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PCR BASED GENE MARKER FOR QUICK DETECTION AND IDENTIFICATION OF *CRONOBACTER SAKAZAKII*

[1]Nikita Sharma, [2]Mansi Mahajan, [3]Alka Prakash

[1]Assistant Professor, [2]Research Scholar, [3]Professor

[1, 2, 3]Department of Zoology, Faculty of Science, Dayalbagh Educational Institute, Agra- 282005, U.P, India.

Abstract

Cronobacter species is acclaimed as one of the major causes of foodborne illness or food poisoning in humans. *Cronobacter sakazakii* is an opportunistic pathogen that can cause potentially fatal infections like meningitis, bacteremia, necrotizing enterocolitis and bloodstream infections mainly in newborns of less than four weeks of age and in adults with a weak immune system. This study targeted one housekeeping gene *GroEL*, two virulent genes *OmpA* and *Cpa*, a ribosomal gene *RpoB* and an enzyme encoding gene *CgcA* in order to develop a PCR based gene marker for rapid detection and identification of *C. sakazakii*. Our results showed that all the five selected genes *GroEL, OmpA, Cpa, RpoB* and *CgcA* amplified in case of *Cronobacter sakazakii* and all the ten isolates from milk and milk products while amplification of only two genes *GroEL* and *RpoB* was observed in *E. coli* and *S. typhimurium*. In this study, the genes *GroEL, OmpA, Cpa, RpoB and CgcA* were selected for identification of *C. sakazakii* as they are useful targets for bacterial identification and phylogenetic studies. The evaluation of the specificity of the primers with *C. sakazakii* and non-specific bacteria were carried out using BLAST program of NCBI. With the selected species-specific primers amplified product was observed *C. sakazakii*. This PCR based assay can be used to precisely identify *C. sakazakii*.

Keywords- *C. sakazakii*, PCR, *OmpA, GroEL, Cpa, RpoB, CgcA*

I. INTRODUCTION

Cronobacter spp. is a rod shaped Gram-negative, facultative-anaerobe. This non-spore forming enteric bacterium is motile by its peritrichous flagella [1]. *Cronobacter* species are opportunistic pathogens that can cause lethal infections in newborns and infants as well as in adults with low immunity [2]. The fatality rate of *Cronobacter* infections ranges from 40 to 80% which includes the clinical syndromes of necrotizing enterocolitis, bacteremia and meningitis [3].

Cronobacter spp. is widely distributed in the environment as it can survive in desiccation, under high heat conditions and high osmotic strain [4]. This bacterium has been isolated from a wide variety of foodstuff including dairy products, infant food, beverages, processed food, plants and spices, fresh produce such as salads, animal products such as poultry, shellfish, shrimp, beef and others [5, 6]. The natural habitat of *Cronobacter* is not known. Powdered Infant Formula (PIF) has been suspected as a vehicle of propagation in numerous cases of *Cronobacter sakazakii* infections in infants [7].

Presently, clinical diagnosis of *Cronobacter* infection is done by laboratory culture which is grown on routine culture media. Microbiological laboratories can detect *Cronobacter* from blood or cerebrospinal fluid (CSF) of

patients with sepsis or meningitis caused by these bacteria [8]. The most frequently isolated species of the *Cronobacter* genus is *Cronobacter sakazakii*. Its characterization is important for distinguishing the disease-causing strains for their epidemiological studies [9].

Cronobacter identification and sub-typing methods include culture based methods like use of chromogenic agars; biochemical detection methods like Voges-Proskauer, motility, production of gas from glucose and others; immune-based methods like ELISA; molecular detection methods like 16s rRNA gene sequencing, DNA-DNA hybridization; Sub-typing methods like PFGE, MALDI-TOF MS, sequencing methods like WGS, plasmid sequencing, MLST; biosensor based detection like fluorescence, calorimetric and electrochemical biosensing [10, 11, 12, 13, 14]. These methods are insufficient and controversial as they have time consuming protocols, are labor intensive and costly. The biosensor technology has not yet commercialized as the researchers are still in the phase of developing it and overcoming some of its limitations.

In the last few years various PCR methods have been devised for detection of *Cronobacter* spp. [15, 16, 17, 18, 19] but only a few can differentiate the various species within the genus *Cronobacter* appropriately. In the present study we have adopted the conventional PCR based method using stable gene markers for rapid detection and identification of *C. sakazakii*.

II. MATERIAL AND METHODS

2.1 Bacterial cultures

C. sakazakii (MTCC 2958) and isolates of *C. sakazakii* from milk and milk products, *E. coli* (MTCC 723) and isolates from water, *Salmonella typhimurium* (MTCC 1252) and isolates from water were used in this study. Standard microbial cultures were procured from Microbial Type Culture Collection and Gene Bank (MTCC) of microbial technology Chandigarh, India.

