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Incidence of *Cronobacter sakazakii* in Powdered Infant Formula Milk Available in Malaysia

(Insidens *Cronobacter sakazakii* dalam Susu Tepung Rumusan Bayi yang Terdapat di Malaysia)

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ABSTRACT

Enterobacter sakazakii *previously known as 'yellow-pigmented* E. cloacae*' has been classified as a new genus '*Cronobacter*' based on taxonomic analysis and geno-and phenotypic evaluation. This pathogenic organism has been associated with rare form of infant meningitis and necrotizing enterocolitis (NEC) with high mortality rate (40-80%). Some cases have been linked to the consumption of contaminated powdered infant formula milk (PIF). The objective of this study was to determine the presence of* Cronobacter *spp. in PIF sold in Malaysia. A selective chromogenic agar, Brilliance* Enterobacter sakazakii *(DFI, Oxoid), was used for detection of* Cronobacter *strains. Presumptive* Cronobacter *isolates were identified using biochemical tests (API 20E and Microgen™) and molecular assays (SYBR Green Real-time PCR and 16S ribosomal DNA sequencing). All presumptive* Cronobacter *strains produced typical blue-green colonies and non-*Cronobacter *strains produced yellow colonies on Brilliance* Enterobacter sakazakii *agar (DFI formulation). A total of 12 presumptive isolates were selected from DFI agar and identified with biochemical and molecular tests. The results indicated prevalence of 12.5%* C. sakazakii *contamination from 72 PIF samples. Molecular detection methods such as Real-time PCR and 16S rDNA proved to have higher identification percentage compared to the biochemical tests. In this study, it was observed that molecular assays were suitable means for sensitive identification of* Cronobacter *strains in PIF samples.*

Keywords: Biochemical test; chromogenic media; Cronobacter sakazakii*; Enterobacteriaceae; Real time-PCR*

ABSTRAK

Enterobacter sakazakii *yang sebelum ini dikenali sebagai '*E. cloacae *berpigmen kuning' telah dikelaskan sebagai genus baru '*Cronobacter*' berdasarkan analisis takson serta semakan secara fenotip dan genotip. Organisma patogen ini dikaitkan dengan penyakit meningitis pada bayi dan enterokolitis nekrosis (NE) dengan kadar kematian yang tinggi (40-80%). Sesetengah kes dikaitkan dengan pengambilan susu tepung rumusan bayi (PIF) yang telah tercemar. Objektif kajian ini adalah untuk menentukan kehadiran* Cronobacter *spp. di dalam PIF di Malaysia. Suatu agar pilihan kromogenik, Brilliance* Enterobacter sakazakii *(DFI, Oxoid) telah digunakan bagi mengenal pasti pencilan* Cronobacter*. Pencilan* Cronobacter *jangkaan telah dikenal pasti dengan menggunakan ujian biokimia (API 20E dan Microgen™) dan asai molekul (SYBR Hijau Masa-nyata PCR dan penjujukan 16S DNA ribosom). Semua pencilan* Cronobacter *jangkaan telah menghasilkan koloni berwarna biru kehijauan manakala pencilan bukan* Cronobacter *menghasilkan koloni berwarna kuning di atas agar Brilliance* Enterobacter sakazakii *(DFI formulation). Sejumlah 12 pencilan jangkaan telah dipilih daripada agar DFI dan dikenal pasti dengan menggunakan ujian biokimia dan molecular. Hasil kajian menunjukkan pelumusan lazim oleh* C. sakazakii *daripada 72 sampel PIF adalah sebanyak 12.5%. Kaedah pengesanan molekul seperti Masa-nyata PCR dan 16S rDNA telah menunjukkan peratus pengenalpastian yang lebih tinggi berbanding dengan hasil ujian biokimia. Kajian ini menunjukkan pengasaian secara molekul adalah suatu cara yang sesuai dan sensitif bagi mengenal pasti pencilan* Cronobacter *dalam sampel PIF.*

Kata kunci: Cronobacter sakazakii*; Enterobacteriaceae; Masa-nyata PCR; medium kromogenik; ujian biokimia*

