Detecting Cronobacter Contamination in Protein Mixture and Biscuit Sample by Conventional PCR Method-A Preliminary Study

Mehal Passi, Nakul Aggarwal and Anu Priya Minhas*

Department of Biotechnology, UIET, Panjab University, Chandigarh, India

*Corresponding author

Abstract

Cronobacter is a gram negative, non-sporulating rod shaped bacteria. It is a known contaminant of different dried milk products, mainly powdered infant formula. Cronobacter is responsible for meningitis and enteritis especially in neonates. However elderly people and children with compromised immunity are also at risk. To detect Cronobacter contamination in food samples, various microbiological, immunological and molecular methods with variable specificity and sensitivity have been developed. This study focus on molecular detection of Cronobacter in some food products employing rpoB and ITS based PCR strategy. Total of six samples were screened with microbiological methods and positive samples were further confirmed using rpoB and ITS based conventional PCR. Although 50% tested sample were found contaminated with such bacteria, still it is a very preliminary study in such food samples requiring further investigation. ITS and rpoB sequence chosen from previous studies were conserved sequences among Cronobacter strains. Therefore probability of primer (based on these sequence) to bind at specific locus in different strains will be more. However only rpoB based primer pair successfully amplified rpoB specific sequence in three out of six food samples tested. No such amplification was observed when ITS based primer pair was used in PCR. In spite of all, such food contaminations are a matter of concern, reflecting poor handling, processing, preparation and storage procedures therefore challenging health of children and immune-compromised patients.

Keywords
Cronobacter, PCR, Enterobacteriaceae, rpoB, ITS.

Introduction

Genus Cronobacter contains gram negative, rod shaped and non-sporulating bacteria and belongs to the family Enterobacteriaceae (Farmer et al., 1980; Iverson et al., 2007a, 2008). Till date only seven species has been identified under the genus Cronobacter. Cronobacter is reclassified form of genus Enterobacter cloacae, which under the genus Cronobacter.

Biochemically Cronobacter spp. has been characterized to have catalase and unique α-D-glucosidase activity with no oxidase activity (Muytjens et al., 1988). It is a food-borne and opportunistic pathogens causing life-threatening infections affecting neonates specially low birth weight babies. However elderly persons and/or children with compromised immunity are also target of
such infection. (Bar-Oz et al., 2001; Gurtler et al., 2005; Anonymous, 2006 a,b; Mullane et al., 2007a). Its ability to form capsular film helps in its adherence to intestinal and macrophages cell surface where in macrophages they can survive for long period of time. All these factors contribute towards its virulence (Iversen and Forsythe, 2004). Clinical syndromes of Cronobacter infection include necrotizing enterocolitis (NEC), bacteraemia and meningitis, with fatality rates between 40% and 80% (Bowen and Braden, 2006; Friedemann, 2009). Cronobacter spp. has been detected and isolated from a wide variety of environmental and food samples including dairy-based foods, dried meats, water, rice and infant formula (Baumgartner et al., 2009; Chap et al., 2009; Healy et al., 2010). For isolation of this bacterium the recommended media by the International Organization for Standardization-International Dairy Federation include DFI medium, OK medium, Enterobacter sakazakii chromogenic plating medium (ESPM), Enterobacter sakazakii isolation agar (ESIA), Violet Red Bile Glucose Agar (VRBGA) (Besse et al., 2006; Iversen et al., 2004; Oh and Kang, 2004; Restaino, et al., 2006). C. sakazakii when grown on nutrient agar at 37°C produces slightly yellow-pigment but at room temperature it forms non-diffusible yellow-gold pigment (Arshan. et al., 2013). Cronobacter spp. has been isolated specifically from powdered infant formulae as this bacterium has characteristic high tolerance to desiccation which helps this bacterium to survive in dry environment of milk factories (Mullane et al., 2006). Different detection strategies include conventional media based, immunological and molecular based method. Among molecular methods Polymerase Chain Reaction (PCR), real-time PCR, DNA microarray-based assays based on unique genes or regions of this pathogen have been employed for Cronobacter diagnostics (Bej et al., 2003; Wang et al., 2009). Some of these unique sequences are 16S rRNA, intergenic region between 16S rRNA-23S rDNA, dnaG, ompA, 1,6 α-glucosidase and RNA polymerase encoding genes Iversen et al., (2004a, 2007a, 2007b), Derzelle et al., (2007), Hassan et al., (2007); Almeida et al., (2009); Seo and Brackett (2005); Drudy et al., (2006); Jaradat et al., (2009); Nair and Venkitanarayanan (2006); Mullane et al., (2008a); Jarvis et al., 2011; Stoop et al., (2009).

