The Potential of *grxB* Gene for Detection of *C. sakazakii* in Infant Formula Milk Using Real-Time Polymerase Chain Reaction

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Abstract. *Cronobacter sakazakii* is one of the bacteria that causes food poisoning that contaminates infant formula. This pathogen causes necrotizing enterocolitis, sepsis, and meningitis in infants or neonates with reported case fatality rates ranging from 40% to 80%. Therefore, it is necessary to develop fast and accurate detection of *C. sakazakii* in infant formula milk. This research aims to develop a method for detecting *C. sakazakii* bacteria using real-time PCR with high sensitivity, specificity, and accuracy. A rapid detection method using real-time PCR with the target gene *grxB* successfully detects the presence of C. sakazakii DNA in artificially contaminated formula milk. The results of the real-time PCR test showed that *C. sakazakii* DNA with a concentration of 53 ng/µL could be amplified by the *grxB* gene primer pair with a Ct value of 12 and a Tm value of 85.8ºC. The specificity test showed that the *grxB* primer could differentiate between target and some non-target bacteria. The sensitivity test showed the ability of the *grxB* primer to detect the smallest concentration of 3,392 pg/µL with a Ct of 24,06. Based on the results obtained, it can be concluded that the *grxB* primer has the potential to be used as rapid detection method for *C. sakazakii* bacteria in infant formula using real-time PCR.

1 Introduction

Microorganisms contamination in food is a major public health issue because of the emergence of foodborne pathogens [1]. The bacterial pathogen *Cronobacter sakazakii* is frequently detected in infant formula and is causes food poisoning as well as illnesses like necrotizing enterocolitis, bacteraemia, meningitis, and septicaemia. Infants with the infection have reported case fatality rates between 40 and 80 percent [2]. Therefore, there is an urgent need to develop fast and accurate methods for detecting foodborne pathogens, especially in detecting *C. sakazakii* in food to ensure food safety. The culturing method is one of the conventional methods used to detect pathogenic bacteria in food products. However, using this method requires a relatively long time and is not specific for detecting pathogenic bacteria. real-time PCR, a molecular-based technique, can be used to identify pathogenic bacteria in food, including *C. sakazakii* a [3,4].

The UNJ Salmonella Team has developed and produced specific primers as target genes for the identification of 9 foodborne pathogenic bacteria in prior research. One of them is a primer pair that has been successful in amplifying a piece of the 95 bp *fim-C* gene fragment using real-time PCR to quickly and accurately detect the pathogenic bacterium *Salmonella typhi* [5]. Using real-time PCR, this study aims to develop a fast, specific, and sensitive method for detecting *C. sakazakii* bacteria with *grxB* target genes. In our previous study, we optimized the ideal annealing temperature for the *grxB* gene at 60°C with a size of 151 base pairs [6].

2 Materials and Methods

2.1 Preparation of Culture Sample.

Tryptic Soya Agar (TSA) media (Merck) was used to culture *Cronobacter sakazakii* ATCC 29544 (Microbiologist, Minnesota) for 18 hours (overnight

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culture). After incubation, one colony was inoculated into Tryptic Soy Broth (TSB) and incubated for 18 hours at 37°C and 150 rpm in an incubation shaker (YIHDER LM-400D). The spread plate method was used to grow bacteria on TSA, and it was incubated at 37°C for 18 hours. The dilution of the bacteria was 10^{-5}-10^{-7}.

2.2 DNA isolation

One milliliter of *C. sakazakii* culture stock from TSB was added to the microtube, then centrifuged (Sorval Legend Micro 17R) at 5000 x g for five minutes. Then, using the Geno Plus Genomic DNA Extraction Miniprep System (Viogene) in accordance with the manufacturer's instructions, gram-negative bacterial DNA from *C. sakazakii* bacteria was isolated from pellets. The A260/A280 ratio and DNA concentration were measured using a nanodrop spectrophotometer (Nanovue Plus) to ensure DNA was successfully isolated.