INTRODUCTION

Recently *Enterobacter sakazakii* has been reclassified as six species in a new genus, *Cronobacter*, based on geno- and phenotypic evaluation (Iversen et al. 2008). *C. sakazakii,* previously referred to as 'yellow-pigmented *E. cloacae'* based on differences in DNA-DNA hybridization, biochemical reactions, production of yellow-pigmented colonies and antibiotic susceptibility (Iversen et al. 2007), is a motile, non-sporeforming, gram-negative foodborne pathogen belonging to the family Enterobacteriaceae (Iversen et al. 2008). This pathogenic organism has been associated with life-threatening outbreaks of a rare form of infant meningitis, necrotizing enterocolitis (NEC), bacteremia and may cause death among neonates (Bar-Oz et al. 2001; Bowen and Braden 2006; Caubilla-Barron & Forsythe 2007; Lai 2001). The groups at particular risk are infants (i.e. children < 1 year) and those who are immunocompromised. Neonates are considered to be at

greatest risk, particularly neonates of low birth weight (FAO/WHO 2006, 2004; Mullane et al. 2007). *C. sakazakii* has been isolated from a wide range of foods including cereals, cheese, fruits, meat, milk, vegetables, grains, herbs and spices as well as their by-products (Friedemann 2007; Iversen & Forsythe 2004). However, its presence in powdered infant formula milk (PIF) as the most common food has raised concern among the food microbiologists (Forsythe 2005).

There are differences between various *Cronobacter* detection and identification methods. Several methods for the specific detection of *Cronobacter* from PIF based on α-glycosidase activity test have been improved recently, including the technical standard ISO/TS 22964 (2006) method from the International Organization for Standardization (ISO) (ISO/TS 22964 2006). Methods available for monitoring *Cronobacter* have been reviewed by Fanning and Forsythe (2007). Culturing methods have improved with the development of chromogenic agars, which helps to distinguish *Cronobacter* from other Enterobacteriaceae that may additionally be present in powdered formula. A test method for isolation of *Cronobacter* developed by Iversen et al. (2004) using chromogenic medium, i.e. Brilliance *Enterobacter sakazakii* agar (Oxoid) formerly known as Druggan Forsythe Iversen (DFI) formulation agar, is based on α-glycosidase activity to give blue-green colonies followed by confirmation with rapid biochemical tests such as API® Gram negative Identification (API 20E) biochemical profile. Meanwhile, the conventional general Enterobacteriaceae method applies to the detection of Enterobacteriaceae on violet red bile glucose agar (VRBGA) followed by yellow pigment production on tryptone soy agar (TSA) after 48-72 h at 25°C (FDA 2002). However, the later method is not specific since not all *Cronobacter* produces yellow-pigmentation.

Molecular assays and DNA-sequence based methods have often proven to be useful as they offer an alternative means of identification of variety of organisms (Keyser et al. 2003). Real time polymerase chain reaction (PCR) offers rapid and quantitative analysis for detection and identification of food-borne pathogens (Zhu et al. 2005) and is able to detect the amount of PCR product (amplicon) using fluorescence. Two approaches have been employed to detect PCR products such as TaqMan and SYBR Green Real-time PCR. The most popular, the 5'-nuclease assay is based on the specific hybridization of a dual-labeled TaqMan probe to the PCR product. SYBR-Green method is based on the binding of the fluorescent dye SYBR-Green into the amplified DNA during the primer annealing and extension steps (Liu et al. 2006).

In this study the prevalence of *Cronobacter* spp. in PIF sold in Malaysia was investigated to determine the presence of *Cronobacter sakazakii*. A *Brilliance Enterobacter sakazakii* agar (DFI formulation) (CM1055; Oxoid) was used for detection of *Cronobacter* strains. Presumptive *Cronobacter* isolates were identified using biochemical tests and molecular assays namely, SYBR Green Real-time PCR and 16S ribosomal DNA (rDNA) sequencing.