rpoB and ITS are the conserved regions among Cronobacter genus with little difference in length and primary sequence. The rpoB gene (~1342 amino acids) encodes β subunit of bacterial RNA polymerase, the second largest polypeptide present in bacterial cell. Internal transcribed spacer (ITS) sequence is the sequence between tRNA-glu and 23S rRNA genes. Real time PCR based on these sequence has been reported to detect multiple species of Cronobacter genus with a mismatch-PCR-based approach (Stoop et al., 2009) (Sylviane Derzelle and Françoise Dilasser, 2006). Simple conventional PCR was performed with microbiologically positive samples. A total of six samples were tested on selective media. For PCR detection, two sets of primers based on above mentioned sequences were employed. Presence or absence of amplified product was analyzed on 0.8% agarose gel for confirming Cronobacter contamination. Samples used in this study were health drinks and high calories protein biscuits.

Materials and Methods

Food sample collection

Six food samples tested were procured from confectionary stores located in vicinity of
Chandigarh, India. Three health drinks sample were A, B, C while three high calories protein biscuits were D, E, F. It was carefully noted that none of samples is expired or seal tampered. All the samples were certified by Food Safety and Standard Authority of India (FSSAI).

**Media and Reagents**

Tryptone Soy Agar (TSA) (HiMedia) and Violet Red Bile Glucose (VRBG) (HiMedia) agar were used as nutrient and selective media respectively for isolating Enterobacteriaceae in this study. TSA contain Pancreatic digest of casein 17g/l, papaic digest of soyabean meal 3g/l, sodium chloride 5g/l, dextrose 2.5g/l, dibasic potassium phosphate 2.5g/l, pH 7.3±0.2. Composition of VRGB include yeast extract 3g/l, pancreatic digest of gelatin 7 g/l, bile salts 1.5 g/l, sodium chloride 5 g/l, glucose monohydrate 10 g/l, neutral red 0.03 g/l, crystal violet 0.002 g/l, pH 7.4±0.2. Buffered Peptone Water (BPW, pH 7±0.2) was used for non selective enrichment of sample. In each microbiological experiment, one day old media plates were used. Each experiment was performed twice and in duplicates. 0.8 % agarose gel in TAE buffer was used for electrophoretic separation of DNA samples. Growth temperature for all cultures was kept at 36 ºC.

**Pre enrichment method**

Pre enrichment of samples was done in Buffered Peptone Water (BPW) as per revised FDA protocol. For this 1g of each sample in 10 ml of sterile BPW was incubated at 36 ºC temperature for ~24 h in shaking (BPW, pH 7±0.2). 1ml of this pre-enriched sample was centrifuged at 6000 rpm for 5 minute and resultant pellet was re-suspended in 300 µl of same buffered peptone water.

**Primary Screening**

Cell suspension in buffered peptone water (obtained in previous step) was spread onto violet red bile glucose (VRBG) (HiMedia) agar plates. Growth was observed for Enterobacteriaceae specific colonies after incubation at 36ºC for ~24h. Obtained colonies were further streaked on tryptone soya agar (TSA) (HiMedia) following incubation at 36ºC for next ~24 h (Steigerwalt et al., 1976). Colonies appearing on TSA plates were tested for presence of Cronobacter contamination following Cronobacter specific PCR.

