African Journal of Laboratory Medicine
ISSN: (Online) 2225-2010, (Print) 2225-2002

In silico and *in vitro* validation of a duplex polymerase chain reaction assay for detecting *Cronobacter sakazakii* in powdered infant milk

Authors:
Danielle Martin[1]
Rispah Chomba[1]
Tshegofatso Pelego[2]
Sanelisiwe T. Duze[1]

Affiliations:
[1]Department of Clinical Microbiology and Infectious Diseases, National Health Laboratory Service and Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa

[2]National Institute of Communicable Diseases, Centre for Tuberculosis, Johannesburg, South Africa

Corresponding author:
Sanelisiwe Duze,
thina.duze@wits.ac.za

Dates:
Received: 13 June 2025
Accepted: 16 Sept. 2025
Published: 30 Nov. 2025

How to cite this article:
Martin D, Chomba R, Pelego T, Duze ST. *In silico* and *in vitro* validation of a duplex polymerase chain reaction assay for detecting *Cronobacter sakazakii* in powdered infant milk. Afr J Lab Med. 2025;14(1), a2902. https://doi.org/10.4102/ajlm.v14i1.2902

Background: *Cronobacter sakazakii* causes life-threatening infections in neonates, primarily transmitted through contaminated powdered infant formula (PIF). In low- and middle-income countries, limited surveillance and diagnostic capacity hinder accurate detection of *C. sakazakii*, highlighting the need for rapid and affordable testing methods.

Objective: To validate a duplex polymerase chain reaction (PCR) assay for rapidly detecting *Cronobacter* spp. and *C. sakazakii* in PIF using both *in silico* and *in vitro* approaches.

Methods: This study was conducted in South Africa between March and August 2022. Seven gene targets and their primers were selected from published literature. To assess sensitivity and specificity, *in silico* PCR was performed using genome sequences of *Cronobacter* and non-*Cronobacter* species from the National Center for Biotechnology Information database. The best-performing primers were selected for an *in vitro* analysis using bacterial isolates and PIF samples from the Infection Control Service Laboratory. The specificity of the assay was assessed using eight foodborne pathogens, and further evaluated using PIF samples artificially contaminated with *Cronobacter* spp., *Bacillus cereus*, and *Salmonella* Typhi.

Results: The best-performing primers, *lpfA_1*, *fimG*, and *fimp1*, showed 100% sensitivity and specificity. Duplex PCR assay successfully detected both *Cronobacter* spp. and *C. sakazakii* with no cross-reactivity with non-*Cronobacter* pathogens, and remained effective in the presence of contaminants such as *B. cereus* and *Salmonella* Typhi.

Conclusion: The validated duplex PCR assay offers a rapid, specific, and affordable assay for detecting *Cronobacter* spp. and *C. sakazakii* in PIF.

What this study adds: This assay combined *in silico* and *in vitro* validation of a rapid and affordable PCR assay for PIF screening in resource-limited settings.

Keywords: *Cronobacter* spp.; *Cronobacter sakazakii*; powdered infant formula; polymerase chain reaction; specificity; sensitivity.

Introduction

Cronobacter sakazakii is a Gram-negative, opportunistic, motile, food-borne bacterium within the Enterobacterales family.[1] Originally described as 'yellow-pigmented *Enterobacter cloacae*', it was renamed *Enterobacter sakazakii* in 1980, and later reclassified under the genus *Cronobacter*.[2,3] The genus currently comprises seven species: *Cronobacter condimenti*, *C. dublinensis*, *C. malonaticus*, *C. muytjensii*, *C. sakazakii*, *C. turicensis*, and *C. universalis*.[3] Among these, only *C. sakazakii*, *C. turicensis*, and *C. malonaticus* are associated with human diseases, particularly *C. sakazakii*, the most clinically significant species, often associated with neonatal meningitis.[4,5,6,7] The majority of *C. malonaticus* infections occur in adults and are less severe.[6]

Cronobacter sakazakii is a clinically significant pathogen, particularly in neonates and infants, where infections can lead to meningitis, necrotising enterocolitis, septicaemia, and even death.[8,9] Premature infants, or those with low birthweight or a compromised immune system, are at an even greater risk of infection, and outbreaks of disease have been reported in hospital units for newborns.[10,11] *Cronobacter* infections are often attributed to contaminated powdered infant formula (PIF), which is a nonsterile product.[1,11,12] *Cronobacter sakazakii* has also been isolated from breast milk expressed with contaminated pump equipment,[13,14] as well as other environmental sources, including pacifiers, bottles, feeding utensils, and kitchen sink surfaces, all of which serve as potential points of contamination for PIF.[14]

Despite its severity, the public health impact of *C. sakazakii* is often underestimated, particularly in low- and middle-income countries because of the absence of active surveillance systems, underreporting of cases, and inadequate diagnostic infrastructure.[15] Although *C. sakazakii* has been detected in various food products and environments across Africa, including South Africa,[16,17,18] Egypt,[19,20,21] Côte d'Ivoire,[22] and Nigeria,[23] epidemiological data remain scarce. This diagnostic gap underscores the need for rapid, accurate, and affordable detection methods to ensure the safety of PIF and enable timely public health interventions, as *C. sakazakii* infections are associated with high morbidity and mortality.[7] Moreover, survivors often suffer irreversible neurological sequelae, such as brain abscesses, quadriplegia, developmental delays, hydrocephalus, and motor impairment.[5,24,25,26]

Traditional culture-based methods, such as those recommended by the US Food and Drug Administration, the International Organization for Standardization, and the International Dairy Federation (ISO/TS 22964:2006), are widely used for detecting *C. sakazakii* in PIF and are considered reliable.[27] However, these methods are labour-intensive, require a pre-enrichment step, and can take up to six days to yield results. Additionally, traditional biochemical tests and chromogenic media used for confirmation are limited by phenotypic overlap between *Cronobacter* spp. and other Enterobacteriaceae, which can lead to misidentification.[27,28] Furthermore, competition from faster-growing organisms and interference from background microflora can reduce recovery rates and hinder accurate detection.[29]

Molecular methods, such as polymerase chain reaction (PCR), offer higher sensitivity and specificity, along with faster turnaround times. Existing PCR assays often lack species-level specificity and may require multiple reactions to differentiate closely related *Cronobacter* spp.[4,30,31] Moreover, the limited availability of rapid and accurate detection methods is compounded by the high cost and technical complexity of the existing assays, making routine testing especially challenging in resource-limited settings. This study, therefore, aimed to optimise and validate an accurate and affordable duplex PCR assay for the rapid and simultaneous detection of *Cronobacter* spp. and *C. sakazakii* in PIF, ideal for use in resource-limited settings.