2.3 Optimization annealing temperature of *grxB* primer pairs

To determine the ideal temperature for the annealing phase, where the primer *grxB* is attached, gradient PCR (Takara PCR Thermal Cycler) was used. The PCR assay was performed with a final volume of 25 µL containing 1 µL of pure *C. sakazakii* DNA isolate, 1 µL of forward and reverse primers, 12.5 µL of Master Mix (NZYTaq), and 9.5 µL of Nuclease Free Water (NFW) contains. The gradient PCR assay protocols were as follows: initial denaturation at 95°C, followed by 40 cycles of denaturation at 95°C, annealing at a temperature between 53 and 62°C, and an extension step at 72°C. The final extension at 72 °C completed the PCR amplification process.

2.4 Confirmation assay of Primer *grxB* using real-time PCR

The assay was performed using a qPCR magnetic induction cycler (biomolecular system) and the total volume used for the reaction mixture was 20 µL. The preparation used consisted of 10 µL of Master Mix SYBR green dye (Smobio), 1 µL each forward and reverse primer, 1 µL *C. sakazakii* DNA isolate template, and 7 µL NFW. In addition, one reaction was also used for Non-Template Control (NTC) as a negative control and negative control containing NFW and Master Mix. Amplification was carried out for 40 cycles with pre-denaturation at 95°C, denaturation at 95°C, followed by annealing at 60°C, extension at 72°C and final extension at 72°C.

2.5 Specificity and sensitivity assay.

Several non-target bacteria, including *Vibrio parahaemolyticus*, *Vibrio alginolyticus*, *Listeria monocytogenes*, *Salmonella typhi*, *Yersinia enterocolitica*, *Staphylococcus aureus* and *klebsiella pneumoniae*, used to assess the specificity of the designed *grxB* primer pair. Each reaction mixture containing target and non-target bacterial DNA samples received a pair of *grxB* primers. The concentration of each bacterial isolate was equalized to 50 ng/ µL. Based on the amplification curve and the corresponding melting curve, the real-time PCR curve findings will be examined. Multilevel dilution sensitivity testing was done on DNA template isolates of the bacteria. The amplification curves and standard curves represent the outcomes of this sensitivity test.

3 Results and discussion

3.1 Cultivation of *C. sakazakii* bacterial

Bacteria on Tryptic Soy Agar (TSA) showed the formation of yellow colonies on the surface of TSA media. The temperature range for *C. sakazakii* bacterial growth is 6-47 °C, with the ideal temperature for growth being 41-45 °C and the minimal growth temperature being 5.5-8.0 °C. On TSA media, it can also produce yellow pigments. The yellow colonies on the TSA, each measuring between 1-2 mm and 2-3 mm, revealed that the bacteria produced were in fact *C. sakazakii* bacteria [7].

3.2 DNA Analysis

The nanodrop spectrophotometer was used for the DNA isolate quantification test. The wavelength ratio of A260nm/A280nm revealed the purity of the DNA isolate. The isolation results were characterized by agarose gel electrophoresis using a 10.000 bp ladder as shown in Fig. 1.

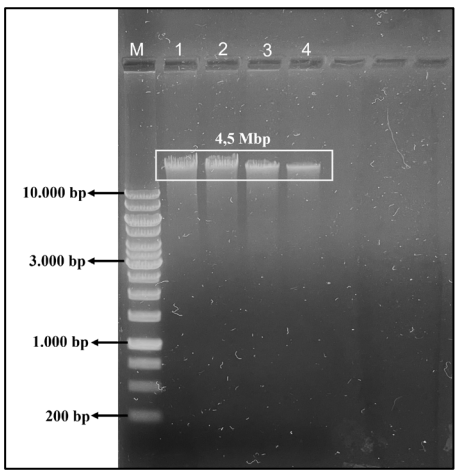

Fig 1. Characterization of *C. sakazakii* DNA on electrphoresis gel agarose. (M) DNA Ladder 10 kb; (1-4) *C. sakazakii* DNA.

