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Rapid Detection of *Enterobacter sakazakii* Using TaqMan Real-Time PCR Assay

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Abstract *Enterobacter sakazakii* is an emerging food pathogen, which induces severe meningitis and sepsis in neonates and infants, with a high fatality rate. The disease is generally associated with the ingestion of contaminated infant formula. In this study, we describe the development of a real-time PCR protocol to identify *E. sakazakii* using a TaqMan probe, predicated on the nucleotide sequence data of the 16S rRNA gene obtained from a variety of pathogens. To detect *E. sakazakii*, four primer sets and one probe were designed. Five strains of *E. sakazakii* and 28 non-*E. sakazakii* bacterial strains were used in order to ensure the accuracy of detection. The PCR protocol successfully identified all of the *E. sakazakii* strains, whereas the 28 non-*E. sakazakii* strains were not detected by this method. The detection limits of this method for *E. sakazakii* cells and purified genomic DNA were 2.3 CFU/assay and 100 fg/assay, respectively. These findings suggest that our newly developed TaqMan real-time PCR method should prove to be a rapid, sensitive, and quantitative method for the detection of *E. sakazakii*.

Keywords: *Enterobacter sakazakii*, real-time PCR, 16S rRNA, rapid detection

Enterobacter sakazakii is a Gram-negative, nonsporulating, motile, rod-shaped bacterium, belonging to the *Enterobacteriaceae* family [15, 25]. This bacterium has been implicated in several illnesses in premature babies and neonates, including meningitis, sepsis, and enteritis [2, 24]. *E. sakazakii* has previously been isolated from rehydrated powdered infant formulas, and has also been detected on equipment used in the preparation of powdered milk-based infant formula [4]. *E. sakazakii* has additionally been found in cheese, minced beef, sausage, and vegetables [10]. One study evaluated 141 powdered formula samples obtained from 35 countries and reported levels of *E. sakazakii* contamination ranging from 0.36 to 66 CFU/100 g [19]. Even minimal *E. sakazakii* contamination in baby food or infant formula can result in fatal disease in sensitive babies [7, 20]. Furthermore, some cases of *E. sakazakii* infections have also been reported in adults [5]. A great deal of effort has been invested in the search for methods to eliminate *E. sakazakii* infection [4]. However, the sources of contamination, ecology, and virulence characteristics of this organism remain poorly understood [4, 6].

In order to reduce or prevent the hazards posed by *E. sakazakii*, it is first necessary to develop an accurate, rapid, and highly sensitive detection method for the identification of *E. sakazakii* in foods. A biochemical method [9] is currently in broad use for the identification of *E. sakazakii*, but this technique is both time and labor intensive. The polymerase chain reaction (PCR), predicated on DNA amplification, is recognized as an extraordinarily useful tool in molecular diagnostics. PCR methods tend to be both more sensitive and more rapid than biochemical methods conducted with individual colonies [3, 11, 21]. Recently, real-time PCR assays using the TaqMan probe have been increasingly exploited [8, 22], and in many cases, these assays have replaced conventional PCR methods. Real-time PCR assays are characterized by a wide dynamic range of quantification, high sensitivity, and a high degree of precision [13]. This technique also saves time, as it obviates the necessity for gel electrophoresis, and eliminates the need to open the reaction tubes during the procedure, thus decreasing the risk of carry-over contamination [3].

Two real-time PCR methods based on the 16S-23S rDNA internal transcribed spacer region [16] and the *rpsU-dnaG* intergenic sequence [23], respectively, were developed to detect *E. sakazakii* in infant formula. The majority of bacteria harbor multiple ribosomal operons at different

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Table 1. Primers and probe sequences for the detection and identification of *E. sakazakii*.

Primers and probe	Sequence (5' to 3')	Amplicon size (bp)
sF1	TAACAGGGAGCAGCTTGCTGCTCTG	426
sR3	CGGGTAACGTCAATTGCTGCGGT	
sF1	TAACAGGGAGCAGCTTGCTGCTCTG	960
sR5	AAGGCACTCCCGCATCTCTGCA	
sF1	TAACAGGGAGCAGCTTGCTGCTCTG	1,031
sR6	AGTCTCCTTTGAGTTCCCGGCCGA	
sF4	CAATTGACGTTACCCGCAGAAGAA	560
sR5	AAGGCACTCCCGCATCTCTGCA	
probe	CCGCATAACGTCTACGGACCAAA	

positions on the chromosome [1, 12, 17]. 16S rDNA sequence variations are one of the more popular targets for microbial identification [14, 18, 26]. Until now, the real-time PCR system based on 16S rRNA sequence for *E. sakazakii* detection has not yet been reported. In this study, we described a newly-developed TaqMan real-time PCR assay, based on 16S rDNA sequences, for the rapid, sensitive, and quantitative detection and identification of *E. sakazakii*.

