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Comparison of In-house and Commercial Real-time PCR Systems for the Detection of Enterobacteriaceae and their Evaluation Within an Interlaboratory Study Using Infant Formula Samples

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Abstract Traditional detection methods for *Enterobacteriaceae* in foods are time-consuming and laborious. The current study assessed the specificity of three real-time PCR primer sets. Set A (*IEC* primers) targeted the conserved flanking regions of the 16S rRNA, the 16S-ITS-23S gene region. Set B (*ENT* primers) annealed to *Escherichia coli* 16S ribosomal RNA gene. The third set (C) used a D-LUX™ (Light Upon eXtension) single FAM-labelled forward primer and a corresponding unlabeled primer. Set A was specific for *E. coli* and for some non-Enterobacteriaceae. SYBR Green-based real-time PCR confirmed the specificity of set B for the Enterobacteriaceae but also detected Vibrionaceae. In contrast, set C was poorly specific. However, set D including the forward LUX™ primer from set C and the reverse primer from set B had a specificity comparable to that of set B, but with higher sensitivity. This combined set was successfully applied to detect Enterobacteriaceae in infant milk formula and compared favourably with a commercial real-time PCR kit.

Keywords Detection · Enterobacteriaceae · Real-time PCR · LUX™ primers · Infant formula milk

Introduction

The Enterobacteriaceae family is composed of widely studied microorganisms and includes several species such as *Escherichia coli*, *Klebsiella pneumoniae* or *Salmonella* Typhimurium which are responsible for food intoxications (Blood and Curtis 1995). Routine monitoring of Enterobacteriaceae serves as a hygiene indicator within food processing plants and their presence typically signifies poor cleaning procedures for process surfaces or post-processing contamination of heat-processed foods. To date, most quality assurance laboratories use agar-based ISO procedures (de Boer 1998) in order to detect and quantify Enterobacteriaceae in food products or swab samples. Combined with enrichment steps, Violet Red Bile Glucose Agar (VRBGA) has been among the most popular media for detecting Enterobacteriacaeae in foods. However, this medium is recognised as having some shortcomings (Baylis 2006) as other strains like *Aeromonas* spp. can also grow. Consequently, the colonies that appear on VRBGA are qualified as presumptive with further confirmatory tests required. Overall, this method can take 5 to 7 days for a definitive result which is not satisfactory for allowing rapid product release. Therefore, it is of commercial interest to accelerate this procedure by investigating alternative rapid DNA-based methods.

Detection of the Enterobacteriaceae by conventional PCR has been previously reported. Bayardelle and Zafarullah (2002) developed PCR protocols for detection of the most frequent species of the Enterobacteriaceae in blood, urine, and water samples using primer sets targeting the *wec* gene cluster involved in the synthesis of the enterobacterial common antigen. Real-time PCR has been widely accepted because of rapidity, sensitivity, reproducibility and reduced carry-over contamination (Mackay et al. 2007). Real-time PCR protocols have also been developed and applied in food samples for the detection of Enterobacteriaceae (Nakano et al. 2003; Qiu et al.

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2009). Nakano et al (2003) used specific primers and SYBR Green I as a detection format. Currently, the foodproof® Enterobacteriaceae plus *E. sakazakii* Detection Kit—5′Nuclease (Biotecon, Potsdam, Germany) uses *TaqMan*® probes for the qualitative detection of the Enterobacteriaceae and *Cronobacter sakazakii* particularly in infant formula.

The LUX™ (Light Upon eXtension) primer system is another commercially available tool for detection of pathogens by real-time PCR. Such primers are labelled with a single fluorophore near the 3′-end in a hairpin structure that intrinsically quenches the fluorescence. When incorporated into double-stranded DNA, the fluorophore is de-quenched, resulting in a significant increase of fluorescence signal. The LUX™ primers are software-designed by entering the targeted DNA sequence and subsequently a range of primers are proposed for use.

The LUX™-based real-time PCR has been recognised as a cost-effective alternative to other fluorescence-based techniques (Nazarenko et al. 2002). Such fluorogenic PCR has the potential to be routinely used in food industries because of rapidity, simplicity, and lower cost compared with real-time PCR systems using probes for instance. LUX™ primers have been designed for use in a number of studies mainly in virology (Aitichou et al. 2005; Antal et al. 2007; Chen et al. 2004; Nordgren et al. 2008; Slavov et al. 2008). However, some applications in bacteriology have been reported (Balcazar et al. 2007; Kunchev et al. 2007; McCrea et al. 2007; Mitchell et al. 2009; Xu et al. 2008).

The use of LUX™ primers for the detection of Enterobacteriaceae in food samples has not been assessed in detail and it was of interest to evaluate their potential as a new molecular tool of analysis.

In this study, the specificity of three separate PCR primer sets was evaluated for the detection of the Enterobacteriaceae. Set A (*IEC* primers) consisted of classic primers, previously tested by Maheux et al. (2009) and Khan et al. (2007), and evaluated in this study using conventional PCR. Set B (*ENT* primers) developed by Nakano et al. (2003) was evaluated in this study by PCR and real-time PCR, using SYBR Green I as a detection format. The LUX™ primers (set C) were designed online using the 16S ribosomal RNA gene of *E. coli*, a sequence previously used by Nakano et al for the design of *ENT* primers (2003). Set C was tested by real-time PCR. Finally, the forward primer from set C and the reverse primer from set B were combined to obtain set D and evaluated for their specificity for detection of the Enterobacteriaceae. All primer sets were tested in parallel using Primer Blast available on the National Centre for Biotechnology Information website. Subsequently, the combined primer set (primer set D) was evaluated on infant formula samples and compared with a commercial kit as part of an interlaboratory study.