The result showed the DNA bands isolated from pure cultures of *C. sakazakii* (lane 1-4) appeared at a position higher than the marker size of 10.000 bp, it can be assumed that the results obtained are in accordance with the size of the whole genome sequence of *Cronobacter sakazakii* ATCC 29544 which is 4,511,265 bp [8]. DNA purity and concentration were measured

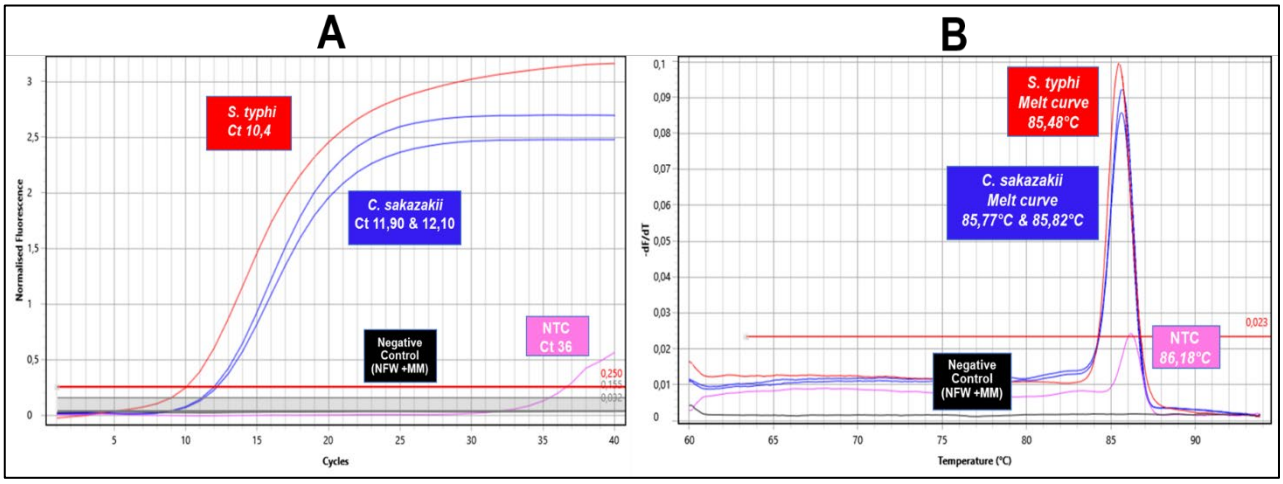

Fig 2. Amplification curve (Fig. 2A.) and melting temperature (Fig. 2B.) of a concentration 53 ng/µL DNA templated *C. sakazakii* bacterial stock culture, Positive control *fimC Salmonella typhi*, and negative control (NTC & NFW+MM).

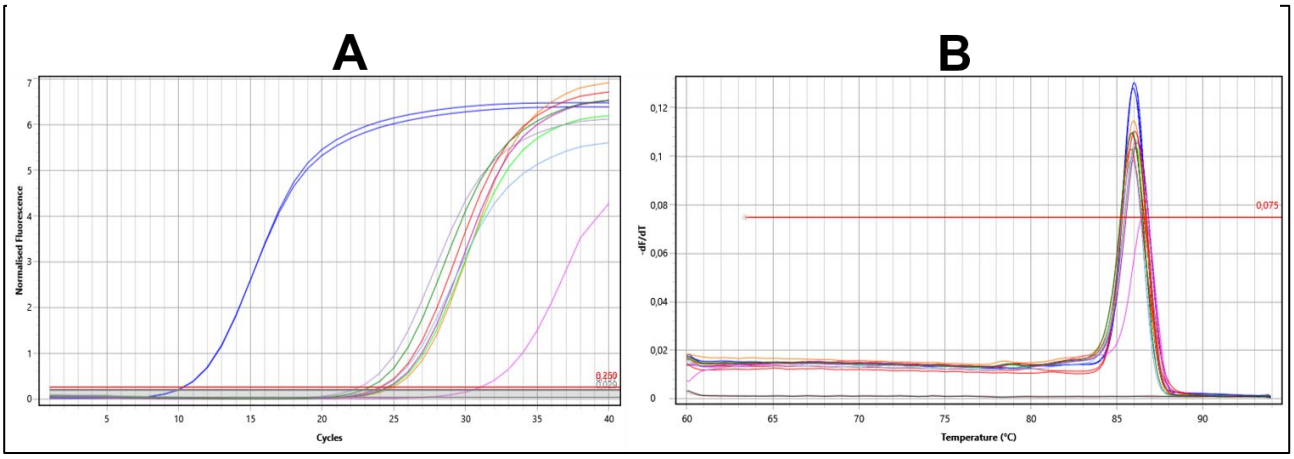

Fig 3. Amplification curve (Fig. 3A.) and meliting curve (Fig. 3B.) for the *C. sakazakii grxB* gene's primer specificity

