

Identification and Virulence of *Enterobacter sakazakii*

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Abstract

Enterobacter sakazakii (formerly known as *Cronobacter sakazakii*) is an opportunistic pathogen that causes necrotizing enterocolitis, bacteremia and meningitis, especially in neonates. This study was an attempt to isolate *E. sakazakii* from different food and environmental sources so as to establish its presence and possible source of transmission. For this, 93 samples (i.e., 37 from dairy and 56 from non-dairy) were collected using standard procedures of sampling. Out of these samples, 45 isolates (i.e., 14 from dairy and 31 from other samples) were taken further on the basis of growth on tryptic soy agar. On further screening on the basis of Gram staining, catalase and oxidase test, only 27 isolates were observed to be positive. The positive isolates were also subjected to PCR based identification using species specific primers. Overall, 11 isolates confirmed as *E. sakazakii* were also tested for virulent characteristics (i.e., hemolytic activity, haemagglutination test and DNase production) and all the isolates were found to be positive showing a potential threat of infection through food commodities.

Keywords: *Enterobacter sakazakii*; Cronobacter; Virulence

Introduction

Enterobacter sakazakii, formerly known as *Cronobacter sakazakii* is a new genus consisting of six genomospecies [1]. The mortality rate of the infants, who develop *Cronobacter* associated neonatal meningitis, is up to 80% [2]. Moreover, infections in elderly and immunocompromised patients have also been. A survey has shown that *Cronobacter spp.* were isolated from 27 products worldwide. Among these, 12% infant foods and drinks were *Cronobacter*-contaminated [3,4]. Both, the source and vehicle of transmission of *Cronobacter* are not always quit clear. The surveillance studies have detected *Cronobacter* in various foods, households, and environmental sites [5]. However, powdered infant formula (PIF) has been epidemiologically linked to the cases of infants' infection [5]; therefore more research has focused on PIF based products. It is assumed that *Cronobacter* contaminate infant foods particularly after pasteurization, including drying and packing, or during vitamins or supplement fortification. Due to the organism's ability to resist drying or osmotic [6-8], *Cronobacter* can persist in PIF for long periods. To establish the routes of contamination from environmental and food sources, researchers isolated *Cronobacter spp.* from dried-milk and related environments [9,10]. Hein et al. [11] and Craven et al. [12] reported the spatial distribution, prevalence and persistence of *Cronobacter* in the environments of milk powder factories. This revealed that the supply air is a potential vehicle for extrinsic contamination and confirmed that *Cronobacter* is widely dispersed in milk powder making factories. Acidification reduced the concentration of *E. sakazakii* in different types of infant formula and vegetable based food products [13-15]. Kim and Beuchat [16] investigated the survival and growth characteristics of *E. sakazakii* on fresh-cut apple, cantaloupe, strawberry, watermelon, cabbage, carrot, cucumber, lettuce, and tomato and in juices prepared from these fruits and vegetables. In juices of vegetables, the reduction of pH after 48 h was correlated with a reduction of the numbers of *E. sakazakii*, but with increasing numbers of *E. sakazakii* in juices of different fruits. Mullane et al. [17] also reported microbial contamination in air filters and proposed that these are linked to contaminated product in powdered milk protein-processing facilities. *C. sakazakii* outer membrane protein A (OmpA) was identified as a major fibronectin-binding protein which plays a significant role in the adherence of this gastrointestinal pathogen to neonatal and immunocompromised hosts [18]. Harouna et al. [19] also reported antibacterial effect of bovine lactoferrin on *C. sakazakii* is mainly due to iron sequestration. However, iron-saturated bovine

lactoferrin showed some effect by reducing the viability of *C. sakazakii* in whey. However, the possible contamination sources and the influence of different processing activities on the contamination levels are not well characterized with respect to the presence of *Cronobacter* in the infant formulae. Therefore, present work was undertaken to evaluate the possible sources of *Cronobacter*.