Methods

Ethical considerations

An ethics waiver (reference number: W-CBP-220422-03) was obtained from the Witwatersrand Human Research Ethics Committee (Medical), University of the Witwatersrand, Johannesburg, South Africa. The study was conducted between March 2022 and August 2022, and involved no human participants or animal research. Bacterial isolates and PIF samples were sourced from the Infection Control Service Laboratory of the National Health Laboratory Service. The PIF samples were tested for contaminants during routine food testing at the Infection Control Service Laboratory before use in artificial contamination experiments. All of the experiments complied with institutional and laboratory biosafety and ethical guidelines. The data generated contained no personal identifiers and were managed following institutional data protection policies.

Study design

This study was a laboratory-based validation combining *in silico* and *in vitro* approaches to evaluate the performance of primers for a duplex PCR assay that can rapidly detect *Cronobacter* spp. and *C. sakazakii* in PIF. The study was conducted at the Infection Control Service Laboratory of the National Health Laboratory Service at the University of the Witwatersrand in Johannesburg, South Africa, between March 2022 and August 2022.

Sample collection

This study used a collection of 27 complete *Cronobacter* genome sequences (20 *C. sakazakii*, 2 *C. malonaticus*, 1 *C. turicensis*, 1 *C. dublinensis*, 1 *C. universalis*, 1 *C. muytjensii*, and 1 *C. condiment*), and 20 complete genome sequences of non-*Cronobacter* foodborne pathogens, including three *Salmonella* Typhi, two *Shigella sonnei*, two *Listeria monocytogenes*, three *E. cloacae*, two *Escherichia coli*, three *Bacillus cereus*, two *Klebsiella pneumoniae*, and three *Staphylococcus aureus.* The genome sequences were downloaded as FASTA files from the National Center for Biotechnology Information database (NCBI: https://www.ncbi.nlm.nih.gov) and were used in the *in silico* PCR analysis. In addition, 11 previously characterised foodborne pathogens and four PIF samples (PIF7069, PIF6748, PIF7070, and PIF6749) from two brands were obtained from the Infection Control Service Laboratory through routine diagnostics; these were used for the *in vitro* PCR analysis. The foodborne pathogens included three *Cronobacter* isolates (*C. turicensis*, *C. sakazakii* M0020, and *C. sakazakii* M0022) and eight non-*Cronobacter* isolates (*Salmonella* Typhi, *S. sonnei*, *L. monocytogenes*, *E. cloacae*, *E. coli*, *B. cereus*, *K. pneumoniae*, and *S. aureus*). Routine food testing confirmed that PIF7069, PIF6748, and PIF7070 were free of bacterial contamination, whereas PIF6749 was positive for *B. cereus.*

Gene targets and primer selection

A literature search through PubMed, Google Scholar, and Scopus was performed to identify gene targets for the molecular detection of *Cronobacter* spp. and *C. sakazakii*. The search was performed using the following terms: '*Cronobacter sakazakii*', '*Cronobacter* spp.', 'PCR', 'gene target', 'species-specific primers', 'genus-specific primers'. Seven gene targets were selected based on published studies that reported their potential diagnostic value in differentiating *Cronobacter* spp., specificity, sensitivity, and availability of the primer sequences. These seven gene targets included six species-specific genes (*cgcA*, *gyrB*, *rpoB*, *fimp1*, *fimG*, *lpfA_1*) and one genus-specific gene (*grxB*). The corresponding primer sequences for each gene target were downloaded and used in the *in silico* PCR analysis (Table 1).

In silico PCR analysis

Genome sequences of the 27 *Cronobacter* species and 20 non-*Cronobacter* foodborne pathogens were used in the *in silico* PCR analysis to determine the sensitivity and specificity of the primer sequences for each gene target. The *in silico* PCR was performed using an online platform available at http://insilico.ehu.es/PCR/, which simulates theoretical PCR amplification based on primer sequences and genome sequences.[35] Each primer pair was tested against each of the 47 genome sequences, using default settings to determine the presence or absence of an amplification product matching the expected amplicon size. The *in silico* PCR analysis provides the predicted start position and size of the amplicon. Results were recorded as positive (+) if the expected product size was detected, and negative (-) if no product was observed. Thereafter, the sensitivity and specificity of each primer set were calculated using Equations 1 and 2. The species-specific primers with the highest sensitivity and specificity for *C. sakazakii* detection were selected for further validation *in vitro*.

PCR optimisation and specificity

Cronobacter turicensis, *C. sakazakii* M0020, and *C. sakazakii* M0022, and eight non-*Cronobacter* foodborne pathogens were cultured on nutrient agar (Thermo Fischer Scientific, Waltham, Massachusetts, United States) and incubated overnight at 36 °C. Crude genomic DNA was extracted by suspending two loopfuls of bacterial colonies in 200 µL of tris-ethylenediaminetetraacetic acid (TE) buffer, followed by incubation at 95 °C for 25 min, vortexing, and centrifugation at 12 000 rpm for 3 min. The supernatant was collected and stored at 8 °C until further use. Two singleplex PCR assays targeting the *lpfA_1* and *grxB* genes, were optimised in a 25 µL reaction volume containing 12.5 µL of 2X PCR Master Mix (Thermo Fischer Scientific, Waltham, Massachusetts, United States), 2 µL of DNA, 0.2 µM primers, and nuclease-free water. The PCR cycling conditions were 95 °C for 3 min, 35 cycles of 95 °C for 30 s, 51 °C for 30 s, 72 °C for 60 s, followed by a final extension at 72 °C for 10 min. Amplicons were resolved by gel electrophoresis on a 2% agarose gel stained with GelRed™ (Anatech, Johannesburg, South Africa), using 1X Tris-Borate-EDTA buffer at 110 V, and visualised using the GelDoc system (Bio-Rad Laboratories, Hercules, California, United States).