2.2 Media and Reagents

Nutrient Broth and EE Broth (*Enterobacteriaceae* Enrichment Broth) were procured from Himedia. PCR kit components like PCR buffer, dNTPs, $MgCl_2$, Taq polymerase and DNase RNase free water were from Genei. Superior Grade Agarose with low EEO was procured from SRL.

2.3 Reviving and streaking of bacterial cultures

All the cultures were revived by inoculating a loopful of culture into nutrient broth which was then incubated for 24 hours at 37℃. The revived culture of *C. sakazakii* was further enriched in specific enrichment broth, the EE Broth. This enriched culture of *C. sakazakii* was streaked on *Enterobacter sakazakii* Isolation Agar plate. Blue-green colonies of *C. sakazakii* were obtained.

2.4 Extraction of bacterial genomic DNA

[a] Genomic DNA extraction from *C. sakazakii*

DNA was extracted from overnight grown pure cultures in EE broth and nutrient broth. 1ml of this culture was boiled in a water bath at 100℃ for 10 minutes, then cooled by placing in finely crushed ice for 10 minutes and centrifuged at 1500g for 1 minute. 200µl supernatant of centrifuged cultures were used as the template. The extracted genomic DNA was estimated spectrophotometrically. The concentration of each sample was recorded at 260nm and their purity was ascertained by their absorbance at 260/280nm [20] (Figure 1). 1% agarose gel electrophoresis was done at 80V for 1 hour using TAE buffer.

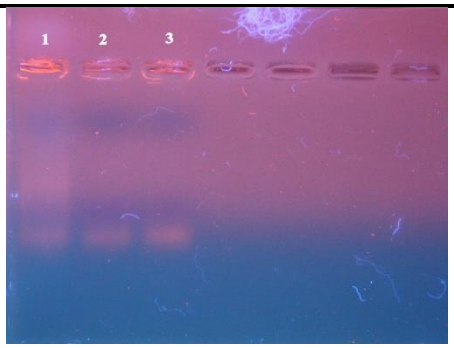

Figure:1. Detection of extracted genomic DNA on 1% agarose gel. Lane 1- genomic DNA of *C. sakazakii*, Lane 2-genomic DNA of *E. coli*, Lane 3- genomic DNA of *S. typhimurium*

2.5 Quick detection and identification of *C. sakazakii* through PCR

Amplification mixtures (total volume 15µl) containing 1X PCR buffer, 1.5mM $MgCl_2$, 2.5U enzyme Taq polymerase, 0.2mM dNTPs each, primers at 1µM each, 10ng of DNA were prepared. Sterile water was used as the blank. The reaction was carried out in SureCycler 8800 (Aiglent Technologies). The amplified products of *GroEL* and *OmpA* were separated on 2% Agarose gel and amplified products of *Cpa*, *CgcA* and *RpoB* were separated on 1.5% agarose gel followed by ethidium bromide staining [21]. The amplified products were visualized under U.V. light and further documented. PCR reaction mixture without DNA sample but an equal volume of distilled water was used as negative control.

Table 1- Primer, sequence and amplification condition

Primer [f/r]	Sequence 5'-3'	Amplicon [bp]	PCR conditions	Reference
GroEL-f	GGTAGAAGAAGGCGTGG TTGC	342bp	94°C, 3 min; 35 cycles at 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min. Final elongation 72°C for 10 min.	[22]
GroEL-r	ATGCATTCGGTGGTGATC ATCAG			
OmpA-f	GGATTTAACCGTGAACTTTTCC	469bp	94°C, 2 min; 35 cycles at 94°C for 15 sec, 53.3°C for 15 sec, and 72°C for 30 sec. Final elongation 72°C for 5 minutes.	[23]
OmpA-r	CGCCAGCGATGTTAGAAGA			
Cpa-f	CTAGGGCGATGATAGCTGCCTCCG	1015bp	94°C, 3 min; 35 cycles at 94°C for 30 sec, 57.7°C for 30 sec, and 72°C for 1 min. Final elongation 72°C for 10 min	[24]
Cpa-r	CTAGGGGGAACAGCCACGAGGAAA			
CgcA-f	GGTGGCGGGGTATGACAAAGAC	492bp	94°C, 3 min; 35 cycles at 94°C for 30 sec, 56°C for 30 sec, and 72°C for 1 min. Final elongation 72°C for 10 min	[25]
CgcA-r	GGCGGACGAAGCCTCAGAGAGT			
RpoB-f	ACGCCAAGCCTATCTCCGCG	514bp	94°C, 3 min; 35 cycles at 94°C for 30 sec, 57°C for 30 sec, and 72°C for 1 min. Final elongation 72°C for 10 min	[10]
RpoB-r	ACGGTTGGCGTCATCGTG			