MATERIALS AND METHODS

ISOLATION OF *CRONOBACTER* FROM POWDERED INFANT FORMULA MILK

A total of 72 PIF samples from 8 different manufacturers in the Malaysian market were evaluated for the presence of *Cronobacter* and other Enterobacteriaceae. For each sample, 25 g was suspended in 225 mL of buffered peptone water (BPW, CM 509, Oxoid) (1: 10, w/v) and homogenized for 1 min at low speed in a Stomacher 400 (Seward, UK) and incubated overnight at 37°C. A 10 mL aliquot of each homogenate was mixed with 90 mL of Enterobacteriaceae Enrichment broth (EE, CM 317, Oxoid), (1:10 w/v) and incubated at 37°C for 24 h. One loopful of incubated EE broth samples were streaked on *Brilliance Enterobacter sakazakii* agar (DFI formulation) (CM 1055, Oxoid) and incubated overnight at 37°C.

IDENTIFICATION OF *CRONOBACTER* ISOLATES USING PHENOTYPING

Colonies that appeared entirely blue-green on DFI agar after 24 h incubation were considered presumptive positive for *Cronobacter*. These typical colonies were then subcultured on Nutrient agar (NA, CM 3, Oxoid) and incubated at 37°C for 24 h and identified using API 20E (bioMérieux France) and Microgen™ GN-ID (GNA+ GNB, Microgen Bioproducts Ltd., UK) biochemical identification systems. The cells were stored in 20% (v/v) glycerol at -20°C. *C. muytjensii* (ATCC 51329) was used as the positive control organism. This was formerly the ATCC Preceptrol™ strain for the quality control of '*Enterobacter sakazakii*' prior to the taxonomic revision.

DNA EXTRACTION

Presumptive *Cronobacter* strains isolated from PIF samples (described above) were also identified using Real-time PCR and 16S rDNA sequencing. In order to extract genomic DNA of the bacteria, a single colony was picked from nutrient agar and added to 1 mL of sterilized Tryptone Soya Broth (TSB, CM 129, Oxoid). The cells were pelleted by centrifuging at 12000 g for 4 min at 4°C (Refrigerator Centrifuge, Eppendorf, Germany) and the bacterial pellets were subjected to DNA extraction MasterPure™ Complete DNA and RNA Purification Kit (EPICENTRE, Madison, Wisconsin, U.S). The genomic DNA samples were re-suspended in Tris-EDTA Buffer (1M Tris-HCl and 0.5 mM EDTA, pH8).

IDENTIFICATION OF *CRONOBACTER* ISOLATES WITH REAL TIME-PCR USING SYBR GREEN

The FailSafe™ Real-time PCR Capillary Pre-Mix Selection Kit (EPICENTRE, Madison, Wisconsin, U.S) containing a unique blend of thermostable DNA polymerases and a set of eight reaction PreMixes (C1-C8) were used for PCR amplification. The *Cronobacter* specific primers used in this study designed by Liu et al. (2006) were: Forward (F) primer:

5'-TATAGGTTGTCTGCGAAAGCG-3' and Reverse (R) primer: 5'-GTCTTCGTGCTGCGAGTTTG-3'. The master mix (20 µL) for PCR amplification containing 8.0 µL sterilized doubled distilled water; 0.5 µL of 10 Pmol of each primer, 0.5 µL of DNA template and 0.5 µL of FailSafe Real-time PCR Enzyme Mix were added to 10 µL of FailSafe Real time-PCR Capillary 2X PreMix (C4) which include SYBR Green I (Molecular Probes, Inc., Eugene, Oregon). PCR was carried out in a Roche thermal cycler (Roche LightCycler 4559, Hoffmann-La Roche Inc., Basel, Switzerland) as follows: An initial 2-step cycling pre-incubation at 40°C for 120 s and 95°C for 60 s for denaturing the templates, 45 cycles consist of denaturing at 95°C for 10 s, 62°C for 10 s for annealing the primers and 72°C for 20 s for extension. Following amplification a melting curve analysis of the amplified DNA was performed at temperatures between 57 and 95°C, with the temperature increasing at a rate of 0.1°C/s, followed by a cooling stage at 45°C for 30 s.