Materials and Methods

Assessment of In-house and Commercial Real-Time PCR Methods Using Pure Cultures

Bacterial Strains

Type strains of Enterobacteriaceae, *E. coli* (ATCC 11775), *Enterobacter aerogenes* (ATCC 13048), *Erwinia persicina* (ATCC 1381), *K. pneumoniae* (ATCC 700603), *S.* Typhimurium (ATCC 13311), *Serratia marcescens* (ATCC 13880), *Shigella flexneri* (ATCC 9199), *Y. enterocolitica* (ATCC 9610); *A. hydrophila* (ATCC 7966), *Campylobacter jejuni* (ATCC 29428), *Listeria monocytogenes* (ATCC 19115) and *Vibrio parahaemolyticus* (ATCC 17802) were obtained from MicroBioLogics Inc, Saint Cloud, USA. *C. sakazakii*, *Enterobacter cloacae*, *Pseudomonas aeruginosa* and *Proteus mirabilis* isolates were obtained from the culture collection at the Department of Life Sciences, University of Limerick. *Staphylococcus aureus* (NCTC 8325) was obtained from the National Collection of Type Cultures (Health Protection Agency Culture Collections, Salisbury, UK). All strains were stored on Protect™ beads 109 (LangenBach Services Ltd, Dublin, Ireland) at −20 °C until cultivation.

Culture Conditions

All Enterobacteriaceae were grown overnight on nutrient agar (NA; Oxoid Ltd, Basingstoke, UK) at 37 °C, except *E. persicina* which was incubated at 30 °C. *L. monocytogenes* and *S. aureus* were incubated at 37 °C overnight on NA. *V. parahaemolyticus* was grown overnight at 35 °C on tryptic soya agar (TSA; Oxoid Ltd). *A. hydrophila* was grown overnight at 35 °C on Columbia blood agar (Oxoid Ltd). *P. aeruginosa* was incubated overnight on NA at 25 °C. *C. jejuni* was grown on Columbia blood agar in a microaerophilic environment (CampyGen 2.5l, Oxoid Ltd) for 72 h at 37 °C. Prior to DNA extraction, a preculture of each strain was made by inoculating one loopful of each culture into a flask of 30 ml of nutrient broth (NB; Oxoid Ltd) with overnight shaking at 37 °C for the Enterobacteriaceae and *S. aureus*. Tryptic Soya Broth (TSB; Oxoid Ltd) was used for growth of *L. monocytogenes* (37 °C), the *Vibrionaceae* (35 °C), and *P. aeruginosa* (25 °C). Following growth, 300 μl of the preculture was transferred into 30 ml of fresh broth. A culture of each strain was obtained under the conditions outlined above and grown to exponential growth phase. *C. jejuni* colonies grown on a Columbia agar plate

were suspended in 1 ml of 0.85% saline sterile water prior to DNA extraction.

DNA Extractions

DNA used for PCR and real-time PCR experiments was extracted following manufacturer's instructions for Gram-negative or -positive bacteria using the DNeasy Tissue Kit (Qiagen, Crawley, UK) based on DNA purification through chromatography columns. For each strain, DNA was extracted from pellets obtained from cultures in the exponential growth phase following centrifugation of 1 ml of a bacterial suspension at 5,000×*g* for 10 min. DNA quantifications were performed by spectrophotometry at 260 nm using a Nanodrop Spectrophotometer ND-1,000 (Thermo Scientific, Wilmington, USA). Finally, DNA concentrations were adjusted to 1 ng per 2 μl. The sensitivity of DNA detection was determined by diluting *E. coli* DNA, assuming that the targeted gene is present in 7 copies in the *E. coli* genome according to the Ribosomal RNA Operon Copy Number Database (Klappenbach et al. 2001), and using 4,990 kb as the size of the *E. coli* genome (Bergthorsson and Ochman 1995). Therefore 1 ng of DNA was calculated to equal 2.6×10^4 genomes.

Commercial Real-time PCR Detection

Enterobacteriaceae were detected using the foodproof® Enterobacteriaceae plus *E. sakazakii* Detection Kit–5′ Nuclease which allows rapid detection of Enterobacteriaceae and simultaneous identification of *C. sakazakii*. A 25-μl reaction included 18 μl of Master mix containing the primers and the Hydrolysis Probes, 1 μl of foodproof® Enzyme solution and 1 μl of foodproof® IC (Internal Control) and 5 μl of sample DNA. A positive control was used for each experiment where the DNA template was replaced by the DNA from the foodproof® Control Template. The reaction purity and cross-contamination were checked by adding a negative control consisting of PCR-grade water, in place of the sample. Experiments were carried out using an AB 7,900 HT (Applied BioSystems Inc, Foster City, USA) or a LightCycler® 480 II (Roche Diagnostics GmbH, Mannheim, Germany). The programme settings included: a single pre-incubation step: 37 °C for 4 min and 95 °C for 5 min, an amplification step of 40 cycles: 95 °C for 10 s, 65 °C for 70 s with a step down at each cycle by 0.1 °C. A Hydrolysis Probe binds specifically to the IC, allowing detection in the ROX/Texas Red channel with the AB 7,900 HT. *C. sakazakii* DNA is detected in the FAM channel and the *Enterobacteriaceae* DNA is detected in the VIC/HEX.

In-House Methods of PCR and Real-time PCR Detection

Primer Selection

Different primer sets were compared for their specificity for the detection of Enterobacteriaceae. All primers were purchased from MWG Eurofins Operon (Ebersberg, Germany). The specificity of the primer sets was tested using in silico PCR analysis against complete genome sequences of Enterobacteriaceae and non-Enterobacteriaceae. All sequences were provided by the National Centre for Biotechnology Information (NCBI) nucleotide database. The Primer BLAST (Basic Local Alignment Search Tool) programme, available on http://www.ncbi.nlm.nih.gov/tools/primer-blast/, was used for simulated PCR with the primer sets for the detection of the Enterobacteriaceae. This software allows searches for primer annealing sites on DNA sequences using the BLAST algorithm which is a segment-to-segment alignment principle.

Primer sets were named sets A, B, C, and D (Table 1). Set A or *IEC* primers were purported to target the conserved flanking regions of the 16S ribosomal RNA gene, the internal transcribed spacer region (ITS) and the 23 ribosomal RNA gene. Set B corresponded to *ENT* primers targeting the DNA sequence coding for *E. coli* 16S ribosomal RNA (accession number J01859). For set C, the LUX™ primers were provided by Invitrogen, Paisley, UK. These included a 6-carboxyfluorescein 3′-labelled forward primer. The online D-LUX Designer software was used for their design by specifying the *E. coli* 16S ribosomal RNA gene (accession number J01859) as the target sequence. For set D, the forward primer from set C and the reverse primer from set B were used in a new real-time PCR assay.