The 16S rDNA sequences of *E. sakazakii* and non-*E. sakazakii* strains were aligned using the ClustalW multiple sequence alignment program, and the conserved regions and variable sequences were determined (data not shown). The primers and probe sequences were analyzed for GC content, Tm value, and primer-dimer formation, using the Primer Express 1.5 program (Applied Biosystems, Foster City, CA, U.S.A.). The probe was labeled with 6-carboxyfluorescein (FAM) as the 5' reporter dye, and quenched with 6-carboxytetramethylrhodamine (TAMRA) at the end.

The 16S rDNA sequences of the *E. sakazakii* strain and the 12 non-*E. sakazakii* strains were obtained from the NCBI's GenBank database. Their accession numbers are as follows: *E. sakazakii* ATCC 29544 (AY803187), *Shigella sonnei* (AY282825), *Yersinia enterocolitica* (AE017130), *Salmonella typhimurium* (AM050347), *Listeria monocytogenes* (AJ417488), *Escherichia coli* (AF550670), *Vibrio parahaemolyticus* (DV181249), *Vibrio cholerae* (AF179596), *Vibrio vulnificus* (DV179578), *Clostridium perfringens* (DQ117160), *Bacillus cereus* (AY920317), *Staphylococcus aureus* (AY894415), and *Camphylobacter jejuni* (AF301164).

Real-time PCR reactions were performed in 20-μl volumes containing 300 nM of each primer, 200 nM of probe, 4 μl of template DNA, 10 μl of 2× TaqMan universal master mix, and deionized water. The PCR reactions were run on an ABI Prism 7700 system (Applied Biosystems, Foster City, CA, U.S.A.), using the following program: 1 cycle of 2 min at 50°C and 10 min at 94°C, 40 cycles of 15 sec at 94°C, and 1 min at 60°C. The results were subsequently analyzed using the Sequence Detection System (Applied Biosystems, Foster City, CA, U.S.A.). Samples were quantified by comparison to a standard regression curve of Ct (threshold cycle at which a significant increase in signal is first detected) values generated from samples of known concentrations. If the Ct value was in excess of 35, no amplification was considered to have occurred. All PCR reactions were conducted in triplicate.

The standard curve for the purified genomic DNA was generated from samples with known quantities of DNA, in a range from 100 fg to 1 ng. The Ct values were plotted relative to the corresponding serial 10-fold dilutions of the template DNA extracted from the pure culture. The standard curve for the *E. sakazakii* cells was constructed using DNA recovered from logarithmic culture broth samples with varying cell numbers. Simultaneously, the number of *E. sakazakii* cells in each of the 10-fold dilutions was determined by plate counting. All determinations were conducted in triplicate. The Ct values were plotted against the colony forming units (CFU).

The alignment of the 16S rDNA sequences of the one *E. sakazakii* and 12 non-*E. sakazakii* strains revealed some regions that appeared suitable for the design of the *E. sakazakii*-specific primers and probe (Table 1). The specificity of the four primer sets was evaluated via the amplification of genomic DNA prepared from five *E. sakazakii* strains and 28 non-*E. sakazakii* strains. A conventional PCR assay, using four primer sets, generated positive reactions for all the *E. sakazakii* strains tested, but not for any of the 28 non-*E. sakazakii* strains (Tables 2 and 3). The sF1/sR3, sF1/sR5, sF1/sR6, and sF4/sR5 primer sets generated PCR amplicons of 426, 960, 1,031, and 560 base pairs, respectively. The sF1/sR3 primer set, which yielded the smallest amplicon, was finally selected and employed in the subsequent real-time PCR assays.

The specificity of the sF1/sR3 primer set and probe was tested against five *E. sakazakii* strains and 28 non-*E. sakazakii* strains. No fluorescent signals were observed in conjunction with any of the tested non-*E. sakazakii* strains, even after 40 cycles (Tables 2 and 3). This finding indicates that the selected primers and probe were useful for specific detection of *E. sakazakii*.

The sensitivity of detection inherent to our real-time PCR assay was evaluated using a template DNA that had

Table 2. Selectivity of conventional PCR and real-time PCR assays for the detection of *Enterobacter* strains.