IDENTIFICATION OF *CRONOBACTER* ISOLATES WITH 16S rDNA SEQUENCING

PCR amplification using primers: Forward: 5'-TATAGGTTGTCTGCGAAAGCG-3' and Reverse: 5'-GTCTTCGTGCTGCGAGTTTG-3' (Liu et al. 2006) was done in a PCR thermal cycler (Gene Amp® PCR Systems 2700, Applied Biosystems, USA) with the following thermal-cycling conditions: Initial incubation at 95°C for 10 min, followed by a 3-step cycling program, performed for 30 cycles: denaturing at 95°C for 30 s, annealing the primers at 60°C for 30 s and 72°C for 45 s for further primer extension. Following amplification, the final extension was performed at 72°C for 10 min and cooled at 4°C. The PCR products were analyzed on a 1.5% agarose gel with ethidium bromide staining. The gel was photographed in a gel visualizer (Alpha imager TM 2200, Alpha innotech, CA) under UV light. A cleanup step was carried out to purify single- or doubled-stranded DNA fragments from PCR and other enzymatic reactions by using QIAquick PCR Purification Kits and was done according to the instructions of manufacturer (QIAGEN, Hoftmann-La Roche, Switzerland). Sequencing of PCR products was carried out in a thermal cycler using the following thermal-cycling conditions, which was a 3–step cycling program, performed for 25 cycles, melting at 96°C for 10 s, annealing the primers at 55°C for 15 s and 60°C for 4 min for extension, followed by the final step of cooling at 4°C. After sequencing, DNA samples were purified with CENTRI-SEP column according to the instructions of manufacturer (Applied Biosystems, USA) and the resulting DNA sequences were analyzed in the sequencer (3130 xl Genetic Analyzer, Applied Biosystems, USA) and compared to the library of National Centre for Biotechnology Information (NCBI) using BLASTn program.

RESULTS AND DISCUSSION

ISOLATION OF *CRONOBACTER* spp.

In this study biochemical and molecular methods were compared for isolation and identification of *Cronobacter* strains from PIF sold in Malaysia. A total of 117 isolates including *Cronobacter* and other members of Enterobacteriaceae were selected from PIF samples using two selective chromogenic media. All the isolates were identified with API 20E and Microgen™ GN-ID identification system. Only 12 of 117 strains (10.3%) appeared blue-green on DFI agar and were identified with 16S rDNA sequencing using MicroSeq®500 16S rDNA Bacterial Identification kit (Table 1). Based on sequencing results, all 12 isolates selected from 9 of 72 PIF samples (12.5%) investigated in this study were identified as *Cronobacter* (Figure 1). The incidence of 12.5% *Cronobacter* contamination of PIF in Malaysia was almost in agreement with reports of Cawthorn et al. (2008) and Witthuhn et al. (2006) who found the prevalence of *Cronobacter* in South African PIF were 14 and 18%, respectively. Muytjens et al. (1988) also reported that 14% of PIF samples were positive for *Cronobacter* contamination.

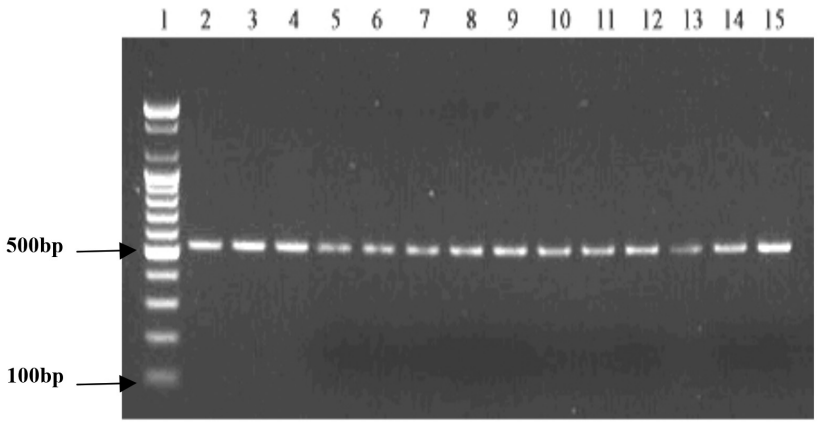
CHROMOGENIC MEDIA

The production of blue-green colonies on DFI agar was unique to *Cronobacter* strains which are due to the presence of α-glycosidase enzyme of all *Cronobacter* strains (Iversen & Forsythe 2004; Iversen et al. 2004). All 12 isolates identified as *Cronobacter* by Real time-PCR and 16S rDNA sequencing gave typical colonies on DFI agar. Cawthorn et al. (2008) reported that sensitivity of this chromogenic media for isolation of *Cronobacter* was 100%. All non-*Cronobacter* strains produced yellow colonies on DFI media and no false positive result was found in this study. In contrast, in other studies, DFI media has been reported to have lower specificity due to occurrence of non-*Cronobacter* strains with α-glycosidase activity (Cawthorn et al. 2008; Iversen et al. 2004).