Materials and Methods

Collection of samples

The faecal samples were taken from the local hospital, Karnal, plant sample like vegetables and fruits, dahi, pasteurized milk; skim milk powder (SMP) and dairy whitener were taken from the local market of Karnal and Bangalore. The cattle and buffalo milk, pasteurized milk samples, paneer, cheese and lassi were taken from cattle yard and Student Experimental Dairy Plant, ICAR-NDRI Karnal. The flours commonly used in the preparation of infant milk formulae like wheat, rice, barley, oat, bajra, soybean, pearl millet, corn and Bengal gram were also taken from the local markets of Karnal, Delhi and Bangalore. A total of 93 different categories of samples including 37 of dairy and 56 of non-dairy were collected and tested for the presence of *E. sakazakii*.

Isolation of *E. sakazakii*

The samples were tested for the presence of *E. sakazakii* with slight modifications as described by Kandhai et al. with brief pre enrichment of samples. For this, 25 g of sample was added to 225 ml buffered peptone water (pH 7.2) and incubated at 37°C for 24 h. After pre-enrichment, 10 ml of pre-enriched sample was used to inoculate 90 ml of Enterobacteriaceae enrichment broth and incubated at 37°C for 24 h. After incubation, a loopful of enriched culture was streaked on VRBG

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(Violet Red Bile Glucose) agar and the plates and re-incubated at 37°C for 24 h. Typical coliform colonies on VRBG agar were streaked on TSA (Tryptic Soya Agar) and the yellow colonies on TSA were selected and tested for Gram's reaction, oxidase and catalase activities. Selective HiCrome *Enterobacter sakazakii* HiVeg Agar was used to identify *E. sakazakii*. Along with these a standard culture of *E. sakazakii* MTCC-659 procured from Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India was also tested.

Identification of *E. sakazakii*

Presumptive identification of isolates was done by API 20E kits (Remel and/or BioMerieux, USA). For that oxidase-negative yellow colonies from TSA were taken. Confirmation was also done by PCR using species specific primers (Table 1). The isolation of DNA was done by Pospiech and Neumann, with some modifications. PCR amplification for detection of *E. sakazakii* was optimized in a 25 µl reaction mixture consisted of 1.0 µl of bacterial genomic DNA, 10X Taq assay containing 15 mM MgCl₂ buffer (2.5 µl), dNTPs (0.5 µl), Taq polymerase (0.25 µl) and primers (0.5 µl). Thermal cycling parameters were as follows: initial denaturation at 94°C for 2 min followed by denaturation at 94°C for 30 sec, annealing at 68°C for 1 min, extension at 72°C for 1.5 min followed by final extension at 72°C for 5 min. The products of PCR (5-µl aliquot) were analyzed by 1% (w/v) agarose gel electrophoresis in 1× TAE buffer (40 mM Tris-HCl, 1.18 ml acetic acid, 2 mM EDTA, pH 8.0) and a constant voltage of 80 V for 45 min to confirm the presence of the amplified DNA.

Virulence

Haemolytic activity

Biochemically confirmed *E. sakazakii* isolates were first evaluated for haemolytic activity using blood agar plates. Overnight grown cultures were streaked on agar plates and incubated at 37°C for 3-4 days [20]. The appearance of zone of clearance around the colony, if any, due to the breakdown of Red Blood Cells (RBC) by the metabolites released by organisms indicated haemolysis and the positive isolates were selected.

Haemagglutination test

Bovine blood was collected using anticoagulant and washed in three volumes of RBC diluent by mixing and centrifuging at 1500 rpm for 15 min and aspirating until the supernatant was completely clear. The pelleted cells were suspended to a 50% suspension in diluent and stored at 4°C and used within 2 weeks. One hundred µl of both the RBC suspension and the test culture were mixed and observed for agglutination after 10 min. Control suspension remained homogenous.

The method used to test haemagglutination was that described by Adegbola and Old [21]. The cultures grown overnight were centrifuged at 2,000 × g for 15 min. The pellet was re-suspended in physiological saline (0.85% NaCl). The test was performed by mixing equal volumes (30 µl) of erythrocytes and cell suspension on a glass slide. It was undisturbed for 10 min and agglutination was observed under microscope.