**DNA isolation**

DNA isolation from Enterobacteriaceae colonies was performed following standard protocol given by Rashidat et al., 2013. Briefly, colonies picked from tryptone soya agar plate were resuspended in 50 µl of autoclaved water. Samples were heated at 80ºC temperature for 10 min to release DNA in supernatant, followed by snap chilling on ice for 10-15 min. Samples were centrifuged at 10,000 rpm for 5 min. Cell pellets were discarded and supernatant containing DNA were transferred to fresh tube. Quality of isolated DNA checked by running 0.8% agarose gel following staining with ethidium bromide.

**Primer used in PCR Reaction**

As discussed above, two set of primers were considered for this study. One primer set was based on rpoB gene encoding β subunit of bacterial RNA polymerase. Various Cronobacter spp. shares significant sequence similarity in their respective rpoB sequence. rpoB based primer pairs from each strain can amplify rpoB sequence from their respective strains from genus Cronobacter (C. sakazakii; C. turicensis; C.
malonicus; C. muytjensii and C. dubliniensis). However, two strains i.e. C. sakazakii and C. malonicus can be amplified using single primer pair with no cross-reaction with non-E. sakazakii strains. Sequence of Cmalf primer is (5'-AAC CAG TTC CGC GTG GGC CTG G-3') while Cmlr is (5'-CCT GAA CAA CAC GCT CGG A-3') as described by (Mollet et al., 1997; Stoop et al., 2009). According to them, size of targeted amplicon should be ~251 bp for both C. sakazakii and C. Malonicus.

Another primer pair was based on internal transcribed spacer (ITS) sequence between between tRNA-glu and 23S rRNA genes. Degenerate primer pair synthesized from ITS region is ESFor (5'ATCTCAAAAMTGACTGTAAAGTCACGTT3') and ESRev is (5'CCGAARAAATMTTTCG KGCTGCGA3') as described by Sylviane Derzelle and Françoise Dilasser (2006). ITS was found to be conserved throughout Eubacteriae, with little difference between genus and species. This can facilitate species differentiation within Cronobacter genus with a mismatch-PCR-based approach. Expected size of targeted amplification with given primer pair is ~158 bp in all strains of E. sakazakii and no cross-reaction with non-E. sakazakii strains.

Detection by PCR

Polymerase chain reaction was carried out with PCR, BioRad system. Targeted amplification was performed in 20 µl PCR reaction containing 1X Thermopol buffer (20 mM Tris-HCl pH 9.2, 10 mM KCl, 10mM ammonium sulfate, 0.1% Triton X-100), 0.2 mM dNTPs, 0.2 µM each of the primers and 0.5 U of Taq DNA polymerase. Primer used in this study were Cmalf (5'-AAC CAG TTC CGC GTG GGC CTG G-3') and Cmlr (5'-CCT GAA CAA CAC 88 GCT CGG A-3') and ESFor (5'ATCTCAAAAMTGACTGTAAAGTCACGTT3') and ESRev (5'CCGAARAAATMTTTCG KGCTGCGA3') (Sylviane Derzelle and Françoise Dilasser (2006, Stoop et al., 2009). PCR conditions include initial denaturation at 95°C for 5 min and repeated amplification for next 25 cycles. Each cycle further comprised of small denaturation at 95°C for 40 sec, primer annealing between 47°C-49°C (depending on melting temperature of the primers), extension at 72°C for 2 hours. Cycles were followed by final extension at 72°C temperature for further 10 min. Amplified product was analysed by running 0.8% agarose gel in 1X TAE buffer and visualized under UV trans-illuminator (after staining with EtBr solution).