All the Enterobacteriaceae members isolated from DFI agar were enriched in EE broth in this study. However, it has been recommended that in addition to isolation media, enrichment broth should also be improved because not all *Cronobacter* strains grow well on enrichment broth such as modified lauryl sulfate tryptose broth (mLST) and Enterobacteriaceae enrichment broth (Guillaume-Gentil et al. 2005; Iversen & Forsythe 2007; Lehner et al. 2006) may give false negative results. *Cronobacter* Screening Broth (CSB, Oxoid), a selective differential enrichment broth has been developed (Iversen et al. 2008) and showed 100% sensitivity for detection of *Cronobacter* strains. All *Cronobacter* strains were able to ferment sucrose in CSB and lower the pH and change purple colour of broth to yellow (Iversen & Forsythe 2007).

TABLE 1. Identification of presumptive *Cronobacter* sp. isolates using biochemical, PCR and 16S rDNA profiling

Presumptive *Cranobacter* isolate	API20E biochemical analysis		Microgen GN-ID analysis (GNA + GNB)		Choromogenic Media		RT-PCR	16S rDNA sequencing analysis		
	Indentification %	Significant Taxon	Identification %	Significant Taxon	DFI	CES	PCR	Significant Taxon	% Sequence identity	NCBI Accession number
1	51.1	*E. sakazakii*	99.96	*Salmonella* group 1	+	+	+	*E. sakazakii*	98	FJ906919
2	Unacceptable		99.91	*E sakazakii*	+	+	+	*E. sakazakii*	95	GU122227
3	95.1	*E. cloacae*	53.87	*E. sakazakii*	+	+	+	*E. sakazakii*	97	GU122218
4	Unacceptable		99.91	*E. sakazakii*	+	+	+	*E. sakazakii*	99	GU586322.1
5	77.6	*E. sakazakii*	53.87	*E. sakazakii*	+	+	+	*E. sakazakii*	99	FJ906919.1
6	51.1	*E. sakazakii*	73.16	*E. sakazakii*	+	+	+	*E. sakazakii*	93	FJ906925.1
7	Unacceptable		96.09	*E. sakazakii*	+	+	+	*E. sakazakii*	98	FN539033.1
8	51.1	*E. sakazakii*	73.16	*E. sakazakii*	+	+	+	*E. sakazakii*	99	EF059831.1
9	Unacceptable		96.09	*E. sakazakii*	+	+	+	*E. sakazakii*	99	GU563940.1
10	51.1	*E. sakazakii*	99.65	*E. sakazakii*	+	+	+	*E. sakazakii*	96	GU227689.1
11	95.1	*E. cloacae*	53.87	*E. sakazakii*	+	+	+	*E. sakazakii*	99	EF059856.1
12	51.1	*E. sakazakii*	99.65	*E. sakazakii*	+	+	+	*E. sakazakii*	99	GU122169.1
ATCC strain 51329	99.9	*E. sakazakii*	96.82	*E. sakazakii*	+	+	+	*E. sakazakii*	99	GU122217



Lane 1, DNA 100 bp marker; lane 2 to 13, twelve isolates of *C. sakazakii* from PIF samples; lane 14, *C. muytjensii* ATCC 51329; lane 15, *E. coli* DNA-positive control

FIGURE 1. Electrophoretic results of PCR amplification to confirm the presence of *Cronobacter sakazakii* in the presumptive isolates from PIF samples