DNase production

To test the production of DNase, the isolates were streaked on DNase test agar and incubated at 37°C [22]. The surface of the plates was flooded with 1 N HCl. Colonies producing DNase hydrolyze the DNA in this medium located in close proximity. The medium is flooded and acidified with 1 N HCl, the DNA precipitates and clear zones appear around DNase-positive colonies.

Results and Discussion

Different samples including dairy, non-dairy and environmental samples were screened, followed by isolation and identification of *E. sakazakii*. A wide variation was observed in the morphology of their colonies (Figure 1A). Some colonies were comparatively bigger in size, bright lemon yellow with rough surface (Figures 1B and 1C). These colonies were somewhat dry, hard and sticky to the agar surface. While the others were small golden yellow colonies with smooth surface. Some were opaque and dull and others were shiny, translucent. Out of 93 samples, 45 samples including 14 (37%) dairy and 31 (55%) non-dairy samples showed the prevalence of *E. sakazakii*. Among 4 commercial infant food formulas tested, 2 samples showed the presence of *E. sakazakii* (Table 2). The selected cultures were identified as *E. sakazakii* with 99.4% ID of RW 3 (Rain Water), 95.5% ID of SMP3 (Skim Milk Powder), 93.6% ID of IMF2 (Infant Milk Formula), 92.2% ID of AS4 (Amul Spray). The standard culture MTCC 659 demonstrated 99.7% ID similarity with *E. sakazakii* (Figure 2). All the 11 isolates were identified as *E. sakazakii* by species specific PCR with an amplified product size of 282 bp together with MTCC 659 (Figure 3). The obtained results in this regard are in complete agreement with Liu et al. [23] who demonstrated the amplification of 282 bp product in the PCR identification of *E. sakazakii*. In the present study all the 11 identified isolates of *E. sakazakii* (S3 (Soil), RM1 (Raw Milk), O1 (Oat), Ba1 (Bread), Cu5 (Cucumber), SMP3, T2 (Tomato), RW3, AS4, FS2 (Faecal Sample), and IMF2 showing β-haemolysis with clear zone on bovine blood agar plates (Figure 4A), were positive for haemagglutination test and also showed delayed DNase production (Figure 4B).

Overall, results revealed the presence of *E. sakazakii* in infant formulae, raw milk, skim milk powder and non-dairy samples [24]. The occurrence of the organism in the infant milk powder indicates it's likely post processing contamination from wash water, equipment's, and utensils or possibly from the infected handlers. The presence of

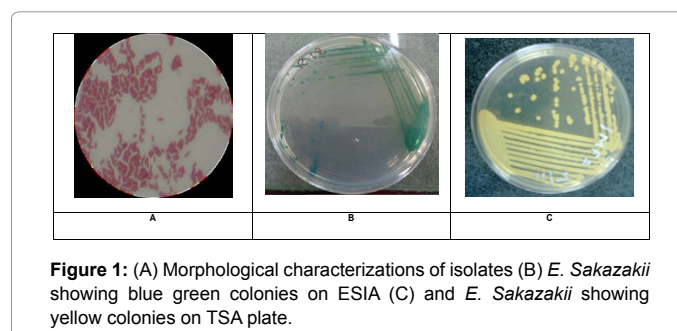


Figure 1: (A) Morphological characterizations of isolates (B) *E. Sakazakii* showing blue green colonies on ESIA (C) and *E. Sakazakii* showing yellow colonies on TSA plate.

Organism and species	Primer	Target region	Primer sequence	Product size	Reference
<i>E. sakazakii</i>	SG-F	ITS-G	GGGTTGTCTGCGAAAGCGAA	282	Liu et al. [23]
	SG-R	ITS-G and ITS-IA	GTCTTCGTGCTGCGAGTTTG		

Table 1: Species specific primers for *E. sakazakii*.

