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SHORT COMMUNICATION

Detection of *Cronobacter sakazakii* in Samples of Milk and Milk Products by PCR Method

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Abstract

Cronobacter sakazakii is a food borne pathogen that causes severe meningoencephalitis, necrotizing enterocolitis and sepsis in neonates and infants, with a high mortality rate. It is ubiquitous and is also prevalent in milk and milk products. In the present study 46 samples of milk and milk products from Agra city were collected and analysed. Out of 200 isolates obtained, 6 were biochemically detected as *C. sakazakii*. The PCR based on *omp A* gene was used to amplify a 496bp DNA fragment specific to *C. sakazakii*, in order to confirm *C. sakazakii* isolates. The study showed that traditional methods take upto 7 days to identify *C. sakazakii*, whereas PCR combined with enrichment culturing, is a rapid alternative method.

Keywords

Cronobacter, Milk, Polymerase Chain Reaction.

Introduction

Enterobacter sakazakii previously referred to as 'yellow pigmented' *E. cloacae* has been defined as a new species in 1980 (Farmer *et al*, 1980) and 15 biogroups have been described based on biochemical characterization. DNA-DNA hybridization revealed several genome species (Iversen *et al*, 2007) and subsequently, led to classification of these bacteria into six species within the new genus, *Cronobacter* (Iversen *et al*, 2008). *C sakazakii* is a gram negative, rod shaped opportunistic pathogen that causes bacteraemia, necrotizing enterocolitis and meningitis. The organism is ubiquitous and has been isolated from a variety of foods including milk, cheese, dried food, water, vegetable, herbs and spices (Iversen *et al*, 2004). Many cases of contamination of powdered infant formula products by *C. sakazakii*, causing severe infections have been reported in UK and US (van Acker *et al*, 2001, Himelright *et al*, 2002). Infections among the infants have also been reported from India (Ray *et al*, 2007). Individuals with a weakened immune response and infants with a low-birth-weight are susceptible to *C. sakazakii* infections. In the present study detection of *C. sakazakii* in samples of milk and milk products collected from Agra city was attempted by the polymerase chain reaction (PCR) method.

Materials and Method

Samples collected from four different regions of Agra i.e. Agra Cant (Southern region), Dayalbagh (Northern region), TajNagri (Eastern region) and LohaMandi (Western region) were included in the study. The samples of cow milk (n = 15) and buffalo milk (n = 15) were collected directly from the udder while milk products like cottage cheese (n = 8) and khoya (n = 8) were collected from local vendors. 0.5 g or 0.5 ml of each sample was added to 4.5 ml of *Enterobacter* enrichment broth (EE) and incubated for 24 hours at 37°C. Culture from EE broth was streaked on violet red bile glucose agar and the pink coloured colonies obtained were examined microscopically. These typical colonies were further streaked on tryptic soya agar plates and incubated for 24 hours at 37°C. Characteristic yellow coloured colonies were picked up and tested biochemically for *C. sakazakii*. Standard strain of *C. sakazakii* (MTCC-2958) was obtained from the Microbial Type Culture Collection (MTCC), Institute of Microbial Technology Chandigarh, India and used as the positive control.

Preparation of Genomic DNA

6 biochemically confirmed isolates of *C. sakazakii* and MTCC-2958 were cultured in EE broth for 24 hours at 37°C. 1.5 ml culture was transferred to a micro centrifuge tube, boiled at 100°C in a heating block for 10 minutes and centrifuged at 1500g for 30 seconds. Primers ESSF and ESSR (Nair and Venkitanarayanan, 2006) were used to amplify a 469 bp fragment of the *ompA* gene specific to *C. sakazakii*. The PCR mix consisted of 1x Gene Amp PCR

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buffer II (50mM potassium chloride and 10mM tris- HCl, pH 8.3), 2.5mM $MgCl_2$, 200µM (each) dNTP, 1µM (each) primer, 1U of Taq DNA polymerase, 50ng of template DNA and sterile deionized water to make the volume up to 50µl. The samples were subjected to PCR cycles consisting of denaturation at 94°C for 15s, 60°C for 15s, and 72°C for 30s, and a final extension at 72°C for 5 min. A 5-µl aliquot of the amplified product was characterized on 1.5% agarose gel. The amplicons were detected by staining with ethidium bromide (0.5µg/ml) and were photographed under a UV transilluminator.