The duplex PCR assay targeting both *lpfA_1* and *grxB* was optimised under the same conditions, with each primer added at a final concentration of 0.2 µM. In all PCR reactions, *C. turicensis* and *C. sakazakii* M0020 were used as positive controls for the *grxB* and *lpfA_1*, and nuclease-free water was used for the negative control. The specificity of the duplex PCR assay was evaluated using genomic DNA from *C. sakazakii* M0022, *C. turicensis*, and eight non-*Cronobacter* foodborne pathogens listed above. The specificity of the assay was calculated using Equation 2.

TABLE 1: Gene targets and primer sequences for detecting *Cronobacter* spp. and *Cronobacter sakazakii*.

Target gene	Primer sequence (5' to 3')	Product size (bp)	Reference numbers
cgcA	F-GGTGGCSGGGTATGACAAAGAC R-GGCGGACGAAGCCTCAGAGAGT	493	32
rpoB	F-ACGCCAAGCCTATCTCCGCG R-ACGGTTGGCGTCATCGTG	554	31
gyrB	F-AGGTAAAATCCACCAGCAAAC R-CAGAATATCGTATTCAAACTC	151	33
fimG	F-GTTACTGCTGCTGATAATGT R-TAAGTAAACGTCACCTGTAC	495	4
lpfA_1†	F-CCGGTAGCCTGTTCTTTATG R-GGTGTCGGCAGTTTGATAGT	881	4
fimp1	F-GADGGWAATGCACTGGTTAACA R-TACCGTTAGGCGTAATCACA	103	5
grxB†	F-AGTTATATATTTACGATCACTGTCCGTT R-CCTCTTTTTTCTCGGTAAAGTAACG	378	34

Note: Please see the full reference list of this article for details on the articles cited: Martin D, Chomba R, Pelego T, Duze ST. *In silico* and *in vitro* validation of a duplex polymerase chain reaction assay for detecting *Cronobacter sakazakii* in powdered infant milk. Afr J Lab Med. 2025;14(1), a2902. https://doi.org/10.4102/ajlm.v14i1.2902

bp, base pair.

†, Selected for *in vitro* validation.

Artificial contamination

To artificially contaminate the PIF samples with *C. sakazakii*, 5 µL of a suspension (10^8 colony-forming units/mL) was added to 10 g of each sample in a sterile glass bottle with 100 mL buffered peptone water, followed by incubation for 6 h at 36 °C. This resulted in an estimated final concentration of approximately 5.0×10^6 colony-forming units/mL in the pre-enrichment. The PIF7070 sample was also inoculated with 5 µL of *Salmonella* Typhi (10^8 colony-forming units/mL). After incubation, 40 mL of the pre-enrichment broth was centrifuged at 3000×g for 10 min. The pellet was resuspended in 200 µL of supernatant, and genomic DNA was extracted using the High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany), followed by PCR amplification using the duplex PCR assay. Powdered infant formula samples PIF7070 and PIF6749 were specifically used to evaluate assay interference. The experiment was performed in duplicate.

Data analysis

All data were collected and recorded using Microsoft Excel® 2010. For both *in silico* and *in vitro* PCR assays, amplification results were recorded based on the presence or absence of the expected amplicon size. Similarly, results from the artificially contaminated PIF samples were classified as positive or negative according to band visualisation on a 2% gel electrophoresis. A true positive was defined as a *C. sakazakii* or *Cronobacter* spp. yielding a positive amplification with the expected amplicon size. A true negative was defined as a non-*Cronobacter* foodborne pathogen (or genome sequence) with no amplification, confirming assay specificity. False positives were recorded when amplification occurred in non-*Cronobacter* spp., and false negatives when the assay failed to amplify the target genes for *Cronobacter* spp. The results were tabulated, and the sensitivity and specificity were calculated using Equation 1 and Equation 2:

$$\text{Specificity} = \left(\frac{\text{true negatives}}{\text{true negatives} + \text{false positives}}\right) \times 100 \quad \text{[Eqn 1]}$$

$$\text{Sensitivity} = \left(\frac{\text{true positives}}{\text{false positives} + \text{true negatives}}\right) \times 100 \quad \text{[Eqn 2]}$$

Results

In silico PCR performance

In total, seven primer pairs corresponding to the seven gene targets for *Cronobacter* spp. and *C. sakazakii* detection were evaluated against 47 genome sequences using *in silico* PCR. Species-specific primers targeting the *lpfA_1*, *fimG*, and *fimp1* genes demonstrated 100% sensitivity and specificity for *C. sakazakii* detection during *in silico* PCR. The *cgcA* and *gyrB* primers had 100% specificity, with sensitivities of 94.7% and 89.5%, each missing one or two *C. sakazakii* sequences during the *in silico* PCR (Table 2). The *rpoB* primers exhibited the lowest performance, with sensitivity and specificity of 78.9% and 92.6%, whereas the genus-specific *grxB* primers detected all *Cronobacter* spp. except *C. dublinensis* and *C. condiment*, resulting in 92.3% sensitivity and 100% specificity (Table 2). Based on these results, *lpfA_1* and *grxB* primers were selected for *in vitro* testing.