BIOCHEMICAL IDENTIFICATION

All members of *Cronobacter* and other Enterobacteriaceae obtained from chromogenic media were identified with API 20E and Microgen GN-ID biochemical identification systems. The highest percentage identification results from both biochemical test kits for *Cronobacter* and other Enterobacteriaceae are shown in Tables 1 and 2. There were some differences in identification of presumptive *Cronobacter* isolates using the two different biochemical systems. Four isolates identified using API 20E biochemical tests as *C. sakazakii* were identified at a higher percentage as *C. sakazakii* using Microgen GN-ID profile. Another four isolates had high percentage of identification as *C. sakazakii* using Microgen GN-ID but was unrecognizable when identified using the API 20E profile. Another two presumptive isolates were identified using API 20E profile as *E. cloacae,* but had higher percentage of identification as *C. sakazakii* when identified with Microgen GN-ID and another one isolate was confirmed as *Salmonella* with Microgen GN-ID, but was identified as *C. sakazakii* using API 20E profile (Table 1). However, all these isolates were identified as *C. sakazakii* using Real time-PCR and 16S rDNA sequencing.

Biochemical kits such as API 20E and Microgen GN-ID systems employ 21 and 24 standardized biochemical substrates, respectively, to identify the family Enterobacteriaceae. The limited number of substrates used for the biochemical tests may be inadequate to identify all *Cronobacter* isolates which may lead to false negative results. In turn, this could lead to errors in the release of batches of PIF and milk powders that would otherwise be withheld from the market. These biochemical identification systems are still in use in many laboratories.

Table 2 shows 105 Enterobacteriaceae strains out of 117 (89.7%) isolates from DFI agar after enrichment in EE broth. All presumptive Enterobacteriaceae isolates were identified using API 20E biochemical kit and Microgen™ GN-ID identification system. Based on biochemical identification, 74 of 117 isolates (64%) were identified as *Enterobacter* spp. Other Enterobacteriaceae members isolated from 72 PIF samples included 46 *E. cloacae* strains (64%), nine *Serratia liquefaciens* strains (12.5%), seven *Klebsiella pneumoniae* spp (9.8%), five *Citrobacter freundii* strains (7%) and five *Salmonella* strains (7%) (Table 2). *Salmonella* was isolated from 6 of 72 powdered PIF samples (8.3%) and one of the isolates was also identified positive for *Cronobacter*. There are also several other reports of isolation of *Cronobacter* (from neonatal intensive care units where *Cronobacter* infections have occurred) and other members of Enterobacteriaceae from reconstituted PIF (Caubilla-Barron & Forsythe 2007; Cawthorn et al. 2008). This shows that infections by *Cronobacter* are possible in neonatal intensive care units and in powdered infant formula milk.

The main microbiological concerns associated with ingredients and PIF samples are the occasional presence of *Salmonella* and Enterobacteriaceae (including coliforms) and *Cronobacter* spp. (FAO/WHO 2004). FAO/WHO (2004) has categorized microorganisms based on their presence in the PIF and the strength of illness in infants into three groups, i.e. *Cronobacter, Salmonella* and Enterobacteriaceae. *Cronobacter* spp. and *Salmonella* are in the first category (category A) due to the clear evidence of illness in infants such as systemic infection, necrotizing enterocolitis (NEC) and severe diarrhea. *Salmonella* contamination of infant formula has been responsible for multiple outbreaks (Bornemann et al. 2002; Olsen et al. 2001). Similar to *Cronobacter*, low-level intrinsic contamination of powdered infant formula with *Salmonella* was epidemiologically and microbiologically associated with infections in infants in these outbreaks. Unlike *Cronobacter* and other Enterobacteriaceae, however, *Salmonella* is rarely found in surveys of powdered infant formula. In the study investigating different powdered

TABLE 2. Identification of 120 Enterobacteriaceae isolated from 72 powdered infant formula milk using biochemical test

Presumptive isolates	API20E analysis identification %	Significant Taxon	Microgen analysis identification%	Percentage of isolates from PIF (72) [a]
1	87	*Enterobacter cloacae*	99.02	64
2	91.1	*E. aerogenes*	94.21	7
3	94.4	*E. agglomerans*	97.27	5.5
4	95.2	*E. gergoviae*	98.00	9.8
5	99	*Escherichia coli*	96.09	4.2
6	96.2	*Citrobacter freundii*	99.65	7
7	92	*C. koseri*	98.28	1.4
8	92	*Hafnia alivei*	92.01	4.2
9	99	*Klebsiella pneumoniae*	97.27	9.8
10	94.5	*Pantoea* spp.	99.15	2.8
11	89.3	*Salmonella* group 1	95.08	7
12	94.3	*Serratia ficaria*	99.14	5.5
13	94.3	*S. liquefaciens*	89.65	12.5
14	89.9	*Shigella* spp.	94.32	2.8
15	92.2	*Xanthomonas maltophilia*	83.87	2.8