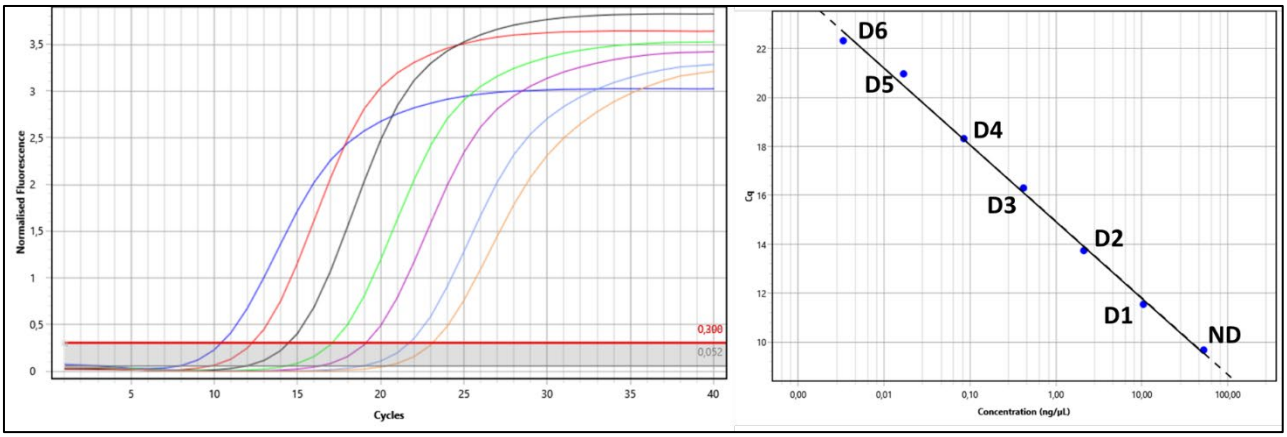

Fig 4. *C. sakazakii grxB* gene sensitivity test amplification curve and standard curve.

using nanodrop (Nanovue plus) with a result as described in Table 1.

Table 1. Value of purity and concentration of *Cronobacter sakazakii* DNA samples

Sample	Concentration (ng/µL)	Purity (A_{260}/A_{280})
C. sakazakii DNA isolated (lane 1 & 2)	85	1.840
C. sakazakii DNA isolated (lane 3 & 4)	53	1.853

The wavelength of A_{260}nm is used because the absorbance of DNA will be optimum as well as protein will absorb maximally at the wavelength of A_{280}nm. The requirements for the purity of a good DNA isolate are 1.8-2.0. There are impurities in the genetic material if it is less than 1.8 or greater than 2.0 [9]. Based on Table 1 it can be assumed that the pure culture results of isolated *C. sakazakii* produce good purity.

3.3 Optimization of *grxB* Primer Pairs Annealing Temperature

The stages in specific gene amplification by PCR are denaturation, annealing and extension. The annealing stage is an important step because it affects the efficiency of the PCR process [10]. DNA amplification by PCR requires a pair of primers (forward and reverse) to limit the area to be amplified. [11]. Smudge-free intact DNA bands have good density and brightness, indicating good amplification results [12]. based on our previous research, it has been found that the optimal annealing temperature for a pair of *grxB* primers is at 57°C to 61°C. This temperature range will be used in the real-time PCR procedure.