In vitro PCR performance

Two singleplex PCR assays targeting the *lpfA_1* and *grxB* genes were successfully optimised and effectively detected the respective genes in *C. sakazakii* M0022, *C. sakazakii* M0020, and *C. turicensis* (Figure 1a). Additionally, a duplex PCR assay targeting both genes was optimised, demonstrating simultaneous detection of both the *lpfA_1* and *grxB* genes in *C. sakazakii* M0022, *C. sakazakii* M0020, while only detecting the *grxB* gene in *C. turicensis* (Figure 1b).

The duplex PCR assay was 100% specific for *Cronobacter* spp. and *C. sakazakii*, with successful amplification of the *grxB* gene in *C. sakazakii* M0022, *C. sakazakii* M0020, and *C. turicensis*, as well as the amplification of the *lpfA_1* gene in *C. sakazakii* M0022 and *C. sakazakii* M0020, and no amplification in any of the eight non-*Cronobacter* foodborne pathogens (Figure 2a). Moreover, the *lpfA_1* and *grxB* genes were successfully detected in the PIF samples artificially contaminated with *C. sakazakii* M0022 (Figure 2b, Table 3), with no interference from the non-*Cronobacter* foodborne pathogens in PIF samples PIF6749 (containing *B. cereus*) and PIF7070 (containing *Salmonella* Typhi). These results demonstrate the assay's reliability and suitability for detecting *Cronobacter* spp. and *C. sakazakii* in PIF, even in the presence of other foodborne pathogens.

Discussion

In silico PCR is a valuable tool used to predict amplification efficiency, specificity, and amplicon size by aligning primers against target genomes, facilitating the selection of optimal primer pairs for PCR assays.[36] In our study, primers targeting the *rpoB*, *cgcA*, and *gyrB* genes exhibited suboptimal sensitivity and specificity for *C. sakazakii* detection. The *rpoB* gene requires two separate PCR reactions to differentiate between *C. sakazakii* and *C. malonaticus*, limiting its diagnostic utility.[31] Our findings are consistent with previous studies reporting limited species-level resolution of the *cgcA* and *gyrB* primers.[31,33]

In contrast, primers targeting the *fimp1*, *fimG*, and *lpfA_1* showed 100% specificity and sensitivity *in silico*. However, the short amplicon size of *fimp1* (103 bp) reduces its suitability for conventional PCR. Prior studies investigating the suitability of *fimG* and *lpfA_1* genes for *C. sakazakii* detection using bioinformatics and *in vitro* approaches demonstrated superior sensitivity for *lpfA_1* over *fimG*, supporting the selection of the *lpfA_1* gene for further validation.[4] The genus-specific *grxB* gene, while slightly less sensitive, showed broad detection across *Cronobacter* genomes and 100% specificity within our study sample, reinforcing its value for genus-level identification. Our findings are consistent with previous studies that have successfully employed the *grxB* gene for the detection of *Cronobacter* spp.[34,36,37,38]

In vitro validation confirmed the efficacy of the selected primers in detecting *Cronobacter* spp. and *C. sakazakii*. Singleplex and duplex PCR assays targeting *lpfA_1* and *grxB* successfully detected *C. sakazakii* and *C. turicensis* with high specificity. The duplex assay enabled simultaneous detection of both targets in artificially contaminated PIF, even in the presence of other foodborne pathogens such as *B. cereus* and *Salmonella* Typhi. These results corroborate the *in silico* findings and demonstrate the assay's robustness and reliability.

Importantly, the duplex PCR assay reduced turnaround time to approximately 12 h, compared to the 3–6-day protocol required by the conventional culture-based method recommended by the United States Food and Drug Administration.[27,39] This time efficiency, combined with high specificity and affordability, makes the assay particularly suitable for resource-limited settings where rapid screening is essential for public health interventions and outbreak response.

Although culture-based methods remain the cornerstone of microbiological diagnostics, particularly for strain isolation, antimicrobial susceptibility testing, and whole-genome sequencing, which are essential for surveillance and outbreak investigations. The duplex PCR assay reported in this study will complement culture-based methods by serving as a rapid screening tool for *C. sakazakii* in PIF, enabling early detection of contaminated PIF products. The assay will facilitate timely interventions and support targeted use of culture-based confirmation, especially in resource-limited settings where laboratory capacity and turnaround time are constrained. By identifying positives quickly, this PCR assay will enable resource-limited laboratories to prioritise samples

TABLE 2: Summary of the sensitivity and specificity of each primer pair based on *in silico* polymerase chain reaction analysis.

