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Short Communication

Characterization and subtyping of *Cronobacter* spp. from imported powdered infant formulae in Argentina

R. Terragno [a,*], A. Salve [a], M. Pichel [a], S. Epszteyn [b], S. Brengi [a], N. Binsztein [a]

[a] Instituto Nacional de Enfermedades Infecciosas (INEI) — ANLIS "Carlos G. Malbrán", Av. Velez Sarsfield 563, C.P. 1281, Buenos Aires, Argentina
[b] Dirección General de Higiene y Seguridad Alimentaria–Gobierno de la Ciudad Autónoma de Buenos Aires, Patricias Argentinas 277 C.P. 1045, Buenos Aires, Argentina

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ABSTRACT

Cronobacter spp. (*Enterobacter sakazakii*), have been associated with severe foodborne infections in neonates and immunocompromised infants. In Argentina, we have isolated *Cronobacter* spp. from three different brands of imported powdered infant formulae (PIF). The objectives of this work were to characterize the recovered isolates phenotypically and to evaluate the use of a Pulsed-Field Gel Electrophoresis (PFGE) protocol for *Cronobacter* spp. subtyping. Out of 23 isolates studied from three brands of PIF (20 of brand A, 1 of brand B and 2 of brand C), 22 were identified as *C. sakazakii* and 1 as *C. malonaticus*. All isolates were susceptible to twelve antimicrobial agents assayed. The 19 *C. sakazakii* isolates of brand A showed five XbaI-PFGE patterns and the genetic clusters revealed by XbaI were confirmed with a second restriction enzyme, SpeI. The isolate from brand B showed the same XbaI and SpeI patterns as those of a group of isolates of brand A, suggesting a possible common source of contamination. The *C. sakazakii* isolates of brand C exhibited two unique XbaI-PFGE patterns, unrelated to the rest. Different genetic subtypes were found among isolates of a single batch of PIF from brand A and the single *C. malonaticus* strain also showed a distinct XbaI-PFGE pattern.

1. Introduction

Cronobacter (*Enterobacter sakazakii*) (Iversen et al., 2008) are opportunistic pathogens associated with necrotizing enterocolitis, septicemia and meningitis. These organisms have a high case fatality rate in vulnerable infants and, in surviving patients, severe neurological sequelae have occurred including hydrocephalus, quadriplegia and developmental delay (Lai, 2001). Although infections have occurred in all age groups, infants less than two months old, preterm or low birth weight infants and those with immune systems weakened by illness are at greatest risk (Bowen and Braden, 2006). Furthermore, *Cronobacter* have been linked to several outbreaks of neonatal meningitis and necrotizing enterocolitis in the USA and Europe (Muytjens et al., 1983; Simmons et al., 1989; van Acker et al., 2001; Caubilla-Barron et al., 2007).

The natural environment of *Cronobacter* is not known, but these appear to be a group of widely distributed microorganisms found in various foods (Iversen and Forsythe, 2004; Friedemann, 2007). They have also been isolated from a number of environments including hospitals, households and factories (Kandhai et al., 2004). *Cronobacter* have been associated with the consumption of powdered infant formulae (PIF) and have been isolated from these products by numerous investigators (van Acker et al., 2001; Iversen and Forsythe, 2004; Mullane et al., 2007; Proudy et al., 2008b). Their presence in a great variety of environments suggests that this opportunistic pathogen is ubiquitous, making control more difficult. This ubiquity highlights the importance of subtyping, to trace the contamination sources and transmission routes of *Cronobacter*. Furthermore, molecular epidemiology tools for bacterial subtyping are also important for public health investigations to determine the genetic relationship of outbreak related isolates and to establish the sources of human infections. Among the molecular epidemiology techniques, Pulsed-Field Gel Electrophoresis (PFGE) has been used successfully to perform comparative chromosomal DNA analysis of several bacterial pathogens. It has a discriminatory ability greater than other methods, is often considered the "gold standard" of molecular typing methods, and is recommended to be used in surveillance and outbreaks investigations (Tenover et al., 1995; Olive and Bean, 1999; Gerner-Smidt et al., 2006). Moreover the PFGE methodology has gained an important role for the subtyping of foodborne pathogens, since the creation of PulseNet (Swaminathan et al., 2001).