*f: forward and r: reverse

III. RESULTS AND DISCUSSION

Detection of genes in *C. sakazakii*, *E. coli* and *S. typhimurium*

The five genes of interest in this study were *GroEL*, *OmpA*, *Cpa*, *CgcA* and *RpoB*. The amplified gene products are shown in Figures 7-11. Wells were loaded with PCR products from test samples (*C. sakazakii*, *E. coli* and *Salmonella typhimurium*) and the negative control. EtBr was added to the gel before electrophoresis followed by separation at 80V for 45 minutes.

C. sakazakii

- ***(a)*** *GroEL*- Fluorescent bands of size 342 bp were obtained at an annealing temperature of 55℃ (Figure- 2).
- ***(b)*** *OmpA*- A bright band of size 469bp was obtained at an annealing temperature of 53.3℃ (Figure- 3).
- **(c)** *Cpa*- A very fluorescent and thick band of size 1015 bp was obtained at an annealing temperature of 57.7℃ (Figure- 4).
- **(d)** *CgcA*- A brilliant band of size 492bp was obtained at an annealing temperature of 56℃ (Figure- 5).
- ***(e)*** *RpoB*- Bands of size 514bp were obtained at an annealing temperature of 57℃ (Figure- 6).

E. coli

- ***(a)*** *GroEL*- Fluorescent bands of size 342 bp were obtained at an annealing temperature of 55℃ (Figure- 2).
- ***(b)*** *RpoB*- Bands of size 514bp were obtained at an annealing temperature of 57℃ (Figure- 6).

S. typhimurium

- ***(a)*** *GroEL*- Fluorescent bands of size 342 bp were obtained at an annealing temperature of 55℃ (Figure- 2).
- ***(b)*** *RpoB*- Bands of size 514bp were obtained at an annealing temperature of 57℃ (Figure- 6).

The results are summarized in the Table 2.

Figure:2. Amplified product of 342bp was seen in various bacteria. Lane 1: 100bp ladder, Lane 2-3: Negative Control, Lane 4: 342bp product of *GroEL* gene in *C. sakazakii*, Lane 5:- 342bp product in *E. coli* and Lane 6:- 342bp product in *Salmonella typhimurium*

Figure:3. Amplified product of 469bp was seen in the various bacteria. Lane 1: 100bp ladder, Lane 2: Negative Control, Lane 3: amplified product of 469bp of *OmpA* gene in *C. sakazakii*, Lane 4 and 5: no amplified product of 469bp of *OmpA* gene in *E. coli* and *Salmonella typhimurium.*

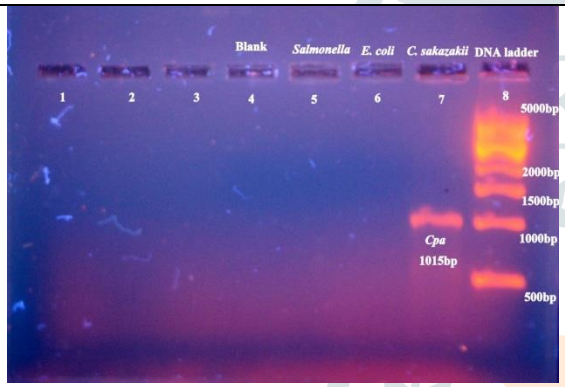

Figure:4. Amplified product of 1015bp was seen in the various bacteria. Lane 8: 500bp ladder, Lane 4: Negative Control, Lane 7: amplified product of 1015bp of *Cpa* gene in *C. sakazakii*, Lane 5 and 6: no amplified product of 1015bp of *Cpa* gene in *E. coli* and *Salmonella typhimurium.*

Figure:5. Amplified product of 492bp was seen in the various bacteria. Lane 2: 100bp ladder, Lane 3: Negative Control, Lane 4: amplified product of 492bp of *CgcA* gene in *C. sakazakii*, Lane 5 and 6: no amplified product of 492bp of *CgcA* gene in *E. coli* and *Salmonella typhimurium.*

Figure:6. Amplified product of 514bp was seen in the various bacteria. Lane 2: 100bp ladder, Lane 3: Negative Control, Lane 4: amplified product of 514bp of *RpoB* gene in *C. sakazakii*, Lane 5 and 6: amplified product of 514bp of *RpoB* gene in *E. coli* and *Salmonella typhimurium.*

Cronobacter sakazakii has been linked with disease infestations and occasional infections, notably in premature newborns and infants with weakened immunity. *Cronobacter sakazakii* can cause foodborne infections such as neonatal meningitis, necrotizing enterocolitis, bacteremia and septicaemia. Consequently, there is an urgent need to control and monitor the *C. sakazakii* in food, especially in powdered infant formula and other baby foods. Thus, in this study, a PCR assay has been developed for the rapid and accurate identification of *C. sakazakii*.