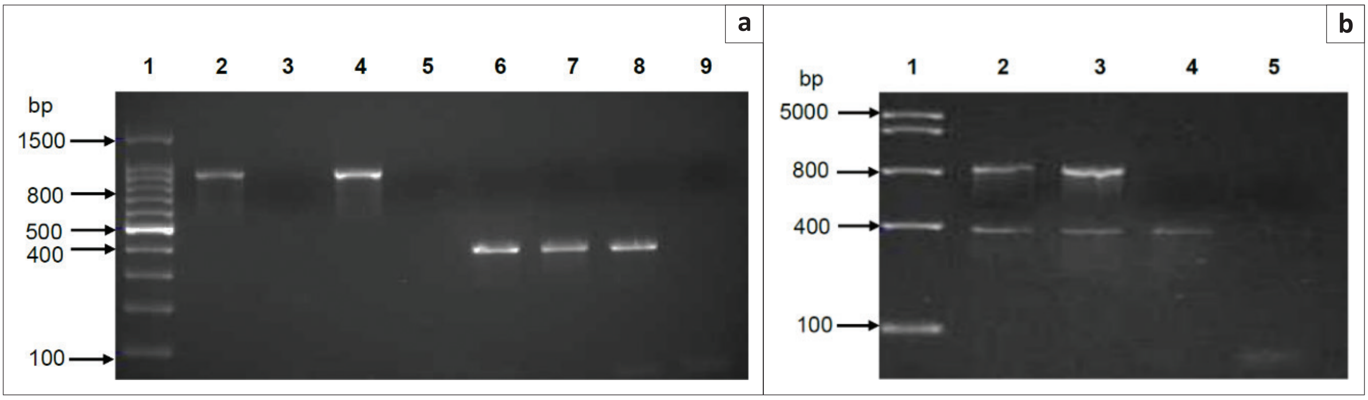
Species	Accession numbers	Target genes						
		grxB	*gyrB*	*cgcA*	*lpfA_1*	*rpoB*	*fimG*	*fimp1*
Cronobacter sakazakii	NZ_CP011047	+	+	+	+	+	+	+
	NZ_CP012253	+	+	+	+	-	+	+
	NC_009778	+	+	+	+	+	+	+
	CP045778	+	+	+	+	+	+	+
	CP078106	+	+	+	+	+	+	+
	NZ_CP027107	+	+	+	+	+	+	+
	CP028974	+	+	+	+	+	+	+
	NC_020260	+	+	+	+	-	+	+
	NC_017933	+	+	+	+	+	+	+
	NZ_CP049154	+	-	-	+	+	+	+
	NZ_CP059045	+	+	+	+	+	+	+
	NZ_CP034340	+	+	+	+	-	+	+
	NZ_CP093376	+	+	+	+	-	+	+
	NZ_CP098777	+	+	+	+	+	+	+
	NZ_CP049150	+	+	+	+	+	+	+
	NZ_CP049147	+	+	+	+	+	+	+
	NZ_CP078110	+	+	+	+	+	+	+
	CP027109	+	+	+	+	+	+	+
	CALF01000071	+	-	+	+	+	+	+
Cronobacter malonaticus	NZ_CP013940	+	-	-	-	+	-	-
	NC_023032	+	-	-	-	+	-	-
Cronobacter turicensis	FN543093	+	-	-	-	-	-	-
Cronobacter dublinensis	NZ_CP012266	-	-	-	-	-	-	-
Cronobacter muytjensii	NZ_CP012268	+	-	-	-	-	-	-
Cronobacter universalis	NZ_CP012257	+	-	-	-	-	-	-
Cronobacter condimenti	NZ_CP012264	-	-	-	-	-	-	-
Shigella sonnei	NZ_CP055292	-	-	-	-	-	-	-
	NZ_CP049169	-	-	-	-	-	-	-
Bacillus cereus	NZ_CP017060	-	-	-	-	-	-	-
	NZ_AP022986	-	-	-	-	-	-	-
	NZ_CP011151	-	-	-	-	-	-	-
Salmonella Typhi	NZ_CP030219	-	-	-	-	-	-	-
	NZ_CP030749	-	-	-	-	-	-	-
	NZ_CP091547	-	-	-	-	-	-	-
Listeria monocytogenes	NZ_CP050027	-	-	-	-	-	-	-
	NZ_CP020827	-	-	-	-	-	-	-
Klebsiella pneumoniae	NZ_CP082009	-	-	-	-	-	-	-
	NZ_CP082000	-	-	-	-	-	-	-
Enterobacter cloacae	NZ_CP053568	-	-	-	-	-	-	-
	CP056460	-	-	-	-	-	-	-
	NC_014107	-	-	-	-	-	-	-
Staphylococcus aureus	NZ_CP014368	-	-	-	-	-	-	-
	NZ_CP058615	-	-	-	-	-	-	-
	NZ_CP013955	-	-	-	-	-	-	-
Escherichia coli	NZ_CP027582	-	-	-	-	-	-	-
	NZ_AP018808	-	-	-	-	-	-	-
Sensitivity (%)		92.3†	89.5	94.7	100†	78.9	100	100
Specificity (%)		100.0†	100.0	100.0	100†	92.6	100	100

+, Detected; -, not detected.
†, grxB (specific for Cronobacter spp.) and ipfA_1 (specific for Cronobacter sakazakii) were selected for in vitro analysis.

for culture, thereby optimising resource use and improving overall diagnostic turnaround time.

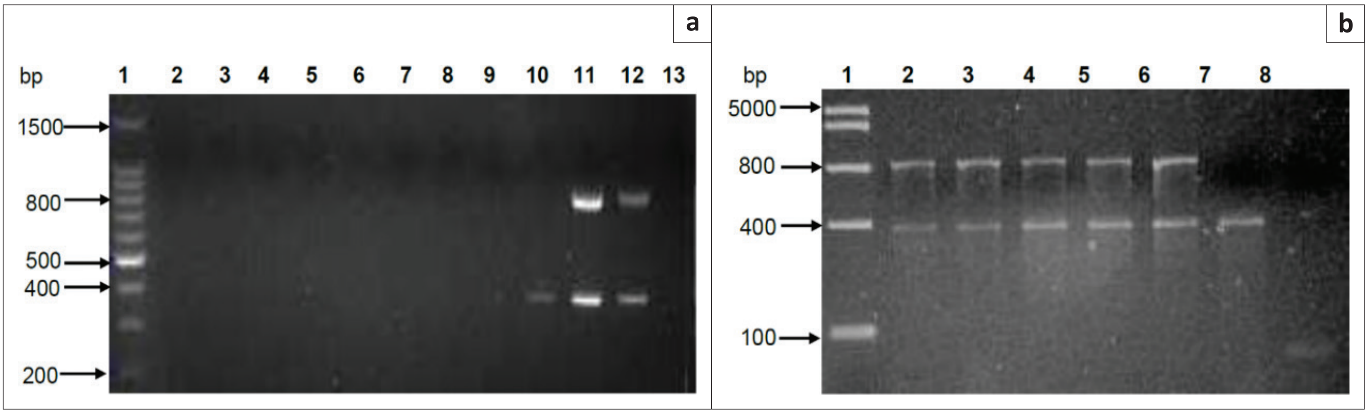
Limitations

This study had a few limitations. The *in vitro* sensitivity of the duplex PCR assay could not be fully assessed because of the limited availability of *C. sakazakii* and other *Cronobacter* spp. isolates, despite efforts to source additional strains from public and private laboratories. However, incorporating *in silico* validation of the primers using publicly available genome sequences from multiple *Cronobacter* species allowed us to evaluate primer specificity across diverse species. *In silico* analysis thus provided a valuable alternative for assay validation. This approach also supports the growing utility of bioinformatics tools in enhancing assay design. Another limitation of this study is that the sensitivity of the assay was determined using high bacterial concentrations,



bp, base pair.