Results and Discussion

Table 1 gives the standard biochemical reactions for *C. sakazakii* and 6 (3 %) out of 200 isolates matched with these biochemical reactions (Fig.1). All the 6 isolates were Gram negative, methyl red negative, VP positive, nitrate positive, indole production negative, catalase positive, oxidase negative, citrate utilization positive, TSI positive, gas production positive, DNase positive, motility test positive and hydrogen sulphide production negative. Fermentation tests revealed that the 6 isolates and MTCC-2958 fermented glucose, maltose and raffinose but not arabinose and adonitol. These biochemically confirmed isolates were further subjected to molecular characterization by PCR method. The primers ESSR and ESSF were used to amplify a 469bp DNA fragment, specific to *C. sakazakii*, to confirm 6 out of 200 isolates (Fig.2). The biochemically confirmed 6 isolates correlated to the PCR amplification.

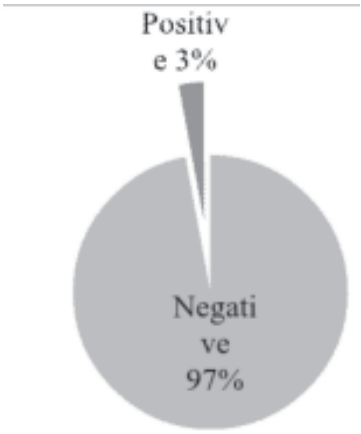

Fig.1. Percentage of isolates confirmed as *C. sakazakii*.

Presence of *C. sakazakii* on or in foods other than powdered infant formula (PIF) raises concern about the safety risks which these foods pose to neonates, infants and vulnerable adults. For persons with diminished immunity, the occurrence of *C. sakazakii* in the environment and food may pose a risk to health (Friedemann, 2007). Very low concentration < 10 cfu/100 ml of *C. sakazakii* has caused infections in neonates (Simmons *et al*, 1989). The isolation of *C. sakazakii* from PIF and PIF production environment indicates that this bacterium has the ability to persist, survive and adapt under desiccated environmental conditions (Drudy *et al*, 2006). Microflora which forms biofilms includes *Klebsiella* spp, *Salmonella* spp.,

Table 1.Biochemical profiles of *Cronobactersakazakii* isolates recovered from analysed samples

Isolate Number	Sample positive for *Cronobactersakazakii*	Region	Gram's staining	Methyl Red	Voges-Proskauer	Nitrate	Indole Production	Catalase	Oxidase	Citrate Utilization	TSI (yellow colour slant)	Gas Production	H_2S Production	Sugar tests (a) Adonitol	(b) Arabinose	(c) Glucose	(d) Maltose	(e) Raffinose	DNase test	Motility test
1	Cow milk	LohaMandi	-	-	+	+	-	+	-	+	+	+	-	-	-	+	+	+	+	+
2	Khoya	Dayalbagh	-	-	+	+	-	+	-	+	+	+	-	-	-	+	+	+	+	+
3	Buffalo milk	LohaMandi	-	-	+	+	-	+	-	+	+	+	-	-	-	+	+	+	+	+
4	Cottage cheese	Dayalbagh	-	-	+	+	-	+	-	+	+	+	-	-	-	+	+	+	+	+
5	Cottage cheese	LohaMandi	-	-	+	+	-	+	-	+	+	+	-	-	-	+	+	+	+	+
6	Buffalo milk	Agra Cant.	-	-	+	+	-	+	-	+	+	+	-	-	-	+	+	+	+	+

(+ = positive biochemical reaction, - = negative biochemical reaction)

Pseudomonas spp., *Listeria* spp., *Escherishia coli* and *Cronobacter* spp. Such bacteria may readily form biofilms on food contact surfaces which in turn may contaminate the ready-to-eat and minimally-processed food products (Kim *et al*, 2006).

