
[1] Bio-Rad Laboratories, Marnes-la-Coquette, France - [2] ADRIA Développement, Quimper, France

Introduction

Cronobacter spp. are Gram-negative, rod-shaped, motile and facultative-anaerobic bacteria originally listed as yellow-pigmented *Enterobacter cloacae*. The bacteria were subsequently called *Enterobacter sakazakii*. Taxonomic studies have determined that *E. sakazakii* comprises a high genetic heterogeneity and should be classified as a novel genus: *Cronobacter* (2).
Cronobacter spp. has been implicated in severe infections such as meningitis, necrotizing enterocolitis, bacteremia and sepsis in neonates (4). In several of these cases, consumption of contaminated powdered infant formula milk PIFM was identified as the source of *Cronobacter* ingestion (3,5).
In response to this hazard, the International Dairy Federation (IDF) and the International Standard Organization (ISO) have standardized the reference method ISO/TS 22964 for the detection of *Cronobacter* spp. which includes of a double enrichment in buffered peptone water (BPW) and modified lauryl sulphate tryptose broth (mLST) followed by isolation on a selective chromogenic agar (1).
To meet this standard the RAPID'*sakazakii* agar has been developed for the detection of *Cronobacter* spp.
Its principle is based on the chromogenic detection of α-D-glucosidase common to all *Cronobacter* species. Hydrolysis of the chromogenic substrate leads to the formation of typical blue to turquoise colonies of *Cronobacter*. A shortened protocol consisting in direct plating on RAPID'*sakazakii* after a single enrichment in BPW has been proposed for the detection of *Cronobacter* spp. in powdered infant formula.
The aim of this study was to assess the RAPID'*sakazakii* short protocol method compared to the double enrichment with the current ISO/TS 22964 standard (Figure 1).

Materials and Methods

All experiments were performed according to technical specifications of the EN ISO 16140 validation standard regarding inclusivity, exclusivity and the comparison study of methods.
Inclusivity, the ability of the RAPID'*sakazakii* method to detect *Cronobacter* spp. with typical colonies was investigated with 24 *Cronobacter* strains (due to the recent change in nomenclature, most strains are always designated *Enterobacter sakazakii*).
Exclusivity, the ability of the method to distinguish non-target strains from *Cronobacter* spp. was tested with 34 strains of *Enterobacteriaceae*.
The RAPID'*sakazakii* short protocol method was also evaluated in comparison with the reference method ISO/TS 22964 by testing 62 powdered infant formula samples including 1 naturally contaminated sample and 30 artificially contaminated samples (6 samples by cross-contamination and 24 samples using a strain of *Cronobacter* stressed by heat treatment at 56°C for 15 minutes).

Results and Discussion

Figure 1: ISO/TS 22964 and RAPID'*sakazakii* protocols

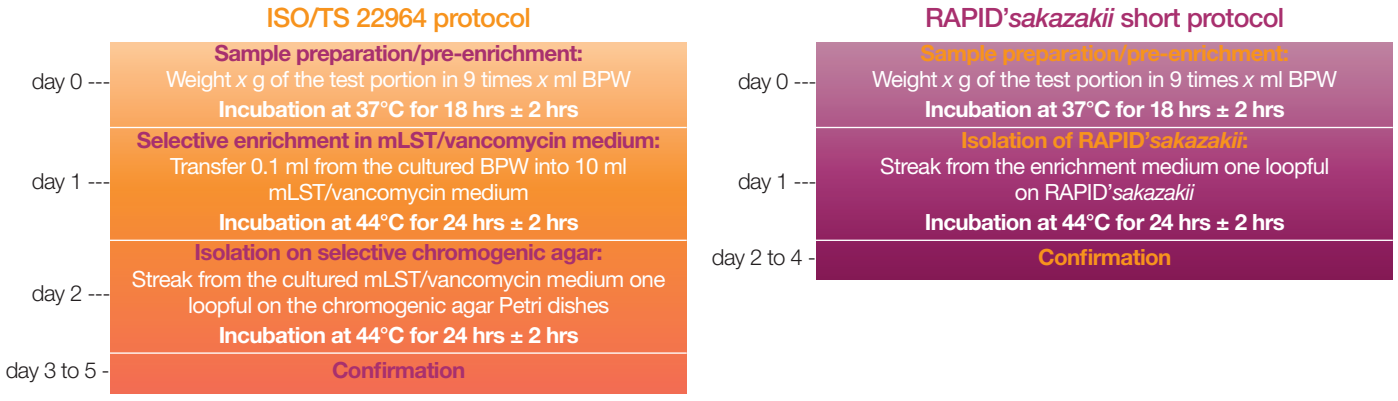

Table 1: Inclusivity study

Microorganism	ID	Growth	Colony color
Enterobacter sakazakii	IPL05/07/019	++	turquoise
Enterobacter sakazakii	IPL06/09/040	++	turquoise
Enterobacter sakazakii	IPL06/26/005	++	turquoise
Enterobacter sakazakii	IPL449076	++	turquoise
Enterobacter sakazakii	IPL480189	++	turquoise
Enterobacter sakazakii	AdriaX36	++	turquoise
Enterobacter sakazakii	AdriaX37	++	turquoise
Enterobacter sakazakii	AdriaX39	++	turquoise
Enterobacter sakazakii	AdriaX40	++	turquoise
Enterobacter sakazakii	AdriaX41	++	turquoise
Enterobacter sakazakii	RDC236	++	turquoise
Enterobacter sakazakii	RDC361	++	turquoise
Enterobacter sakazakii	RDC362	++	turquoise
Enterobacter sakazakii	RDC363	++	turquoise
Enterobacter sakazakii	RDC364	++	turquoise
Enterobacter sakazakii	RDC365	++	turquoise
Enterobacter sakazakii	RDC366	++	turquoise
Enterobacter sakazakii	RDC367	++	turquoise
Enterobacter sakazakii	LMG5740	++	turquoise
Enterobacter sakazakii	ATCC51329	++	turquoise
Cronobacter sakazakii	LMG23826T	++	turquoise
Cronobacter dublinensis	DSM 18705	++	turquoise
Cronobacter malonaticus	DSM18702	+	turquoise
Cronobacter muytjensii	CIP103581	+	turquoise