[a] Number of analysed PIF samples

infant formulas and other food products, Iversen and Forsythe (2004) and Muytjens et al. (1988) did not isolate any *Salmonella* serovars. This has proven that the presence of *Salmonella* in PIF samples used in this experiment is not unusual since it has been reported to contaminate PIF and has caused multiple outbreaks in the past.

Organisms such as *E. agglomerans;* recently renamed as *Pantoea agglomerans* (Brenner & Farmer 2005) and *E. vulneris*, *Hafnia alvei*, *K. pneumoniae*, *C. koseri*, *C. freundii*, *K. oxytoca* and *E. cloacae* are in category B according to FAO/WHO (2004) categorization, as their presence in PIF has been reported but contaminated PIF has not been shown to be the source of infant infections. Twelve cases of NEC occurred within three weeks in the Neonatal Intensive Care Unit (NICU). *E. cloacae* strain was found in stool and blood cultures from Twelve cases of affected babies to NEC prior to the onset of the disease (Powell et al. 1980). Enterobacteriaceae such as *E. gergoviae* and *E. aerogenes* are important causes of nosocomial bacteremia in the hospitalized pediatric and neonatal population. In a nosocomial outbreak in the NICU of a general hospital in Johor Bahru, Malaysia, *E. gergoviae* was isolated from 11 infected babies and was also isolated from the dextrose saline used for the dilution of parenteral antibiotics (Ganeswire et al. 2003). *E. aerogenes* was isolated from blood culture of a 5-day-old neonate of which 12 more cases of nosocomial infections were discovered in the next 70 days in that ward (Loiwal et al. 1999). *C. freundii* has been also related to nosocomial infections, meningitis, sepsis, brain abscess, pneumonia arthritis and endocarditis in newborns in several outbreaks (Thurm & Gericke 1994).

REAL-TIME PCR IDENTIFICATION

All twelve presumptive *Cronobacter* isolates from dried PIF and *C. muytjensii* type strain ATCC 51329 were confirmed as *Cronobacter* spp. using SYBR Green Real-time PCR. Figure 2(a) and 2(b) shows the presence or absence of these presumptive *Cronobacter* isolates in Real-time PCR melting curve analysis performed in channel 1. The results are represented by a graph of number of Derivative value (-d (fluorescence units/d (time)) Temperature. Analysis of melting temperature (Tm) on SYBR Green Real-time PCR in Figure 2(a) and 2(b) shows a Tm value of 86°C for *Cronobacter* control culture (*C. muytjensii* ATCC 51329). All 12 presumptive isolates showed same melting peak at 86 ± 2°C and confirmed as *C. sakazakii* using 16S rDNA sequence analysis.

In another study specific PCR with six different primers pairs was used for the detection of *Cronobacter* from PIF samples. The results showed large degree of variation in the specificity of the PCR primers and that not all primers were suitable for accurate detection and identification of *Cronobacter* (Cawthorn et al. 2008). Liu et al. (2006) developed a Real-time PCR assay using TaqMan and SYBR Green for detection and enumeration of this pathogen after selective enrichment. The newly developed assays would detect 1.1 cfu/g infant formula and both could be accomplished within 2 days, while standard methods such as conventional culture and biochemical-based assays used to enumerate and identify *Cronobacter* as '*E. sakazakii*' from different sources requires at least 5 days. However, SYBR Green Real-time PCR has advantage over the 5'-nuclease assay with TaqMan probes because it provides accurate diagnostic results in less time for clinical applications, from less biopsy material and at less cost than assays currently employed such as FISH or Southern blotting (Ponchel et al. 2003).