PCR Conditions

For conventional PCR, mixes were prepared with the ready-to-use kit FastStart Taq DNA Polymerase, dNTPack 5 U/μl purchased from Roche Diagnostics GmbH. On the basis of a final 50-μl reaction volume, the master mixture contained 48 μl of 4 mM $MgCl_2$ (2 mM from 10× PCR buffer and 2 mM from $MgCl_2$ solution), 500 nM of forward primer, 500 nM of reverse primer, dNTP mixture and the FastStart Taq DNA Polymerase. Two μl of DNA sample was added to each reaction. The PCR programmes were carried out as shown in Table 2. The specificity of the primer sets was tested against the bacterial strains described above.

PCR was performed on a G-Storm GS2 Thermal Cycler (Genetic Research Instrumentation Ltd, Braintree, UK). Each PCR product was subsequently run on a 2% agarose electrophoresis gel, stained using SYBR Safe™ (Molecular Probes, Eugene, USA) and visualised with a transilluminator G-BOX (Syngen, Frederick, USA) under UV light. The

Table 1 Primers used in PCR and real-time PCR

Genetic target	Set	Primer	Primer sequence	*a*	*b*	Reference
16SrRNA–ITS–23SrRNA	A	IEC—F[a]	5′-CAATTTTCGTGTCCCCTTCG-3′	57	450	Khan et al. 2007
		IEC—R[b]	5′-GTTAATGATAGTGTGTCGAAAC-3′			Maheux et al. 2009
16S rRNA	B	ENT—F	5′-GTTGTAAAGCACTTT CAGTGGTGAGGAAGG-3′	59	424	Nakano et al. 2003
		ENT—R	5′-GCCTCAAGGGCACAACCTCCAAG-3′			
	C	LUX—F	5′-CGGTGTACCCGCAG AAGAAGCAC[FAM]G-3′	55	69	This study, generated by D-LUX™ software
		LUX—R	5′-GCTTGCACCCTCCGTATTACC-3′			
	D	LUX—F	5′-CGGTGTACCCGCAG AAGAAGCAC[FAM]G-3′	55	368	This study, primer combinations
		ENT—R	5′-GCCTCAAGGGCACAACCTCCAAG-3′			

a Annealing temperature (°C), *b* Amplicon size (bp)

[a] Forward

[b] Reverse

Perfect DNA™ 100 bp ladder (Novagen, Madison, USA) was used as a molecular marker.

Real-time PCR Conditions

When testing the *ENT* primers, conventional PCR procedures were adapted to a real-time procedure on a LightCycler® 1.2 (Roche Diagnostics GmbH), using LightCycler® FastStart DNA Master SYBR Green I (Roche Diagnostics GmbH). In each capillary, a 20 µl reaction mix contained 1× concentration LightCycler® FastStart Reaction Mix SYBR Green; 4 mM $MgCl_2$; 500 nM concentration of each primer; and 2 µl of the template and carried out under conditions outlined in Table 2. Real-time DNA amplifications were observed in Channel 1 at 530 nm of the LightCycler® 1.2. The *ENT* primers were also tested using a LightCycler® 480 with the same PCR mix in channel FAM/SYBR Green, except that the volume per reaction was 10 µl, using the same programme settings.

The detection of Enterobacteriaceae using LUX™ primers (sets C and D) was first assayed with a LightCycler® 1.2, using glass capillaries. In each capillary, the 20 µl reaction mix contained 10 µl of Platinum® qPCR SuperMix-UDG (Invitrogen), 5 mM $MgCl_2$ (final concentration); 500 nM concentration of each primer; 1 µl of Bovine Serum Albumin; 0.12 µl of FastStart Taq DNA Polymerase (Roche Diagnostics GmbH); and 2 µl of the template. Real-time PCR conditions are shown in Table 2.

The LUX™ primers (sets C and D) were also evaluated on the LightCycler® 480 in channel FAM/SYBR Green with 10 µl reaction of the same PCR mix.

Analysis of Enterobacteriaceae in Infant Formula Milk Samples

An interlaboratory study was performed across Europe as part of the MicroVal EN ISO 16140:2003 procedure for validation of an alternative method (Anonymous 2003a).

Table 2 PCR and real-time PCR conditions using primer sets A, B, C, and D

Programme	PCR	SYBR green-based real-time PCR	LUX™-based real-time PCR
Pre-incubation	95 °C, 6 min	95 °C, 10 min	50 °C, 2 min
			95 °C, 2 min
Amplification	28 cycles	35 cycles	35 cycles
	95 °C, 30 s	95 °C, 5 s	94 °C, 5 s
	°C, 15 s[a]	°C, 10 s[a]	55 °C, 10 s (single)
	72 °C, 30 s	72 °C, 20 s (single)	72 °C, 10 s
Melting	N/A[b]	95 °C, 0 s	95 °C, 0 s
		65 °C, 10 s	55 °C, 15 s
		95 °C, 0 s	95 °C, 0 s
		0.2 °C/s (continuous)	0.1 °C/s (continuous)
Cooling	N/A[b]	40 °C, 30 s	30 °C, 30 s

[a] Specific to each primer set used, similar to conventional PCR annealing temperatures

[b] Non applicable

This study evaluated specifically the foodproof® kit for the detection of *Enterobacteriaceae* and *C. sakazakii* in powdered infant formula samples. The 40 samples of 100 g powdered infant formula in sterile stomacher bags were provided by RIKILT—Institute of Food Safety, Wageningen, The Netherlands. A blind study was undertaken using whereby all samples labelled EBES 1 to 40 with no further information provided to the participants.

Each sample was enriched with 900 ml pre-warmed Buffered Peptone Water (BPW; Bio Trading, Mijdrecht, The Netherlands) at 37 °C and incubated for 18 h at 37 °C. One milliliter of BPW culture was added to a separate tube containing 10 ml Enterobacteriaceae Enrichment broth (EE; Bio Trading) and incubated for 24 h at 37 °C. Then, 50 μl of BPW culture were pipetted into 450 μl of fresh BPW and incubated for 3 h at 37 °C. DNA from dead cells (100 μl of each 3 h culture) was eliminated by adding 300 μl of Reagent D (Biotecon, Potsdam, Germany) to remove DNA from dead bacterial cells and avoid false-positive PCR results. DNA was prepared following the manufacturer's instructions using a Start Prep One kit (Biotecon) by heating a cell pellet resuspended in 200 μl of the provided lysis buffer at 95 °C.