In Argentina, no human cases of *Cronobacter* spp. infections have been documented up to now. However, between 2005 and 2008, the pathogen was found in three brands of imported PIF from three separate companies in different countries. These results led us to focus on the study of this pathogen, as well as to implement and stimulate its surveillance in the routine analysis of PIF in Argentina. In the present study, we describe the biochemical characterization, the antimicrobial

* Corresponding author. Servicio Enterobacterias, Instituto Nacional de Enfermedades Infecciosas — ANLIS "Carlos G. Malbrán", Av. Velez Sarsfield 563, C.P. 1281, Buenos Aires, Argentina. Tel./fax: +54 11 43032333.
E-mail address: rterragno@anlis.gov.ar (R. Terragno).

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susceptibility and the PFGE subtyping of a collection of *Cronobacter* isolates recovered in our country.

2. Materials and methods

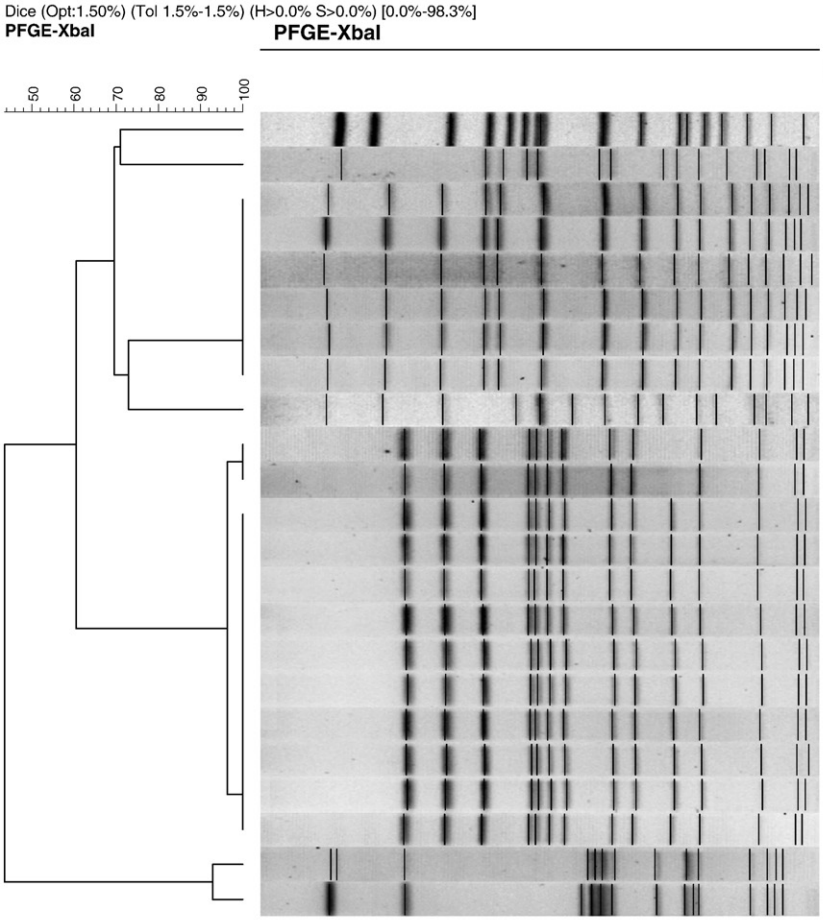
2.1. Bacterial strains

Twenty-three *Cronobacter* spp. isolates were studied, recovered in Argentina between 2005 and 2008 from PIF samples of three different brands, produced by three separate companies. Twenty isolates were from samples of brand A, imported from a Latin American country. One isolate of brand B, from a different Latin American country and 2 isolates of brand C, from Europe. The samples from brand A were analyzed in 2005 as an initiative to search for *Cronobacter* spp., after the FAO/WHO designation of this pathogen as category A for PIF (FAO/WHO, 2004). The first samples of brand A were obtained for routine inspection; others after a subsequent recall due to the finding of *Cronobacter* spp. and some other samples were submitted by customers after a public alert. One of the batches from brand A had been widely distributed in the country and, therefore, more samples were analyzed from this one than from the rest of the batches. In 2006, *Cronobacter* spp. was found only in samples from a batch of brand B during routine inspection. The isolates identified in 2007 and 2008 belonged to brand C and were submitted to the National Reference Laboratory, Instituto Nacional de Enfermedades Infecciosas — ANLIS "Carlos G. Malbrán", by the Instituto Nacional de Alimentos, Ministerio de Salud de la Nación.