Result and Discussion

Microbiological detection

Total of six food samples as mentioned in materials and methods were used in this study. Three health drink samples (A, B and D) while three biscuit samples (C, E and F) were used. Initial screening on violet red blue glucose agar (VRBGA) plates resulted in ~500 Enterobacteriaceae specific red coloured colonies in health drink sample A. Similar colonies were also observed in E and F biscuit samples. Sample B, D and C didn’t show such colonies on VRBGA plate. These red coloured colonies were specific for Enterobacteriaceae contamination (Figure I). Obtained cultures were further streaked on Tryptone Soya Aga (TSA) plates for pure culture.

Secondary Screening of colonies on Tryptone Soya Aga (TSA)

Obtained Enterobacteriaceae specific red colored colonies from VRBGA plates were
further streaked on Tryptone Soya Aga (TSA) agar plates for further confirmation. For this, five red colored colonies (numbering 1, 2, 3, 4, 5) from each VRBGA plates (Health drink A; A1, A2, A3, A4, A5 Biscuit E; E1, E2, E3, E4, E5 and Biscuit F; F1, F2, F3, F4, F5) were streaked on TSA plates. Pale yellow colored colonies appearing on TSA plates further confirmed Enterobacteriaceae contamination these sample (Figure II).

**Polymerase chain reaction**

DNA was isolated from five colonies i.e. A1, A2, A3, A4, A5 from Health drink A sample as described in materials and methods. Isolated DNA was used as template in PCR Separate PCR reactions were set up with both rpoB and ITS specific primer pairs. rpoB based PCR should results in 251 bp product while ITS based PCR reaction will give 158 bp amplified product in Cronobacter positive samples (Stoop et al., 2009, Sylviane Derzelle and Françoise Dilasser, 2006). Reactions were performed in duplicates with both pairs of primers. Total of seven Cronobacter spp. characterized, this primer pair (CmalF and CmalR) is capable of amplifying 251 bp rpoB gene region from either of C. sakazakii and C. malonaticus if present. PCR reaction performed with five colonies from sample A. Samples were analysed on 0.8 % agarose gel stained with EtBr. Cronobacter specific amplification (251bp) was observed in three (A3, A4 and A5) DNA samples. No such amplification in A1 and A2 colonies was observed (Figure III). Although all five colonies were microbiologically tested on specific media, negative PCR for A1 and A2 indicate presence of other Enterobacteriaceae spp. whose amplification is beyond these primer pairs. Similar PCR reaction was also carried out with five colonies each from sample E and F. Only two colonies from sample E and F i.e. E4, E5 (Figure IV), F4, F5 (data not shown) respectively resulted in rpoB based amplification. This could be the because of same reason i.e. presence of other Enterobacteriaceae spp. whose amplification is beyond this primer pairs. Separate PCR with ITS base primer pair was also carried out with similar template DNA. However, none of the template from any of the sample resulted in amplification even when gradient PCR was carried out (Figure III). Therefore according to these findings, microbiological detection followed by rho based conventional PCR can be exploited as the simple but effective Cronobacter detection strategy.

Cronobacter, a multi-species, complex, gram negative, non-sporulating rod shaped bacteria. It has been found responsible for outbreaks causing meningitis and enteritis, especially in infants. Powdered infant formula (PIF) is the most common vehicle of infection resulting in number of neonatal infections. Although Cronobacter do not sustain pasteurization process but heat sensitive nutrients and un-pasteurized raw material can re-contaminate the final powdered infant formula. Other foods found contaminated with this bacterium are skimmed milk powder, herbal teas, starches, wastewater as well as other food supplements. However natural habitat is not well clearly understood. Infants especially less than two months of age, elderly people or infants with compromised immune system are most vulnerable. Associated illness includes neonatal meningitis, necrotising enterocolitis (NEC), bacteraemia, necrotising meningencephalitis, neurological disorders or even 20% to 50% high mortality rate. Various microbiological immuological and molecular methods are already employed in Cronobacter detection.
Fig.1 Screening food samples on violet red bile glucose (VRBG) agar media plates- Samples suspended in buffered peptone water was either spread or streaked onto violet red bile glucose (VRBG) (HiMedia) agar plates and plates were incubated at 37°C for ~24h. Red colour colonies indicative of Enterobacteriaceae contaminations appeared on sample (A), (E) and (F) while samples (B), (C) and (D) didn’t show any such growth. Sample A, B, C (Health drinks) and D, E, F (High calories Protein Biscuits)
**Fig. 2** Streaking of Enterobacteriaceae colonies on nutrient media i.e. tryptone soya agar (TSA) plates- Red colour colonies appearing in sample A (on violet red bile glucose agar plates) were carefully streaked on nutrient media i.e. tryptone soya agar (TSA) and same were incubated at 37°C temperature for 24 h for confirmation. Same procedure was also performed with colonies appearing in sample E and F.