In parallel, reference methods were used for comparison purposes with the suitable commercial and in-house real-time PCR systems.

For the detection of Enterobacteriaceae, the ISO 21528-1:2004 method was used (Anonymous 2004). A loopful of each EE broth tube was plated out on the selective isolation medium, VRBGA (Bio Trading). Characteristic colonies on VRBGA plates were pink to red or purple, with or without precipitation haloes. At least one representative colony from each VRBGA plate was subcultured onto NA plates incubated for 24 h at 37 °C. An oxidase test was performed on each subculture and a colony from each plate was stabbed into a glucose agar tube for incubation 24 h at 37 °C.

C. sakazakii was detected using the ISO/TS 22964:2006 (Anonymous 2006). After pre-enrichment of samples with BPW for 18 h at 37 °C, 100 μl of each BPW preculture was transferred into a 10 ml tube of modified Lauryl Sulphate Tryptose broth, (mLST/v; Bio Trading) containing 0.1% of vancomycin and incubated for 24 h at 44 °C. Each of the mLST/v broth tubes was plated out on a *C. sakazakii* Isolation Medium (CSIM; Bio Trading) and incubated for 24 h at 44 °C. Typical colonies on CSIM plates appeared small to medium in size (1–3 mm) and were green to blue-green colonies. Atypical colonies were slightly transparent and violet coloured. A presumptive colony from each plate was then subcultured on TSA (Bio Trading) and incubated for 48 h at 25 °C. A single yellow pigmented colony from each TSA plate was tested on an ID 32 E System (Biomerieux, Craponne, France) for the identification of Enterobacteriaceae and other non-fastidious Gram-negative rods according to the manufacturer's instructions.

The total viable count was obtained following the ISO 4833:2003 method (Anonymous 2003b). A 10-g reference powdered infant formula sample was dissolved into 90 ml of Peptone Physiological Salt (PFZ) (Bio Trading) and diluted to 1/100 dilution. One milliliter of each dilution (1/10, 1/100) was plated in duplicate with pre-melted Plate Count Agar (PCA; Bio Trading). The plates were incubated at 30 °C for 72 h.

Results and Discussion

Evaluation of the Commercial Detection Kit

The occurrence of Enterobacteriaceae including *Salmonella* spp. (Cahill et al. 2008), *Pantoea* spp., *E. hermanii*, *E. cloacae* (Estuningsih et al. 2006) or *Klebsiella* spp. (Gao et al. 2010) has been previously demonstrated in infant formula milks. The occurrence of *C. sakazakii* is of major concern to infant formula manufacturers (Chap et al. 2009; Drudy et al. 2006; Giovannini et al. 2008). In-house Real-time PCR systems have recently been developed for detection of *C. sakazakii* (Krascsenicsova et al. 2008; Lehmacher et al. 2007) or *K. pneumoniae* (Sun et al. 2010) in infant powdered milks. In addition, the foodproof® Enterobacteriaceae plus *E. sakazakii* Detection Kit–5′ Nuclease on the LightCycler® 480 is also commercially available for these applications. As manufacturers of the commercial kit, Biotecon purport to have tested a range of 121 *C. sakazakii* strains and 120 non-*C. sakazakii* strains including various species. All *C. sakazakii* gave a signal in the FAM and VIC/HEX channels while all the non-*C. sakazakii* Enterobacteriaceae produced a signal in VIC/HEX channel only. For the detection of Enterobacteriaceae, this exclusivity was confirmed by Biotecon with more than 60 non-Enterobacteriaceae including most of the closely related genera of *Aeromonas* and *Vibrio*. None of the non-Enterobacteriaceae was detected in any channel. After an enrichment step, 1 to 10 cells per 25 to 100 g of relevant type of food sample could be detected.

In the present study, the foodproof® Enterobacteriaceae plus *E. sakazakii* Detection Kit—5′Nuclease was assayed using DNA samples from Enterobacteriaceae and non-Enterobacteriaceae species. All the tested Enterobacteriaceae, including *P. mirabilis*, were detected in the VIC channel. *C. sakazakii* DNA had a Ct value equal to 18.77 whereas *K. pneumoniae* DNA was detected at a Ct equal to 20.73. In the FAM channel, the identification of *C. sakazakii* DNA was confirmed with a Ct equal to 19.90. Data obtained from real-time PCR on serial dilutions of copy numbers of *E. coli* DNA indicated a detection limit of

4 copies in a pure culture at a Ct equal to 30.08. This real-time PCR system was easy to use and appeared highly specific for the Enterobacteriaceae. To date, it is the only commercial kit available for the detection of Enterobacteriaceae by real-time PCR. In a single kit, 96 real-time PCR reactions can be performed; however the cost per reaction remains high. Therefore, if real-time PCR determinations are required to be performed in duplicate or triplicate to obtain statistical data it may not be economically feasible to use this kit but this obviously depends on the particular end user application. For this reason, modifications to these actual protocols and in-house methods were investigated and some general cost comparisons per real-time PCR reaction were subsequently made.

Comparison of Primer Sets

Figure 1 shows the PCR products run on a SYBR Safe™ pre-stained 2% agarose electrophoresis gel obtained after PCR using set A. Among the range of species tested, these primers amplified only DNA from *E. coli*, a finding not in agreement with that of Maheux et al. (2009). These workers reported that the *IEC* primers, tested by Khan et al.,