This study shows that SYBR Green Real-time PCR assay is an important tool for rapid identification of *Cronobacter* strains isolated from infant formula milk. The LightCycler capillary system incorporated SYBR Green I dye for the detection of double-stranded DNA generated during PCR. During each phase of DNA synthesis, the SYBR Green I dye bound to the amplified PCR products and the amplicon quantity was monitored in real-time by measuring its

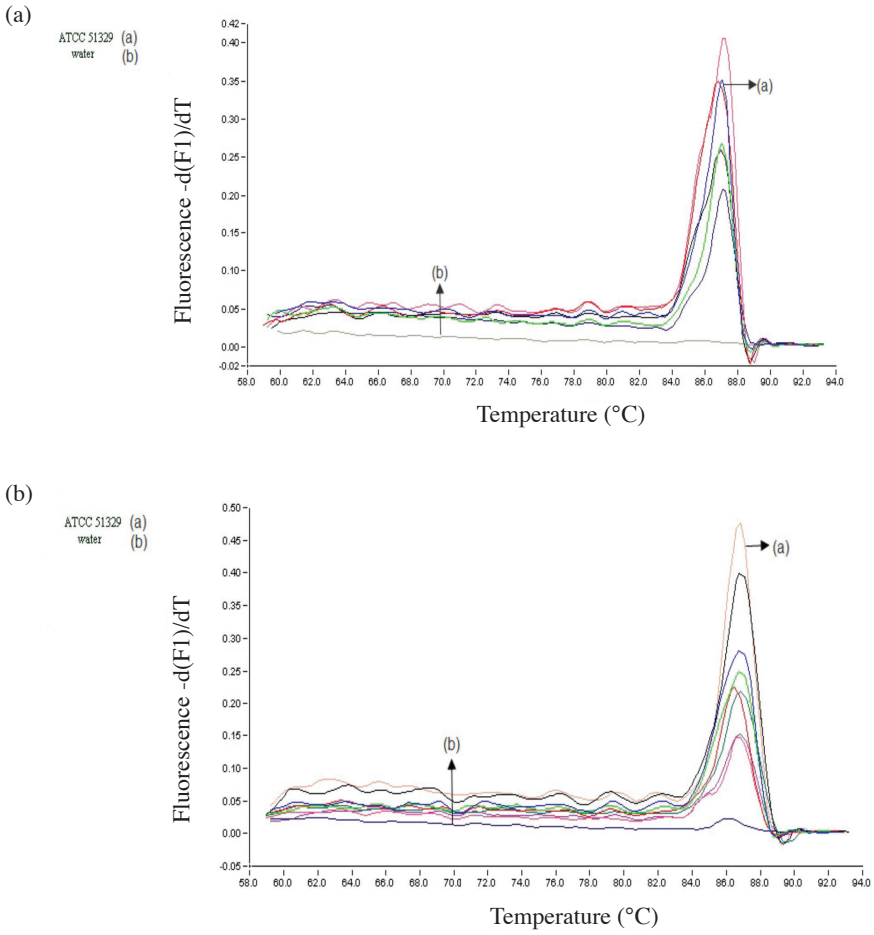


FIGURE 2. Lightcycler melting peaks analysis of five (a) and seven (b) *Cronobacter* isolates from IFM and *C. muytjensii* type strain ATCC 51329

fluorescence. The advantage of molecular assays such as SYBR Green Real-time PCR is that it does not require post PCR handling by running agarose gel electrophoresis and thus reduces the chances of contamination.

CONCLUSION

In this study all non-*Cronobacter* strains isolated from PIF produced yellow colonies on DFI agar and those appeared blue-green on DFI agar were identified as *Cronobacter* using PCR and DNA sequencing with no false positive result which makes chromogenic agar a more sensitive and accurate medium for detection of *Cronobacter*. Other Enterobacteriaceae associated with NEC, the most common gastrointestinal emergency in the newborns, such as *E. coli*, *K. pneumoniae*, *E. cloacae* and *Salmonella* spp. were also present in Malaysian PIF samples. There are differences in the identification of *Cronobacter* isolates using biochemical kits and molecular assays. It was shown that molecular detection methods such as Real time-PCR and 16S rDNA had higher identification percentage compared to the biochemical tests and were useful for rapid identification of *Cronobacter*.

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