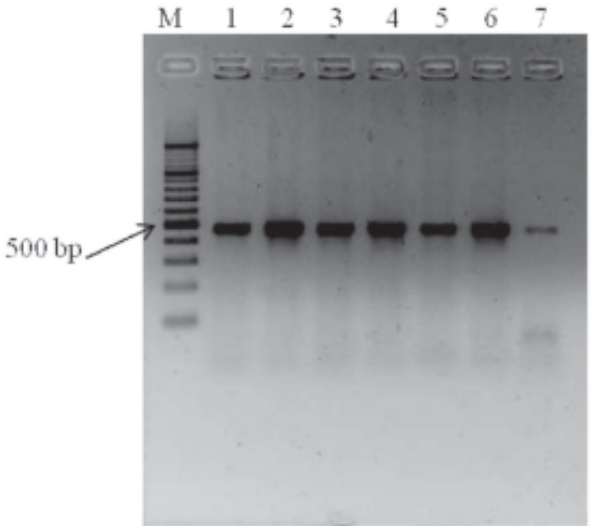

Fig.2.Detection of *C. sakazakii* by PCR amplification of the *omp A*gene. Lanes M: 100 bp ladder, Lane1: PCR *C. sakazakii* (MTCC-2958) DNA, Lane 2 to 7: *C. sakazakii* isolate numbers 1-6.

Since, *C. sakazakii* is a budding food borne pathogen and has a serious impact on human health; it has gained the interest and concern of food industry, scientific community and regulatory agencies. Limited information is available about the ecology of *C. sakazakii* and uncertainty concerning the source of infection in neonates, infants and immune compromised individuals requires the study to identify the foods that may contain *C. sakazakii*. This will help to disclose the possible routes for transmission of infection. Foods other than PIF have rarely been investigated for the occurrence of *C. sakazakii*. The present study was carried out by isolation and identification of *C. sakazakii* based on standard microbiological methods of enrichment followed by isolation of colonies on differential and selective media. Colonies were confirmed by identification of isolates based on biochemical tests. Biochemically confirmed isolates were subjected for molecular identification of the confirmed isolates. For this, PCR was conducted for amplification of 469 bp virulent gene fragment which is unique to *C. sakazakii*. PCR combined with enrichment culturing is a rapid tool for the detection of *C. sakazakii* in milk and milk products. The severity of infection in infants, neonates and immunocompromised individuals and infants, the scarcity of information available on the routes of transmission other than PIF motivated to take us the present study which focused on the isolation of *C. sakazakii* from milk and milk products available in Agra city. The primary goal of this study was to rapidly detect and confirm *C. sakazakii* from milk and milk products. The cultural procedure for detection of *C. sakazakii* is laborious, taking up to 7 days for completion whereas PCR combined with enrichment culturing, can detect *C. sakazakii* in about 12 hours and thus has the potential to be used as a rapid tool for detecting its presence.

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sp. nov., *Cronobacter turicensis* sp. nov. *Cronobacter muytjensii* sp. nov., *Cronobacter dublinensis* sp. nov., *Cronobacter genome species*1 and three subspecies, *Cronobacter dulinensis subsp. Lausannensis* sub sp. nov. and *Cronobacter dublinensis* sub sp. *lactaridi* sub sp. *nov*. *Int J Syst Evol Microbiol* **58**:1442-1447. doi: 10.1099/ijs.0.65577-0 IJSEM June 2008 vol. 58 no. 6 1442-1447

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