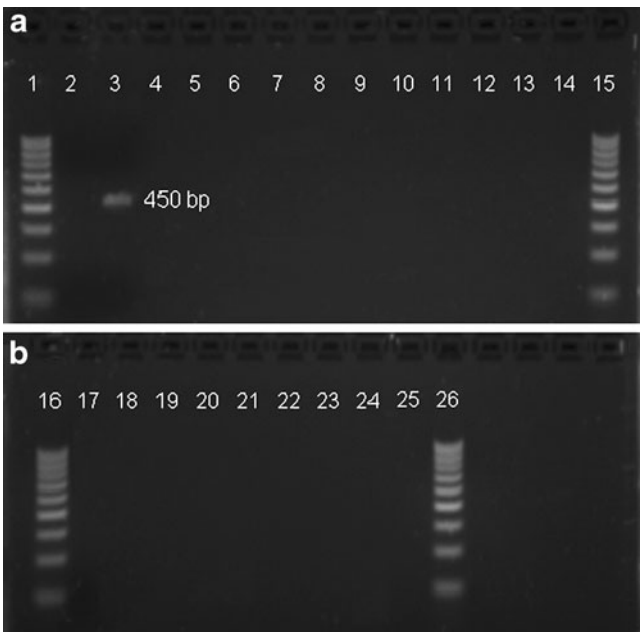

Fig. 1 Amplification results obtained by PCR using the *IEC* primers (set A). **a** *Lanes 1*, *15*, *16*, *26*, Perfect DNA™ 100 bp ladder; *lane 2*, *17*, negative control (no DNA template in the PCR reaction); *lane 3*, *E. coli*; *lane 4*, *E. aerogenes*; *lane 5*, *E. cloacae*; *lane 6*, *C. sakazakii*; *lane 7*, *S.* Typhimurium; *lane 8*, *S. flexneri*; *lane 9*, *K. pneumoniae*; *lane 10*, *Y. enterocolitica*; *lane 11*, *E. percisina*; *lane 12*, *S. marcescens*; *lane 13*, *P. mirabilis*; *lane 14*, *V. parahaemolyticus*. **b** *Lanes 16*, *26*, Perfect DNA™ 100 bp ladder *lane 18*, *A. hydrophila*; *lane 19*, *P. aeruginosa*; *lane 20*, *L. monocytogenes*; *lane 21*, *S. aureus*; *lane 22*, *S. xylosus*; *lane 23*, *Micrococcus* spp.; *lane 24*, *B. cereus*; *lane 25*, *C. jejuni*

amplified DNA belonging to species from the Enterobacteriaceae family that are phylogenetically relatively close to *E. coli* and *Shigella* spp. According to Khan et al. (2007), the ITS region allows discrimination of bacterial species, and the flanking highly conserved 16S and 23S ribosomal RNA genes can be targeted by primers for specific amplification of *E. coli* strains. Data in the present study would support the findings of Khan et al. (2007) and would suggest that set A may not be suitable for detection of the entire Enterobacteriaceae family. In silico PCR analysis using Blast Primers confirmed this statement (Table 3). PCR procedures can be developed for the specific detection of *E. coli*, as described by Khan et al. (2007). However, other species like *S. aureus* or *P. fluorescens* may also be detected according to Primer Blast data. In the present study, when specificity was tested over a range of Enterobacteriaceae and non-Enterobacteriaceae, *S. aureus* was not detected using this primer set. Therefore, the specificity of the *IEC* primers for *E. coli* requires further analysis by testing of DNA extracted from other non-Enterobacteriaceae.

Set B (*ENT* primers) amplified DNA from all the Enterobacteriaceae tested except for *P. mirabilis*. However, DNA from *V. parahaemolyticus* (424-bp product) was also detected using this primer set by agarose gel electrophoresis (Fig. 2.). Nakano et al. (2003) evaluated the specificity of *ENT* primers with over 72 different bacterial species and noted amplifications for every tested strain belonging to the Vibrionaceae or Enterobacteriaceae family except *P. mirabilis*. For all other bacterial strains tested, results were negative. Data obtained in this study following PCR with the same set indicated similar results by conventional PCR, where the Enterobacteriaceae, *V. parahaemolyticus* and *A. hydrophila* were detected. Real-time PCR assays using mixes prepared with *ENT* primers were performed on both LightCycler® 1.2 and 480 instruments. The amplification curves obtained with the LightCycler® 480 confirmed that all the Enterobacteriaceae tested were detected as well as *P. mirabilis*, *V. parahaemolyticus*, and *A. hydrophila* were positive using the LightCycler® 1.2 (Table 4). Ct data were retrieved from real-time PCR using the LightCycler® 1.2 (Table 4). Primer set B appeared to be highly specific, as all the Enterobacteriaceae DNA samples tested would generate a PCR product based on Primer Blast analysis, except *Pantoea agglomerans* and *P. mirabilis*. However, the NCBI software showed detection of species from the *Vibrio* and *Aeromonas* genera, as confirmed by PCR and real-time PCR. Overall, this primer set would appear to be a good option for the detection of the Enterobacteriaceae, compared to the designed LUX™ primers (set C) which lacked specificity, as described below.

Initial assays using LUX™ primers targeting the 16S rRNA gene designed using the D-LUX™ software (set

Table 3 In silico PCR analysis using primer sets A, B, C and D with Primer Blast software

Bacterial species	Set			
	A	B	C	D
Budvicia aquatica	−	+	+	+
Cedecea davisae	−	+	+	+
Citrobacter braakii	−	+	+	+
Citrobacter farmeri	−	+	+	+
Citrobacter freundii	−	+	+	+
Citrobacter koseri	−	+	+	+
Cronobacter sakazakii	−	+	+	+
Enterobacter aerogenes	−	+	+	+
Enterobacter cloacae	−	+	+	+
Erwinia persicina	−	+	+	+
Escherichia coli	+	+	+	+
Escherichia coli 0157:H7	−	+	+	+
Ewingella americana	−	+	+	+
Hafnia alvei	−	+	+	+
Klebsiella pneumoniae	−	+	+	+
Klebsiella oxytoca	−	+	+	+
Kluyvera ascorbata	−	+	+	+
Kluyvera intermedia	−	+	+	+
Pantoea agglomerans	−	−	−	−
Plesiomonas shigelloides	−	+	+	+
Proteus mirabilis	−	−	−	−
Proteus vulgaris	−	+	+	+
Salmonella Typhimurium	−	+	+	+
Salmonella enteritidis	−	+	+	+
Serratia liquefaciens	−	+	+	+
Serratia marcescens	−	+	+	+
Shigella flexneri	−	+	+	+
Shigella sonnei	−	+	+	+
Yersinia enterocolitica	−	+	+	+
Yersinia pseudotuberculosis	−	+	+	+
Yersinia rohdei	−	+	+	+
Acinetobacter baumanii	−	−	+	−
Aeromonas hydrophila	−	+	+	−
Aeromonas punctata	−	+	+	+
Aeromonas sobria	−	+	+	+
Alcaligenes faecalis	−	−	+	−
Bacillus cereus	+	−	−	−
Bacillus subtilis	+	−	−	−
Campylobacter coli	−	−	+	−
Campylobacter jejuni	−	−	+	−
Enterococcus faecalis	+	−	−	−
Listeria innocua	−	−	−	−
Listeria monocytogenes	−	−	−	−
Micrococcus spp.	−	−	+	−
Pseudomonas aeruginosa	−	−	+	−
Pseudomonas fluorescens	+	−	+	−
Pseudomonas putida	−	−	+	−
Staphylococcus aureus	+	−	+	−
Staphyloccoccus capitis	−	−	−	−
Staphyloccoccus lentus	−	−	−	−
Staphyloccoccus xylosus	−	−	−	−
Vibrio parahaemolyticus	−	+	+	+
Vibrio vulnificus	−	+	+	+