IPL: Strain from Institut Pasteur, France
Adria, Ad: Adria Collection, France
RDC: Bio-Rad R&D Collection, France

Table 2: Exclusivity study

Microorganism	ID	Growth	Colony color
Enterobacter aerogenes	ATCC13048	++	purple
Enterobacter aerogenes	RDC227	++	purple
Enterobacter aerogenes	Ad889	+/-	Faint purple
Enterobacter agglomerans	Adria11	+/-	faint purple
Enterobacter amnigenus	RDC222	-	/
Enterobacter amnigenus	Adria129	-	/
Enterobacter amnigenus	A00C068	-	/
Enterobacter cloacae	LMG2742	++	purple
Enterobacter cloacae	RDC22	++	purple
Enterobacter cloacae	Adria10	-	/
Enterobacter cloacae	RDC225	++	purple
Enterobacter fergusonii	Adria2876	++	faint purple
Enterobacter gergoviae	LMG5739	+/-	purple
Enterobacter gergoviae	CIP76.1T	-	/
Enterobacter intermedius	RDC234	-	/
Enterobacter intermedius	Adria60	-	/
Enterobacter hormaechei	Ad990	++	faint purple
Enterobacter kobei	RDC221	+	purple
Enterobacter kobei	Ad706	+/-	purple
Enterobacter spp	RDC205	++	purple
Citrobacter freudii	ATCC8090	+	purple
Citrobacter koseri	RDC186	+/-	purple
Escherichia coli	ATCC25922	++	grey blue to purple
Escherichia coli	DSMZ1576	++	grey blue to purple
Escherichia hermanii	RDC72	++	purple
Escherichia vulneris	RDC195	-	/
Hafnia alvei	CIP57.31	-	/
Klebsiella oxytoca	RDC30	++	opalescent blue
Klebsiella pneumoniae	ATCC13883	++	opalescent blue
Leclercia adecarboxylata	Ad707	-	/
Pantoea agglomerans	LMG1286T	-	/
Salmonella Enteritidis	ATCC13076	++	purple
Salmonella Typhimurium	ATCC14028	++	purple
Serratia marcescens	ATCC8100	-	/

Inclusivity and exclusivity

In pure culture testing, all *Cronobacter/E. sakazakii* strains produced typical turquoise colonies within 22 hours of incubation (Table 1). Most of the strains showed large colonies with a shimmering, highly visible turquoise color.
None of the non-target strains gave typical colonies. Among 34 strains of *Enterobacteriaceae* tested, 2 strains belonging to the genus *Klebsiella* produced opalescent blue colonies easily distinguishable from *Cronobacter* colonies (Table 2).

Methods comparison study

Among 62 samples tested for *Cronobacter* spp. detection, 29 positive results were found with at least one method.
Each method detected 30 positive samples and failed to detect 1 positive sample (Table 3).

Table 3: Paired results of the reference method ISO/TS 22964 and the RAPID'*sakazakii* method

	ISO/TS 22964 meth. +	ISO/TS 22964 meth. -
RAPID'*sakazakii* meth. +	29*	1**
RAPID'*sakazakii* meth. -	1†	31††

* Positive Agreement (PA) ** Positive Deviation (PD)
† Negative Deviation (ND) †† Negative Agreement (NA)

These results enabled the calculation of relative accuracy, relative specificity and relative sensitivity of the RAPID'*sakazakii* short protocol method compared to the reference method ISO/TS 22964 (Table 4).

Table 4: Relative accuracy, relative specificity and relative sensitivity of the RAPID'*sakazakii* method compared to ISO/TS 22964

	Rel. accuracy	Rel. specificity	Rel. sensitivity
Response to *Cronobacter* spp.	96.8%	96.9%	96.7%

The sensitivities of both methods, by taking into account all obtained positive results, were re-evaluated according to the EN ISO 16140 standard (Table 5).

Table 5: Sensitivities of the reference ISO/TS 22964 and the RAPID'*sakazakii* methods

	Ref. method	RAPID'*sakazakii* meth.
Response to *Cronobacter* spp.	96.7%	96.7%

Both methods provided exactly the same results for the detection of *Cronobacter* in powdered infant formulas.

Conclusion

The RAPID'*sakazakii* short protocol method was evaluated for the detection of Cronobacter spp. compared to the standardized reference method ISO/TS 22964.
The inclusivity/exclusivity study demonstrated the superior specificity of the RAPID'*sakazakii* method.
Regarding sensitivity, the performances of the RAPID'*sakazakii* method were similar to the reference method. Removing the 2nd enrichment step of the reference method, the RAPID'*sakazakii* method reduces the time to result. Moreover, *Cronobacter* colonies are often larger and more colorful on RAPID'*sakazakii* agar compared to the reference chromogenic agar.
In conclusion, the RAPID'*sakazakii* short protocol method has been demonstrated to be an efficient, convenient, cost and time-saving method for the detection of *Cronobacter* spp. in powdered infant formula milk.

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