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**Fig. 3** Detection of *C. sakazakii* in five colonies from sample A by PCR with two sets of primers separately. The PCR products were analyzed by electrophoresis in 0.8% agarose gel. (A) A ~251bp DNA fragment was amplified from rpoB region with rpoBF and rpoBR primers specific for *C. sakazakii* in three (A3, A4 and A5) out of five selected colonies. (B) No PCR amplification was observed in any of above five colonies with ITS specific primers. Lane M-1kb Marker, Lane 2- A1, Lane 3- A2, Lane 4- A3, Lane 5- A4, Lane 6- A5.
Fig.4 Detection of *C. sakazakii* in five colonies from sample E by PCR with two sets of primers separately. The PCR products were analyzed by electrophoresis in 0.8% agarose gel. A ~251bp DNA fragment was amplified from rpoB region with rpoBF and rpoBR primers specific for *C. sakazakii* in three (E3, E4 and E5) out of five picked colonies. Lane M-1kb Marker, Lane 2- E1, Lane 3- E2, Lane 4- E3, Lane 5- E4, Lane 6- E5.

Table 1 Samples used and summary of results obtained from this study

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Sample code/name</th>
<th>VRBGA plates</th>
<th>TSA Plates</th>
<th>Specific PCR amplification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>With rpoB based primer</td>
<td>With ITS based primer</td>
</tr>
<tr>
<td>1</td>
<td>A/ Health drink</td>
<td>Red colour colonies appeared</td>
<td>Pale yellow colour colonies appeared</td>
<td>+VE</td>
</tr>
<tr>
<td>2</td>
<td>B/ Health drink</td>
<td>No Colonies</td>
<td>No Colonies</td>
<td>-VE</td>
</tr>
<tr>
<td>3</td>
<td>D/ High calories Protein Biscuits</td>
<td>No Colonies</td>
<td>No Colonies</td>
<td>-VE</td>
</tr>
<tr>
<td>4</td>
<td>C/ Health drink</td>
<td>No Colonies</td>
<td>No Colonies</td>
<td>-VE</td>
</tr>
<tr>
<td>5</td>
<td>E/ High calories Protein Biscuits</td>
<td>Red colour colonies appeared</td>
<td>Pale yellow colour colonies appeared</td>
<td>+VE</td>
</tr>
<tr>
<td>6</td>
<td>F/ High calories Protein Biscuits</td>
<td>Red colour colonies appeared</td>
<td>Pale yellow colour colonies appeared</td>
<td>+VE</td>
</tr>
</tbody>
</table>

All the employed methods differ in their specificity and sensitivity.

This study re-evaluated two separate methods based on rpoB and ITS sequence (Table 1. Summary of result). Although both studies reported fast and reliable *Cronobacter* detection, we found rpoB based PCR approach as quite reliable for *Cronobacter* detection. Mostly infant powder formula milk and infant foods has been screened for *Cronobacter* detection. This small study tested health drinks and high calories biscuit especially consumed by children. Finding such food samples below acceptable international standards is still a
matter of concern. Therefore food processing, preparation, handling and storage steps should be scanned by the authority.

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