+ target templates found, − target templates not found

C) were performed using the LightCycler® 1.2 with Enterobacteriaceae and non-Enterobacteriaceae. All the non-Enterobacteriaceae were detected before 35 cycles (Table 4). As a consequence, the primers appeared to lack a satisfactory degree of specificity over the range of strains tested. For example, *P. aeruginosa* DNA could be detected at a Ct equal to 27.22, which was quite close to the value for *E. percisina* DNA (Ct=26.47). Moreover, fluorescence gains were observed for the negative control

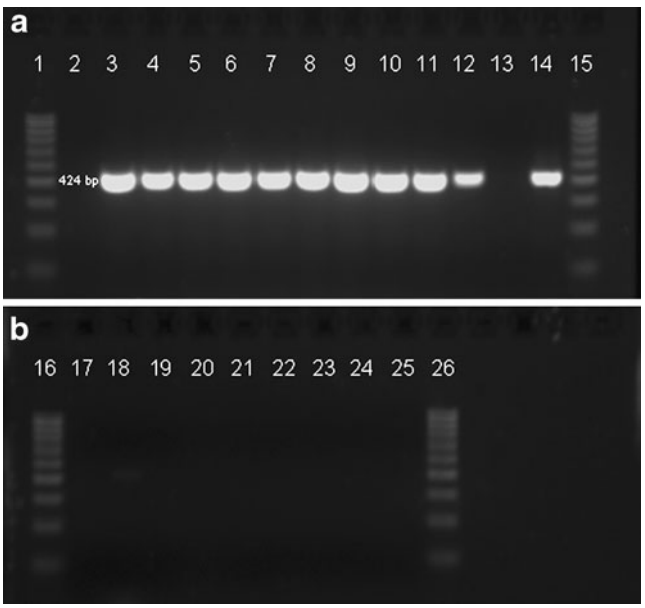

Fig. 2 PCR products obtained with the *ENT* primers (set B). **a** *Lanes 1*, *15*, *16*, *26*, Perfect DNA™ 100 bp ladder; *lane 2*, *17*, negative control (no DNA template in the PCR reaction); *lane 3*, *E. coli*; *lane 4*, *E. aerogenes*; *lane 5*, *E. cloacae*; *lane 6*, *C. sakazakii*; *lane 7*, *S.* Typhimurium; *lane 8*, *S. flexneri*; *lane 9*, *K. pneumoniae*; *lane 10*, *Y. enterocolitica*; *lane 11*, *E. percisina*; *lane 12*, *S. marcescens*; *lane 13*, *P. mirabilis*. **b** *Lanes 16*, *26*, Perfect DNA™ 100 bp ladder; *lane 14*, *V. parahaemolyticus*; *lane 18*, *A. hydrophila*; *lane 19*, *P. aeruginosa*; *lane 20*, *L. monocytogenes*; *lane 21*, *S. aureus*; *lane 22*, *S. xylosus*; *lane 23*, *Micrococcus* spp; *lane 24*, *B. cereus*; *lane 25*, *C. jejuni*

Table 4 Sensitivity of primer sets B, C and D against test strains as determined by mean Ct (Cycle threshold) values determined using the automated method provided by the LightCycler® 4.1 software

Tested strains	Primer set used		
	B	C	D
Escherichia coli	23.14	25.51	24.30
Enterobacter aerogenes	27.47	25.64	23.43
Enterobacter cloacae	28.67	25.79	26.08
Serratia marcescens	28.26	25.00	23.50
Erwinia percisina	26.85	26.46	25.68
Klebsiella pneumoniae	27.27	25.63	22.80
Shigella flexneri	22.74	24.83	24.29
Yersinia enterocolitica	24.71	25.01	22.95
Salmonella Typhimurium	29.00	25.32	25.19
Cronobacter sakazakii	23.52	25.17	24.81
Listeria monocytogenes	34.49	31.48	37.01
Staphylococcus aureus	31.34	32.28	37.84
Staphylococcus lentus	36.82	31.39	>41.00
Staphylococcus xylosus	35.70	31.80	>41.00
Staphylococcus capitis	33.71	31.89	37.92
Micrococcus spp.	–	29.86	38.04
Campylobacter jejuni	38.09	31.96	36.96
Bacillus cereus	33.02	32.68	>41.00
Pseudomonas aeruginosa	34.14	27.21	36.79
Vibrio parahaemolyticus	22.73	22.79	21.18
Aeromonas hydrophila	29.44	25.33	34.99

and the other non-Enterobacteriaceae, suggesting that forward and reverse primers were possibly forming primer-dimers beyond 30 cycles. It is reasonable to speculate that the design aspects of the LUX™ primers can impact on the specificity even if a particular DNA sequence recognised to be highly conserved among species is entered into the software.

Set D appeared to give higher specificity for the Enterobacteriaceae, as all the Enterobacteriaceae tested were detected except *P. mirabilis*. All tested non-Enterobacteriaceae, including *A. hydrophila*, were detected at or beyond 35 cycles. Therefore, cycle number should not exceed 35 in order to avoid generation of false-positive data. However, Enterobacteriaceae and *V. parahaemolyticus* were detected within similar Ct values, between 21.18 and 26.08. Therefore, the specificity of primer set D appeared limited by the detection of *V. parahaemolyticus*, as confirmed by Primer Blast (Table 3). Using the LightCycler® 1.2, this combined primer set showed greater sensitivity as Ct values for the Enterobacteriaceae were lower than those obtained using the *ENT* primer set (Table 4). The detection limit was four cells per PCR reaction at a mean Ct equal to 32.32 for a pure culture. Non-Enterobacteriaceae species had Ct values greater than 30, except for *V. parahaemolyticus* (Ct= 21.18), as shown in Table 4.