3.4 Real-time PCR amplification

3.4.1 Confirmation assay of Primer grxB

The molecular method is more efficient, more accurate, and more sensitive than the culture method for detecting *C. sakazakii*. In this study, we used real-time PCR to develop a detection method. real-time PCR data are the number of PCR cycles required to reach a given level of fluorescence (the "cycle threshold" Ct) [13]. A confirmation assay was carried out to find out that the *grxB* primer could recognize the target bacteria by amplifying the *grxB* DNA in the target bacteria *C. sakazakii*. The *grxB* gene primer with a concentration of 2 pmol was used at this stage with the target bacteria *C. sakazakii* which had a concentration of 53 ng/µL.

Confirmation assay resulted from that *grxB*-f and *grxB*-r primer pairs can amplify *C. sakazakii* DNA at Ct 11,90 and 12,10, as shown in Fig. 2A. The melting curve Fig. 2B. of *C. sakazakii* was at 85,77°C and 85,82°C. Non template control amplified ct 36 with the differences 24 cycles and different melt curve with low peak at 86,18 °C. The results of NTC amplification considered as non-target as evidenced by the difference in Ct/cycle distance between *C. sakazakii* and NTC target bacteria samples >10 cycles [14]. Furthermore, the melting curve results showed no mispriming. This means that the primers amplified only the target DNA as indicated by the formation of one peak [15].

3.4.2 Specificity and Sensitivity assay

The *grxB* primer pair was tested with non-target bacteria and showed good results, as shown in Fig. 3A. There are differences in ct values up to 12-24 different cycles between target and non-target bacteria. If the ct value between target and non-target bacteria has a range of 10 different cycles, then the non-target bacteria is considered as a negative control. [16]. *C. sakazakii* bacteria and non-target bacteria have a different melting curve (Fig. 3B and Table 2). From the Ct value and the melting curve, it can be concluded that the *grxB* primer is specific for detecting *C. sakazakii* bacteria.

Table 2. The results of the specificity test *grxB* primers

Line	Sample	Ct	Tm
	C. sakazakii	11.05	85.85
	V. parahaemolyticus	24.89	86.00
	V. alginolyticus	24.03	86.10
	L. monocytogenes	22.50	86.10
	S. typhi	24.47	86.19
	Y. enterocolitica	24.03	86.00
	S. aureus	24.13	85.50
	K. pneumoniae	23.35	85.50
	NTC	34	86.54

The sensitivity test aims to obtain a standard curve so that the Limit of Detection (LoD) of primer *grxB* can be identified in detecting *C. sakazakii* bacterial DNA. LoD is a parameter used to describe the sensitivity of an analytical method [17]. *C. sakazakii* isolate was diluted as much as five times of dilution.

Table 3. The results of the sensitivity test *grxB* primers

Line	Concentration (ng/µL)	Ct
	53	11.33
	10.6	13.16
	2.12	15.34
	0.424	18.01
	0.0848	20.04
	0.01696	22.64
	0.003392	24.06

This assay gave the results that *C. sakazakii* DNA template can still detect at a lower concentration of 0,003392 ng/µL or 3,392 pg/µL at 24,06 with LoD 0,0045 CFU/mL (Fig. 4 and Table 3). The standard curve is obtained by plotting the Ct value (y axis) against the transformation concentration of the target nucleic acid dilution (x axis). Standard curve has a regression value of R^2= 0,9964 and equation y = - 3,132 x + 14,93.

3.4.3 Confirmation Test on Food Samples with real-time PCR

The *grxB* primer pair can amplify *C. sakazakii* DNA in artificially contaminated formula milk samples at Ct 13 to 14 (Fig. 5A). The appearance of a sigmoid curve in