Strain	Origin	Conventional PCR				Real-time PCR
		sF1/sR3	sF1/sR5	sF1/sR6	sF4/sR5	
E. sakazakii	ATCC[a] 29544	+	+	+	+	+
E. sakazakii	ATCC 29004	+	+	+	+	+
E. sakazakii	ATCC 51329	+	+	+	+	+
E. sakazakii	ATCC 12868	+	+	+	+	+
E. sakazakii	KCTC[b] 2949	+	+	+	+	+
E. cloacae	KCTC 1685	–	–	–	–	–
E. cloacae	KCTC 1943	–	–	–	–	–
E. cloacae	KCTC 2361	–	–	–	–	–
E. cloacae	KCTC 2519	–	–	–	–	–
E. leracium	KCTC 2873	–	–	–	–	–

[a]American Type Culture Collection.
[b]Korean Collection for Type Cultures. PCR results: +, positive; –, negative.

been isolated from overnight cultures of *E. sakazakii* ATCC 29544. The real-time PCR assays were conducted using ten-fold dilutions of column-purified genomic DNA (100 fg to 1 ng/assay). An inverse relationship was detected between the Ct values and the template DNA concentrations. The minimum detectable limit of the genomic DNA assays was 100 fg, and the corresponding Ct value was 30.96±0.49 (Table 4). We also determined the detection limit of the real-time PCR assay, using template DNA prepared from ten-fold serial dilutions (2.3×10^0 to 2.3×10^4 CFU/tube) of the same *E. sakazakii* strain. Ten-fold dilutions of a logarithmic culture of *E. sakazakii* ATCC 29544 were utilized as templates in the assay, and were plated on LB agar in parallel, in order to enable a bacterial CFU count. A minimum detectable limit of 2.3×10^0 CFU/tube was determined when using the *E. sakazakii*-specific primer set sF1/sR3 and probe. The Ct value corresponding to 2.3×10^0 CFU was 30.29±0.37 (Table 4) and no Ct value was detected for the negative control, which contained no template DNA.

Table 3. Non-*E. sakazakii* bacterial strains evaluated by conventional PCR and real-time PCR.

Strain	Origin	PCR sF1/sR3	Real-time PCR
Bacillus cereus	ATCC[a] 49063	–	–
Bacillus cereus	KCCM[b] 11204	–	–
Bacillus licheniformis	KCTC[c] 3006	–	–
Bacillus subtilis	KCTC 2213	–	–
Campylobacter jejuni	KCCM[c] 41773	–	–
Clostridium perfringens	KCTC 3269	–	–
Escherichia coli O157:H7	ATCC 43894	–	–
Escherichia coli W3110		–	–
Listeria monocytogenes	ATCC 19113	–	–
Listeria monocytogenes	ATCC 35152	–	–
Salmonella enteritica	ATCC 10376	–	–
Salmonella enteritidis	ATCC 13076	–	–
Salmonella typhimurium	ATCC 14028	–	–
Salmonella typhimurium	ATCC 29629	–	–
Salmonella typhimurium	KCTC 2421	–	–
Shigella flexneri	KCTC 2517	–	–
Shigella sonnei	KCTC 2518	–	–
Staphylococcus aureus	KCTC 2199	–	–
Staphylococcus aureus	ATCC 65389	–	–
Vibrio cholerae	KCCM 41626	–	–
Vibrio vulnificus	KCTC 2980	–	–
Vibrio parahemolyticus	KCCM 11965	–	–
Yersinia enterocolitica	KCCM 91657	–	–

[a]American Type Culture Collection.
[b]Korean Culture Center of Microorganisms.
[c]Korean Collection for Type Cultures. PCR results: +, positive; –, negative.

In a recent study, two studies using methods for the rapid detection of *E. sakazakii* via TaqMan real-time PCR were reported. When the 16S-23S rDNA internal transcribed spacer (ITS) region was employed as the target DNA [16], the detection limit was found to be 1.8×10^1 CFU/tube, with a corresponding Ct value of 36.51. In the second report, the detection limit for the *rpsU-dnaG* intergenic sequence was found to be 1.0×10^2 CFU/ml, with a corresponding Ct value of 45 [23]. Our method resulted in a comparable detection limit of 2.3×10^0 CFU/tube at a Ct value of 30.29±0.37.

Table 4. Detection limits for detection of *E. sakazakii* in pure culture.

E. sakazakii	
CFU/assay (Ct[a]±SD)	fg DNA/assay (Ct±SD)
2.3×10^4 (16.55±0.11)	1.0×10^6 (17.46±0.41)
2.3×10^3 (20.08±0.52)	1.0×10^5 (20.14±0.52)
2.3×10^2 (23.48±0.83)	1.0×10^4 (24.79±0.48)
2.3×10^1 (26.54±0.45)	1.0×10^3 (27.20±0.44)
2.3×10^0 (30.29±0.37)	1.0×10^2 (30.96±0.49)

[a]The Ct values are means±standard deviations for 3 independent experiments.

In summary, we have developed a TaqMan real-time PCR assay based on 16S rDNA sequences. Our method was shown to be able to detect as little as 100 fg of *E. sakazakii*-specific DNA, or 2.3 CFU per assay. This real-time PCR assay technique proved to be both specific and sensitive and may in the future be used for the rapid detection of *E. sakazakii* in infant formula and other foods.

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