Comparative studies between LUX™ primers and other detection formats have been reported recently. Xu et al. (2008) developed a LUX™-based real-time PCR for the detection of *V. parahaemolyticus* in seafood and obtained comparable results for rapidity, specificity and sensitivity to a *TaqMan*® probe-based real-time PCR procedure. However, Mitchell et al. (2009) found that the application of LUX™ primers used in the detection of *Chlamydophila pneumoniae* in clinical specimens displayed a log less sensitivity than their designed *TaqMan*-based assay. In their comparative analysis using SYBR Green I, *TaqMan*® probe and LUX™ primers as the detection format, McCrea et al. (2007) confirmed that the hairpin structure of the LUX™ primers may improve the specificity of PCR by reducing mispriming and primer–dimer formation. The primers used by Castillo et al. (2006) were reported as being suitable for real-time PCR in the detection of Enterobacteriaceae using primers targeting the 16S ribosomal RNA gene. However, these workers used SYBR Green I technology which is purported to be less specific than LUX™ primers (Anonymous 2010). Moreover, the LUX™ detection

format could not be adapted on pre-designed sequences such as those of Castillo et al. (2006).

Limitations of the Assays Using Primer Sets B and D

Generally, with a LUX™-based real-time PCR, PCR products can be identified based to their melting temperature, Tm, on condition that the amplified region of the targeted gene is sufficiently variable from one species to another. In their study, Mitchell et al. (2009) confirmed their positive samples using melting curve analysis to ensure the specificity of the LUX™ primers. However, when using primer set D in a real-time PCR run, DNA from a range of Enterobacteriaceae, *V. parahaemolyticus* and *A. hydrophila* were amplified and melting curve analysis showed a very low variability of Tm from one species to another. The Tm values obtained were between 89 °C and 90 °C and indicated that the amplified region of the 16S ribosomal RNA gene may be highly conserved among the Enterobacteriaceae and the Vibrionaceae. Therefore, a melting curve analysis would not allow species identification and discrimination using this primer set. The main issue in the use of primer sets B and D is that the Vibrionaceae family was also detected. The 16S ribosomal RNA gene has been used for phylogenetic analyses (Olivier et al. 2005; Wertz et al. 2003). Some sequences of the gene include hypervariable regions and conserved regions. Sequence alignment using Blastn (Basic Local Alignment Search Tool nucleotide) of 16S ribosomal RNA genes of Enterobacteriaceae and Vibrionaceae showed sequences with homologies, which explains the detection of these families by the primer sets B and D.

According to Drake et al. (2007), all *Vibrio* species are ubiquitous in the marine environment and all species except *V. cholerae* and *V. mimicus* require sodium chloride supplementation of the media for growth. Therefore, PCR assays using primer sets B and D may not be applied for the analysis of certain samples including seafood. However, no outbreaks related to contamination with Vibrionaceae have, to date, been reported in infant formula milks. The growth of the Vibrionaceae may be prevented using Brain Heart Infusion (BHI), as noted by Nakano et al. (2003). However, Wong et al. (2004) reported that another low salt medium such as Morita Mineral Salt (MMS)-0.5% NaCl allowed resuscitation of *V. parahaemolyticus* which was present in a viable but non-culturable state. Hence, this may provide false-positive data with real-time PCR using primer sets B and D. Therefore, the use of BHI should be further tested in order to confirm the statement by Nakano et al. (2003).

The ISO method 21528-1:2004 for the detection of Enterobacteriaceae in foods requires an enrichment step in EE broth, which was used in the interlaboratory study. Gurtler and Beuchat (2005) and Iversen and Forsythe (2007) have reported that some *Cronobacter* strains do not grow in EE broth, which can lead to false negative results. Joosten et al. (2008) advised an enrichment of the food samples with BPW only. However, Gram-positive flora may interfere with the recovery of Enterobacteriaceae. Weber et al. (2009) supplemented the BPW with 40 μM 8-hydroxyquinoline, 0.5 g/L ammonium iron (III) citrate, 0.1 g/L sodium deoxycholate and 0.1 g/L sodium pyruvate in order to optimise the enrichment and the selection of the Enterobacteriaceae.

Comparative Analysis of Milk Samples

The results of the analysis of milk samples are shown in Table 5, and provide comparative data between the LUX™-based method (set D), the foodproof® Enterobacteriaceae plus *E. sakazakii* Detection Kit 5′-nuclease and ISO methods. In the interlaboratory study, five spiking modes

Table 5 Comparison of percentage (%) of positive results obtained in the analysis of 40 samples of 100 g powdered infant formula after spiking and detected using ISO and alternative real-time PCR methods

Spiking mode	Detection enterobacteriaceae			Detection *C. sakazakii*	
	Set D	foodproof® kit	ISO 21528-1:2004	foodproof® kit	ISO/TS 22964:2006
EBES0[a]	0	0	12.5	0	0
EB1[b]	100	100	100	0	37.5
EB2[c]	100	100	100	0	0
CS1[d]	37.5	37.5	37.5	37.5	37.5
CS2[e]	100	100	100	100	100

[a] Non-spiked blank samples

[b] Samples spiked with low level of Enterobacteriaceae

[c] Samples spiked with higher level of Enterobacteriaceae

[d] Samples spiked with very low levels of *C. sakazakii*

[e] Samples spiked with higher levels of *C. sakazakii*

were decided by RIKILT who gave the identification of the samples after the pooling of results: blank samples or no spiking (EBES0), samples spiked with low levels (EB1) and higher level of Enterobacteriaceae (EB2), and samples spiked with low levels (CS1) and higher levels of *C. sakazakii* (CS2). For each spiking mode, eight samples were tested. Twenty seven out of 40 samples tested positive for the Enterobacteriaceae and 11 out of these 27 samples tested positive for *C. sakazakii* using the foodproof® Enterobacteriaceae plus *E. sakazakii* Detection Kit 5′-nuclease. The samples were analysed with the LightCycler® 480 (Fig. 3). Overall, the data obtained was in good agreement among all participants in the interlaboratory study. However, three false negative results were obtained for some CS1 samples among the three methods tested (also found by other participating laboratories). The occurrence of false negative results could be explained by a failure to detect *C. sakazakii*. As described by Edson et al. (2009) the bacteria may have failed to grow because of issues with media or incubation conditions.