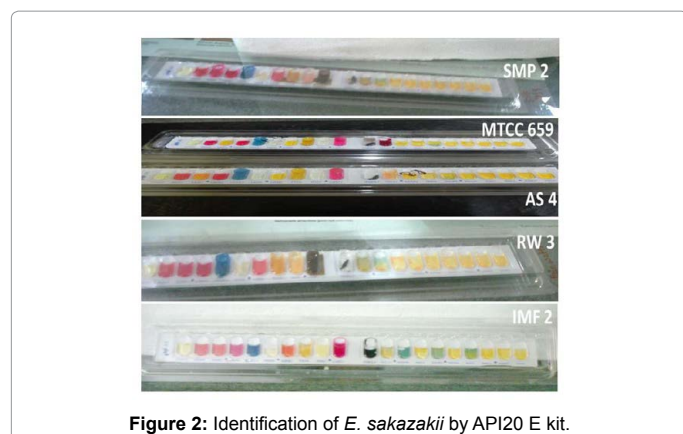


Figure 2: Identification of *E. sakazakii* by API20 E kit.

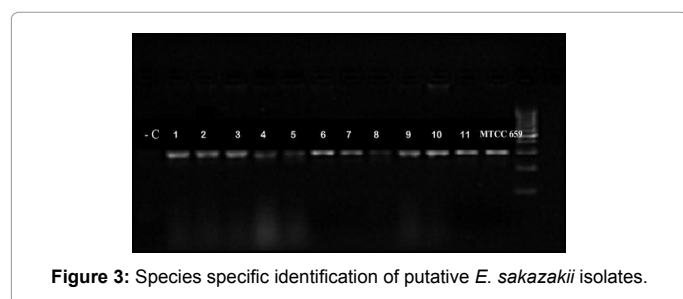


Figure 3: Species specific identification of putative *E. sakazakii* isolates.

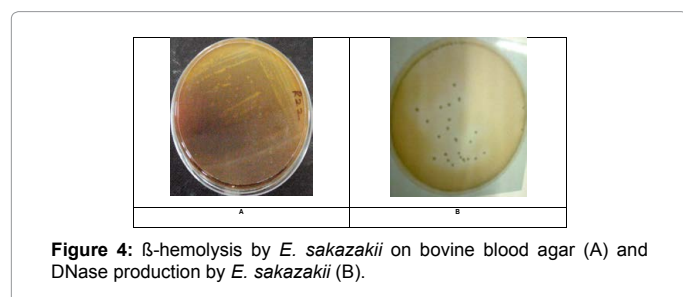


Figure 4: β -hemolysis by *E. sakazakii* on bovine blood agar (A) and DNase production by *E. sakazakii* (B).

the organism in infant milk powder alerted us about their use for the neonates and the young ones. The occurrence of the organism in the stools of neonates and raw milk also causes concern about the overall sanitary conditions prevailing at different levels of production, processing, transportation, storage and consumption that have so far not reported earlier.

Conclusions

The present study is likely to have a far reaching implication in quality assurance measures for foods, particularly of the dairy origin in India. However a detailed study about the interaction among different virulence determinants of Cronobacter is needed, aside from its pathogenesis, prevention, prophylaxis and control before reaching a real conclusion.

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Sample	Source(s)	Number of samples	Samples showed growth on TSA
Infant formulae	Karnal market	4	2
Milk powders	Karnal market, Experimental dairy plant and Bangalore	6	4
Raw milk	Experimental dairy plant	4	2
	Cattle yard	4	2
Pasteurized milk	Experimental dairy plant	3	Nil
	Other organized dairies	6	2
Pond/ lake/ Rain water	Dairy Technology/ Microbiology Division; Karn lake	3	3
Vegetables	Tomato	Karnal and ICAR-NDRI market	6
	Carrot		2
	Cabbage		3
	Cucumber		6
	Green leaves		4
Fermented products	Bread	Karnal and ICAR-NDRI market	3
	Dahi		6
Flours	Karnal market, Delhi, Bangalore	11	8
Cheese	Experimental dairy plant	2	Nil
Soil	Hostel, ICAR-NDRI	3	2
Paneer	Experimental dairy plant	2	Nil
Faecal sample	Civil hospital, Karnal	15	8

Table 2: Collection of dairy, non-dairy and environmental samples and screening for the presence of *E. sakazakii*.

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