FIGURE 1: (a) Gel electrophoresis of the singleplex polymerase chain reaction (PCR) assays targeting the *lpfA_1* (881 bp; Lanes 2–5) and *grxB* (378 bp; Lanes 6–9) genes: Lane 1, DNA ladder (100 bp, Thermo Fischer Scientific, Waltham, Massachusetts, United States); Lane 2, *Cronobacter sakazakii* M0022; Lane 3, *C. turicensis*; Lane 4, *C. sakazakii* M0020; Lane 5, negative control; Lane 6, *C. sakazakii* M0022; Lane 7, *C. sakazakii* M0020; Lane 8, *C. turicensis;* and Lane 9, negative control. (b) Duplex PCR assay targeting *C. sakazakii* and *Cronobacter* spp. in a single reaction: Lane 1, DNA ladder (Fastruler, Thermo Fischer Scientific, Waltham, Massachusetts, United States); Lane 2, *C. sakazakii* M0022; Lane 3, *C. sakazakii* M0020; Lane 4, *C. turicensis;* and Lane 5, negative control.



bp, base pair.

FIGURE 2: (a) Gel electrophoresis showing the specificity of the polymerase chain reaction (PCR) assay: Lane 1, DNA ladder (100 bp, Promega, Madison, Wisconsin, United States); Lanes 2–9, non-*Cronobacter* species (*Escherichia coli, Listeria monocytogenes, Bacillus cereus, Salmonella Typhi, Klebsiella pneumoniae, Staphylococcus aureus, Enterobacter cloacae, Shigella sonnei*); Lane 10, *Cronobacter turicensis*; Lane 11, *C. sakazakii* M0022*;* Lane 12, *C. sakazakii* M0020; and Lane 13, negative control. (b) Polymerase chain reaction results from the artificially contaminated powdered infant formula (PIF) samples: Lane 1, DNA ladder (Fastruler, Thermo Fischer Scientific, Waltham, Massachusetts, United States); Lane 2 and Lane 5, PIF contaminated with *C. sakazakii* M0022; Lane 3, PIF with *B. cereus* and *C. sakazakii* M0022; Lane 4, PIF with *Salmonella* Typhi and *C. sakazakii* M0022; Lane 6, *C. sakazakii* M0020; Lane 7, *C. turicensis*; and Lane 8, negative control.

TABLE 3: *Cronobacter sakazakii* detection in artificially contaminated powdered infant formula samples.

Sample ID	PIF brand	Pre-existing contaminants	Artificial contaminants	PCR result
PIF6748	1	None	*Cronobacter sakazakii* M0022	+
PIF7069	1	None	*Cronobacter sakazakii* M0022	+
PIF7070	1	None	*Cronobacter sakazakii* M0022 and *Salmonella* Typhi	+
PIF6749	2	*B. cereus*	*Cronobacter sakazakii* M0022	+

PCR, polymerase chain reaction; PIF, powdered infant formula.

+, Detected.

which may not fully represent the variable contamination levels of *C. sakazakii* typically found in PIF, as contamination may occur at low levels, and accurate detection under such conditions is critical for food safety. Future work should therefore include the determination of the assay's limit of detection using serial dilutions of *C. sakazakii* spiked into PIF. While this study focused on conventional PCR to ensure affordability and accessibility in basic laboratory settings, it may not fully meet the throughput and automation needs of modern food safety laboratories. Adapting the assay into a real-time multiplex PCR assay will allow for quantitative detection, improved sensitivity, and reduced turnaround time. Additionally, integrating automated DNA extraction workflows could streamline sample processing and minimise human error, making the assay more suitable for high-throughput environments and outbreak response scenarios. These enhancements would support broader implementation in both resource-limited and well-equipped laboratories, contributing to more efficient monitoring of *Cronobacter* contamination in infant formula.

Conclusion

This assay combined *in silico* and *in vitro* validation of a rapid and affordable duplex PCR assay for the simultaneous detection of *Cronobacter* spp. and *C. sakazakii* in PIF. The performance of the assay was not affected by the presence of non-*Cronobacter* species such as *B. cereus* and *Salmonella* Typhi. This duplex PCR assay can facilitate the diagnosis of *C. sakazakii* in PIF, particularly in resource-limited settings, contributing to improved epidemiological surveillance and screening of *Cronobacter* spp. in PIF. Its adaptability for routine diagnostics and outbreak investigations further underscores its potential for integration into national food safety monitoring programmes.

Acknowledgements

The authors would like to acknowledge the staff of the Infection Control Service Laboratory of the National Health Laboratory Service for providing us with the bacterial isolates and powdered infant formula samples used in this study. This article is partially based on Danielle Martin's thesis entitled, 'Optimizing a PCR assay for rapid detection of *Cronobacter sakazakii* in powdered infant formula', towards the degree of BHSc Honours in the Department of Clinical Microbiology and Infectious Diseases, University of the Witwatersrand, South Africa, submitted in December 2022, with supervisors Rispah Chomba, Tshegofatso Pelego and Sanelisiwe T. Duze. The thesis is not publicly available.

Competing interests

The author reported that they received funding from National Research Foundation which may be affected by the research reported in the enclosed publication. The author has disclosed those interests fully and has implemented an approved plan for managing any potential conflicts arising from their involvement. The terms of these funding arrangements have been reviewed and approved by the affiliated university in accordance with its policy on objectivity in research.

Authors' contributions

All of the authors contributed to this manuscript. S.T.D. and R.C. conceptualised the original idea for the study and supervised this study. D.M. and T.P. carried out the laboratory work, data collection, and analysis; D.M. and S.T.D. wrote the manuscript; and R.C., D.M., T.P., and S.T.D. reviewed the final draft of the article.