One false-positive result for Enterobacteriaceae was obtained among the EBES 0 samples using the ISO 21528-1:2004 method only, while both alternative methods provided the expected results. Similarly, one false-positive result for *C.*

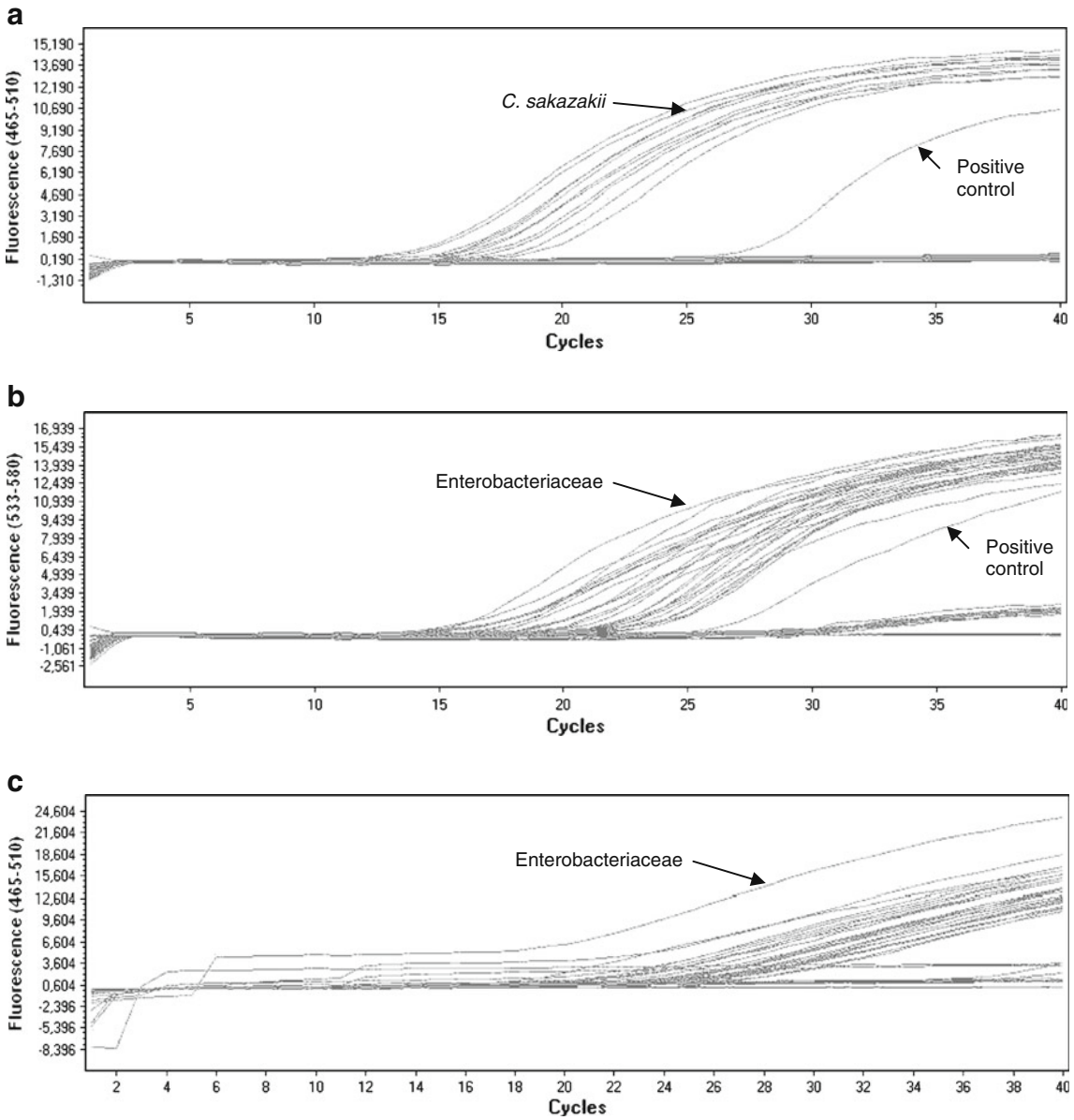

Fig. 3 Amplification curves for the detection of Enterobacteriaceae using the foodproof® Enterobacteriaceae plus *E. sakazakii* Detection Kit 5′-nuclease on FAM channel for the detection of *C. sakazakii* on FAM channel (**a**) and for the Enterobacteriaceae on VIC channel (**b**), and using primer set D on FAM channel (**c**)

sakazakii was retrieved among the EB1 samples using the ISO/TS 22964:2006. As a hypothesis, both false-positive results may have arisen from cross-contamination in the laboratory while performing the ISO procedures, more than likely after the pre-enrichment step with BPW. This indicates that the real-time PCR methods used may reduce the occurrence of such false-positive results.

In summary, Enterobacteriaceae could be detected simultaneously using primer set D or the foodproof® Enterobacteriaceae plus *E. sakazakii* Detection Kit. The real-time PCR with set D appeared to be as rapid and sensitive as the commercial detection kit. However, the specificity of set D was limited by the detection of the Vibrionaceae family and the non-detection of *P. mirabilis*. As shown with real-time PCR and in silico PCR data, primer set D cannot be used for the detection of *P. mirabilis*. However, the presence of this species in food samples remains very rare and *P. mirabilis* has not been reported to date in infant formula. The identification of *C. sakazakii* was not possible using primer set D by melting curve analysis. The main commercial interest in the use of this primer set arises from its' potential lower cost based on current retail prices for the commercial kit and the costs of the individual components of the method developed in this study. Therefore, the choice is given to the user: either a lower specificity at low cost or higher specificity at higher cost with the guarantee to identify *C. sakazakii* from other Enterobacteriaceae. It should be stated that no other in-house methods or other commercial real-time PCR systems are available with the specificity of the foodproof® Enterobacteriaceae plus *E. sakazakii* Detection Kit for safety control in milk samples. Despite the lower specificity compared with the commercial real-time PCR kit, the LUX™-based real-time PCR system with set D may be considered as a low-cost screening method for the detection of Enterobacteriaceae. This primer set could directly discriminate negative samples, while presumptive positive samples could be further analysed using the foodproof® Enterobacteriaceae plus *E. sakazakii* Detection Kit for Enterobacteriaceae confirmation, or even identification of *C. sakazakii*.

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