Identification based on PCR amplification of target genes is considered to be a reliable technique for detection of pathogens. With the distinct advantages of rapidity, specificity and sensitivity over culture based methods, many PCR assays for detection and validation of foodborne bacteria in food has been developed and applied.

In 2013, Wang *et al.* showed that the molecular chaperone *GroEL* can cause a strong immune response and is highly expressed in *C. sakazakii* [26]. Therefore, it can act as potential virulent target gene for molecular characterization of *C. sakazakii*. *C. sakazakii* and *C. malonaticus* are the only species with which neonatal deaths have been associated in most cases of infections till date. Holy *et al.* (2019) reported that *Cpa* gene was absent in all the clinically isolated 11 strains of *C. malonaticus* but present in all the clinically isolated 25 strains of *C. sakazakii* [23]. This gene can act as a distinctive biomarker between *C. sakazakii* and *C. malonaticus* strains which cannot be distinguished by the 16s rRNA gene. But further studies involving large number of isolates of *Cronobacter* isolated from clinical, food and environmental samples is still needed to confirm this fact. According to a study conducted by Arslan and Erturk (2021), at the genus level *OmpA* gene was present in all the 54 Cronobacter isolates (100%), *CgcA* gene was more successful than *RpoB* gene in identifying *Cronobacter* because out of the 64 suspected isolates *RpoB* gene amplified in 10 non-*Cronobacter* isolates while *CgcA* gene did not amplified in most of the *non-Cronobacter* isolates making *OmpA* and *CgcA* better targets for *C. skazakii* detection and identification [27].

In the present study *GroEL, OmpA, Cpa, CgcA* and *RpoB* genes were selected from literature for identification of *C. sakazakii*. These have been employed as useful targets for bacterial identification and phylogenetic classification (Table 2). A conventional PCR system was developed to enable strains of *Cronobacter* to be discriminated up to the species level.

One of the genes of interest in this study *GroEL,* is reported as a housekeeping gene which remains conserved across *Enterobacter* species and is used for the classification and study of phylogenetic relationships among *Cronobacter* species. The second gene of interest tested was the outer membrane protein A (*OmpA*) gene. It is apparently the best characterized virulence marker of *Cronobacter* [23, 28]. *OmpA* is involved in colonization of gastrointestinal tract by *C. sakazakii*. *OmpA* also plays a critical role in penetration of Blood Brain Barrier [29]. *Cronobacter* plasminogen activator (*Cpa*) is the third gene of interest which encodes a protease that provides resistance against bactericidal activity of serum and helps in spreading and invading *C. sakazakii* into the host organism. In 2009, Jaradat *et al.* reported that all of the *Cronobacter* spp. harbored *OmpA* gene followed by virulence gene *Cpa* (present in only 98% of *Cronobacter* spp.) [30]. RNA polymerase beta subunit (*RpoB*) gene is reported as a new molecular marker for identification of *Cronobacter* species. This genus is more easily discriminated by *RpoB* than 16S rRNA. The fifth gene of interest is cyclic diguanylate (*CgcA*) gene. It is recognized as second messenger during signal transduction which is involved in complex physiological processes such as virulence, biofilm formation and persistence under unfavorable conditions. This gene is reported as 100% specific and 100% sensitive for identification of *Cronobacter* sp. strains [25]. Further researches can be done to develop similar PCR kits for other foodborne pathogens.

IV. CONCLUSION

This study demonstrates the development of a PCR assay that can be used to identify *C. sakazakii* precisely. This PCR assay here can be used as an important tool for detecting and identifying suspected *C. sakazakii* isolates from clinical, environment and food samples, rapidly and precisely.

Table 2:- Presence/absence of the selected genes in the various bacteria.

Gene	Presence of selected gene		
	***C. sakazakii* isolates+ *C. sakazakii* [MTCC 2958]**	***E. coli* isolates+ *E. coli* [MTCC 723]**	***Salmonella typhimurium* isolates+ *S. tyhimurium* [MTCC 1252]**
GroEL	+	+	+
OmpA	+	-	-
Cpa	+	-	-

CgcA	+	-	-
RpoB	+	+	+

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