Funding information

This work was supported by the National Research Foundation (NRF) under Sanelisiwe T. Duze (Grant Number 138279).

Data availability

The authors confirm that the data supporting the findings of this study are available within the article.

Disclaimer

The views and opinions expressed in this article are those of the authors and are the product of professional research. The article does not necessarily reflect the official policy or position of any affiliated institution, funder, agency, or that of the publisher. The authors are responsible for this article's results, findings, and content.

References

1. Jason J. Prevention of invasive *Cronobacter* infections in young infants fed powdered infant formulas. Pediatrics. 2012;130(5):e1076–e1084. https://doi.org/10.1542/peds.2011-3855
2. Farmer JJ, 3rd, Asbury MA, Hickman FW, Brenner DJ, Enterobacteriaceae Study Group. *Enterobacter sakazakii*: A new species of 'Enterobacteriaceae' isolated from clinical specimens. Int J Syst Bacteriol. 1980;30(4):569–584. https://doi.org/10.1099/00207713-30-4-569
3. Iversen C, Mullane N, McCardell B, et al. Cronobacter gen. nov., a new genus to accommodate the biogroups of *Enterobacter sakazakii*, and proposal of *Cronobacter sakazakii* gen. nov., comb. nov., *Cronobacter malonaticus* sp. nov., *Cronobacter turicensis* sp. nov., *Cronobacter muytjensii* sp. nov., *Cronobacter dublinensis* sp. nov., *Cronobacter* genomospecies 1, and three subspecies of *Cronobacter dublinensis*. Int J Syst Evol Microbiol. 2008;58(Pt 6):1442–1447. https://doi.org/10.1099/ijs.0.65577-0
4. Shang Y, Ye Q, Wu Q, et al. PCR and multiplex PCR assays for the detection of *Cronobacter* species using specific targets obtained by a bioinformatics approach. Food Control. 2021;125:107896. https://doi.org/10.1016/j.foodcont.2021.107896
5. Kim SS. Application of comparative genomics for the development of PCR primers for the detection of harmful or beneficial microorganisms in food: Mini-review. Foods. 2025;14(6):1060. https://doi.org/10.3390/foods14061060
6. Otake S, Okada Y, Forsythe SJ, Kasai M. Meningitis and brain abscess formation caused by *Cronobacter malonaticus* sequence type 440 in a full-term neonate. J Infect Chemother. 2021;27(11):1648–52. https://doi.org/10.1016/j.jiac.2021.06.002
7. Holý O, Forsythe S. *Cronobacter* spp. as emerging causes of healthcare-associated infection. J Hosp Infect. 2014;86(3):169–177. https://doi.org/10.1016/j.jhin.2013.09.011
8. Strysko J, Cope JR, Martin H, et al. Food safety and invasive *Cronobacter* infections during early infancy, 1961-2018. Emerg Infect Dis. 2020;26(5):857–865. https://doi.org/10.3201/eid2605.190858
9. Mousavi ZE, Hunt K, Koolman L, Butler F, Fanning S. *Cronobacter* species in the built food production environment: A review on persistence, pathogenicity, regulation and detection methods. Microorganisms. 2023;11(6):1379. https://doi.org/10.3390/microorganisms11061379
10. Henry M, Fouladkhah A. Outbreak history, biofilm formation, and preventive measures for control of *Cronobacter sakazakii* in infant formula and infant care settings. Microorganisms. 2019;7(3):77. https://doi.org/10.3390/microorganisms7030077
11. Bowen AB, Braden CR. Invasive *Enterobacter sakazakii* disease in infants. Emerg Infect Dis. 2006;12(8):1185–1189. https://doi.org/10.3201/eid1208.051509
12. World Health Organization. Safe preparation, storage, and handling of powdered infant formula: Guidelines [homepage on the Internet]. Geneva: WHO/FAO; 2007 [cited 2025 May 02]. Available from: https://www.who.int/publications/i/item/9789241597889
13. McMullan R, Menon V, Beukers AG, Jensen SO, Van Hal SJ, Davis R. *Cronobacter sakazakii* infection from expressed breast milk, Australia. Emerg Infect Dis. 2018;24(2):393–394. https://doi.org/10.3201/eid2402.171411
14. Haston JC, Miko S, Cope JR, et al. *Cronobacter sakazakii* infections in two infants linked to powdered infant formula and breast pump equipment – United States, 2021 and 2022. MMWR Morb Mortal Wkly Rep. 2023;72(9):223–226. https://doi.org/10.15585/mmwr.mm7209a2
15. Mazi IM, Onyeaka H, Nnaji ND. Foodborne pathogens in Africa: Understanding *Cronobacter sakazakii*. Public Health Challenges. 2023;2(1):e53. https://doi.org/10.1002/puh2.53
16. Strydom A, Cameron M, Witthuhn RC. Phylogenetic analysis of *Cronobacter* isolates based on the rpoA and 16S rRNA genes. Curr Microbiol. 2012;64(3):251–258. https://doi.org/10.1007/s00284-011-0061-8
17. Cawthorn DM, Botha S, Witthuhn RC. Evaluation of different methods for the detection and identification of *Enterobacter sakazakii* isolated from South African infant formula milks and the processing environment. Int J Food Microbiol. 2008;127(1–2):129–138. https://doi.org/10.1016/j.ijfoodmicro.2008.06.024
18. Witthuhn RC, Kemp F, Britz TJ. Isolation and PCR detection of *Enterobacter sakazakii* in South African food products, specifically infant formula milks. World J Microbiol Biotechnol. 2007;23(2):151–157. https://doi.org/10.1007/s11274-006-9203-8
19. El-Gamal MS, El Dairouty RK, Okda AY, Salah SH, El-Shamy SM. Incidence and interrelation of *Cronobacter sakazakii* and other foodborne bacteria in some milk products and infant formula milks in Cairo and Giza area. World Appl Sci J [serial online]. 2013 [cited n.d.];26(9):1129–1141. Available from: https://www.idosi.org/wasj/wasj26(9)13/1.pdf
20. Elkhawaga AA, Hetta HF, Osman NS, Hosni A, El-Mokhtar MA. Emergence of *Cronobacter sakazakii* in cases of neonatal sepsis in Upper Egypt: First report in North Africa. Front Microbiol. 2020;11:215. https://doi.org/10.3389/fmicb.2020.00215
21. Mohammed MA, Sallam KI, Tamura T. Prevalence, identification and molecular characterization of *Cronobacter sakazakii* isolated from retail meat products. Food Control. 2015;53:206–211. https://doi.org/10.1016/j.foodcont.2015.01.010
22. Yao K, Zinzendorf N, Bohoua G, et al. Occurrence of *Enterobacter sakazakii* (*Cronobacter*) and other Enterobacteriaceae in commercial powdered infant formula in Abidjan, Ivory Coast. Food Nutr Sci. 2012;3(6):822–826. https://doi.org/10.4236/fns.2012.36110