The isolation and identification of *Cronobacter* spp. from PIF samples of brands A and B were carried out according to the method of US FDA (Anonymous, 2002), briefly: triplicates of 100 g, 10 g, and 1 g samples from each single can were suspended in pre-warmed sterile distilled water to obtain 1:10 dilutions and incubated 18 h at 37 °C. After a secondary enrichment in Enterobacteriaceae enrichment broth, duplicate Violet Red Brilliant Green (VRBG) agar plates were inoculated for isolation of Enterobacteriaceae; 5 presumptive colonies were subcultured by streaking each colony onto a single trypticase soy agar plate (TSA). The resulting isolated colonies from the TSA plates were identified using the API 20E biochemical identification system according to the manufacturer's instructions. At the National Reference Laboratory, Instituto Nacional de Enfermedades Infecciosas —ANLIS "Carlos G. Malbrán", the isolates were confirmed by biochemical characterization with individual tests and by the performance of a PCR for amplification of the *ompA* gene, as described below.

2.2. Biochemical characterization

Biochemical tests were performed as follows, with negative tests being discarded after 7 days incubation. Acid production from carbohydrates (adonitol, dulcitol, glucose, lactose, raffinose, sucrose



# isolate	# Pattern-*XbaI*	# Pattern-*SpeI*	Brand (Batch)	species
ARG-1	1 - XbaI	NR	C (1)	s
ARG-2	2 - XbaI	NR	C (2)	s
ARG-3	3 - XbaI	3 - SpeI	A (1)	s
ARG-4	3 - XbaI	3 - SpeI	A (2)	s
ARG-5	3 - XbaI	3 - SpeI	A (1)	s
ARG-6	3 - XbaI	3 - SpeI	A (3)	s
ARG-7	3 - XbaI	3 - SpeI	A (1)	s
ARG-8	3 - XbaI	3 - SpeI	A (1)	s
ARG-9	4 - XbaI	NR	A (1)	m
ARG-10	5 - XbaI	2 - SpeI	A (1)	s
ARG-11	5 - XbaI	1 - SpeI	A (1)	s
ARG-12	6 - XbaI	1 - SpeI	B	s
ARG-13	6 - XbaI	1 - SpeI	A (1)	s
ARG-14	6 - XbaI	1 - SpeI	A (1)	s
ARG-15	6 - XbaI	1 - SpeI	A (1)	s
ARG-16	6 - XbaI	1 - SpeI	A (1)	s
ARG-17	6 - XbaI	1 - SpeI	A (1)	s
ARG-18	6 - XbaI	1 - SpeI	A (1)	s
ARG-19	6 - XbaI	1 - SpeI	A (4)	s
ARG-20	6 - XbaI	1 - SpeI	A (1)	s
ARG-21	6 - XbaI	1 - SpeI	A (1)	s
ARG-22	7 - XbaI	NR	A (1)	s
ARG-23	8 - XbaI	NR	A (5)	s

( ) The identification of the batches was arbitrarily assigned.

s: *C. sakazakii* – **m:** *C. malonaticus*

NR: not realized

Fig. 1. XbaI-PFGE dendrogram of the 23 isolates of *Cronobacter* spp. recovered from imported powdered infant formulae in Argentina.