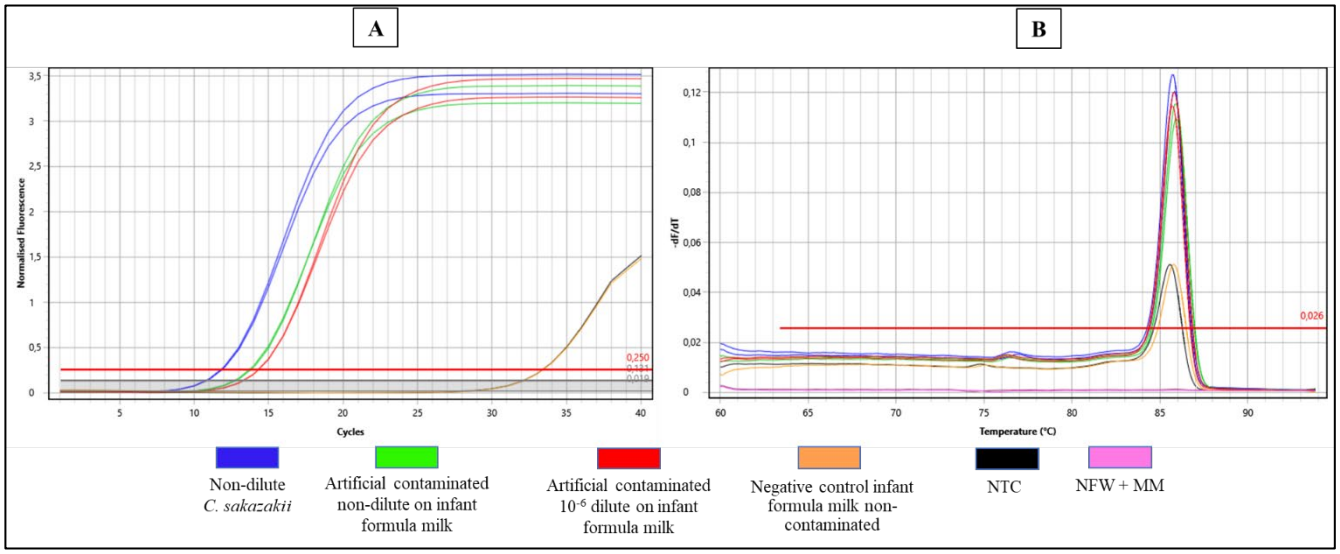

Fig 5. Amplification curve (Fig. 5A.) and melting curve (Fig. 5B.) confirmation assay in infant formula milk of *grxB* primer.

infant formula samples indicates that *C. sakazakii* is able to live and reproduce in formula milk samples so that bacterial DNA can be recognized with real-time PCR. *C. sakazakii* contamination in infant formula has been attributed to biofilms, which are communities of microorganisms that attach to biological or abiotic surfaces. *C. sakazakii* can form biofilms on food, food processing surfaces and equipment leading to cross-contamination [18]. The melting curve in Figure 5B shows that the pure culture sample and the artificially contaminated infant formula have nearly the same melting temperature. Real-time PCR results on artificially contaminated infant formula are presented in the table 4. These results can be concluded that this method can be developed as a detection model kit to detect *C. sakazakii* bacteria quickly, sensitively, and specifically and shows accurate results.

Table 4. The results of confirmation assay in infant formula milk with real-time PCR.

Line	Sample	*grxB*	
		Ct	Tm (°C)
	Pure culture of *C. sakazakii*	11.74 & 11.82	85.72 & 85.81
	Non-dilute bacterial + infant formula milk	13.76 & 13.64	85.90 & 85.93
	10^{-6} dilution bacterial + infant formula milk	14.27 & 14.26	85.81 & 85.71
	Infant formula milk without artificial contamination	33.36	85.78
	NFW+MM	-	-
	NTC	33.34	85.56

4 Conclusion

This research has successfully developed a detection method that is rapid, specific and accurate through real-time PCR method using a primer pair *grxB* for the *C. sakazaki*i bacteria in infant formula milk. *C. sakazakii* DNA with a concentration of 53 ng/µL could be amplified by the *grxB* gene primer pair with a Ct value of 12 and a Tm value of 85.7ºC. The *grxB* primer that we have designed is specific for detecting the target *C. sakazakii* bacteria and can detect *C. sakazakii* bacteria as low as 0.0045 CFU/mL or the equivalent of a sample DNA concentration of 3.392 pg/µL at Ct 24.06. Furthermore, the results of this study will be used for the development of a detection method for *C. sakazakii* in formula milk samples in validation tests to improve the detection kit that is being developed.

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