23. Obasi A, Nwachukwu SC, Ugoji E. First detection of augmentin and colistin resistant *Cronobacter sakazakii* from a pharmaceutical wastewater in South-Western Nigeria. Am J Chem Biochem Eng. 2018;2(1):1. https://doi.org/10.11648/j.ajcbe.20180201.11
24. Song X, Teng H, Chen L, Kim M. *Cronobacter* species in powdered infant formula and their detection methods. Korean J Food Sci Anim Resour. 2018;38(2):376–390. https://doi.org/10.5851/kosfa.2018.38.2.376
25. Ling N, Jiang X, Forsythe S, et al. Food safety risks and contributing factors of *Cronobacter* spp. Engineering. 2022;12:128–138. https://doi.org/10.1016/j.eng.2021.03.021
26. Drudy D, Mullane NR, Quinn T, Wall PG, Fanning S. *Enterobacter sakazakii*: An emerging pathogen in powdered infant formula. Clin Infect Dis. 2006;42(7):996–1002. https://doi.org/10.1086/501019
27. Yan QQ, Condell O, Power K, Butler F, Tall BD, Fanning S. *Cronobacter* species (formerly known as *Enterobacter sakazakii*) in powdered infant formula: A review of our current understanding of the biology of this bacterium. J Appl Microbiol. 2012;113(1):1–15. https://doi.org/10.1111/j.1365-2672.2012.05281.x
28. Iversen C, Lehner A, Mullane N, et al. Identification of 'Cronobacter' spp. (*Enterobacter sakazakii*). J Clin Microbiol. 2007;45(11):3814–3816. https://doi.org/10.1128/jcm.01026-07
29. Shukla S, Lee G, Song X, et al. Detection of *Cronobacter sakazakii* in powdered infant formula using an immunoliposome-based immunomagnetic concentration and separation assay. Sci Rep. 2016;6:34721. https://doi.org/10.1038/srep34721
30. Iversen C, Forsythe SJ. Isolation of *Enterobacter sakazakii* and other Enterobacteriaceae from powdered infant formula milk and related products. Food Microbiol. 2004;21(7):771–776. https://doi.org/10.1016/j.fm.2004.01.009
31. Stoop B, Lehner A, Iversen C, Fanning S, Stephan R. Development and evaluation of *rpoB* based PCR systems to differentiate the six proposed species within the genus *Cronobacter*. Int J Food Microbiol. 2009;136(2):165–168. https://doi.org/10.1016/j.ijfoodmicro.2009.04.023
32. Carter L, Lindsey LA, Grim CJ, et al. Multiplex PCR assay targeting a diguanylate cyclase-encoding gene, *cgcA*, to differentiate species within the genus *Cronobacter*. Appl Environ Microbiol. 2013;79(2):734–737. https://doi.org/10.1128/AEM.02898-12
33. Huang CH, Chang MT, Huang L. Use of novel species-specific PCR primers targeted to DNA gyrase subunit B (*gyrB*) gene for species identification of *the Cronobacter sakazakii* and *Cronobacter dublinensis*. Mol Cell Probes. 2013;27(1):15–18. https://doi.org/10.1016/j.mcp.2012.08.004
34. Dong X, Wu Q, Zhang J, Mo S, Kou X, Guo W. Sequencing of the *grxB* gene of *Cronobacter* spp. and the development of a PCR assay for its identification. Foodborne Pathog Dis. 2013;10(8):711–717. https://doi.org/10.1089/fpd.2012.1431
35. San Millán RM, Martínez-Ballesteros I, Rementeria A, Garaizar J, Bikandi J. Online exercise for the design and simulation of PCR and PCR-RFLP experiments. BMC Res Notes. 2013;6:513. https://doi.org/10.1186/1756-0500-6-513
36. Kalendar R, Shevtsov A, Otarbay Z, Ismailova A. *In silico* PCR analysis: A comprehensive bioinformatics tool for enhancing nucleic acid amplification assays. Front Bioinform. 2024;4:1464197. https://doi.org/10.3389/fbinf.2024.1464197
37. Kireina D, Parreira VR, Goodridge L, Farber JM. Survival and expression of *rpoS* and *grxB* of *Cronobacter sakazakii* in powdered infant formula under simulated gastric conditions of newborns. J Food Prot. 2024;87(5):100269. https://doi.org/10.1016/j.jfp.2024.100269
38. Juliansyah DA, Nurjayadi M, Declan JL, et al. The potential of *grxB* gene for detection of *Cronobacter sakazakii* in infant formula milk using real-time polymerase chain reaction. E3S Web Conf. 2023;400:04010. https://doi.org/10.1051/e3sconf/202340004010
39. Yan Q, Fanning S. Strategies for the identification and tracking of *Cronobacter* species: An opportunistic pathogen of concern to neonatal health. Front Pediatr. 2015;3:38. https://doi.org/10.3389/fped.2015.00038