

Original Research Article

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

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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 has 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.

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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 *Chronobacter 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 VRBG 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 °C.

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 °C 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 36°C for ~24h. Obtained colonies were further streaked on tryptone soya agar (TSA) (HiMedia) following incubation at 36°C 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 80°C 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.*

malonaticus; *C. muytjensii* and *C. dublinensis*). However, two strains i.e. *C. sakazakii* and *C. malonaticus* 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 GTT GGC CTG G-3') while Cmalr 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. Malonaticus*.

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'ATCTCAAAMTGACTGTAAAGTCACGTT3') and ESRev is (5'CCGAARAAGTMTTCGKGCTGCGA3') 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 GTT GGC CTG G-3') and Cmalr (5'-CCT GAA CAA CAC 88

GCT CGG A-3') and ESFor (5'ATCTCAAAMTGACTGTAAAGTCACGTT3') and ESRev (5'CCGAARAAGTMTTCGKGCTGCGA3') (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 Agar (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).

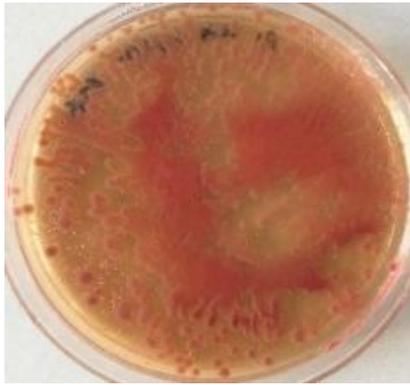
Polymerase chain reaction

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. malonicus* if present. PCR reaction performed with five colonies from sample A. Samples were analyzed 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

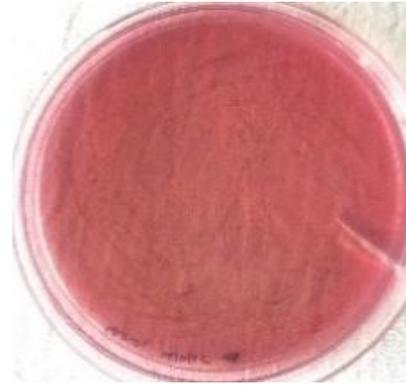
F. Only two colonies from sample E and F i.e. E4, E5 (Figure IV), F4, F5 (data not shown) respectively resulted in *rpoB* based amplification. This could be because of same reason i.e. presence of other *Enterobacteriaceae spp.* whose amplification is beyond this primer pairs. Separate PCR with ITS base 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 *rpoB* 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 meningo-encephalitis, neurological disorders or even 20% to 50% high mortality rate. Various microbiological immunological 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)



(Sample A)



(Sample D)



(Sample B)



(Sample E)



(Sample C)



(Sample F)

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.



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.

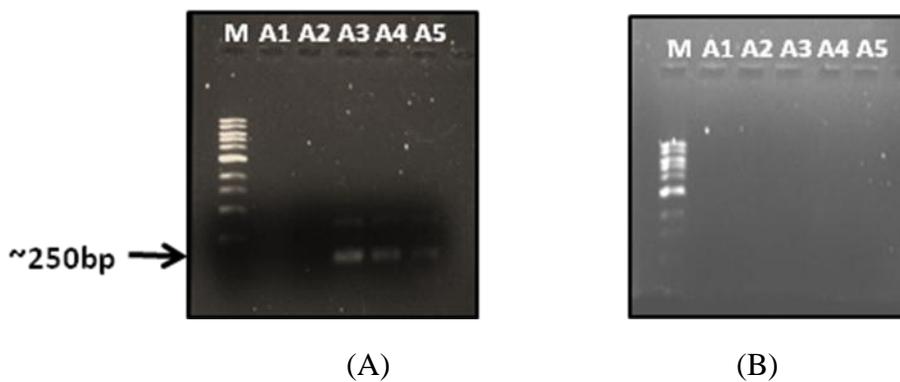


Fig.4 Detection of *C. sakazakii* in five colonies from sample E by PCR with two sets of primers separately. The PCR products were analyzed by electrophoresis in 0.8% agarose gel. A ~251bp DNA fragment was amplified from rpoB region with rpoBF and rpoBR primers specific for *C. sakazakii* in three (E3, 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.

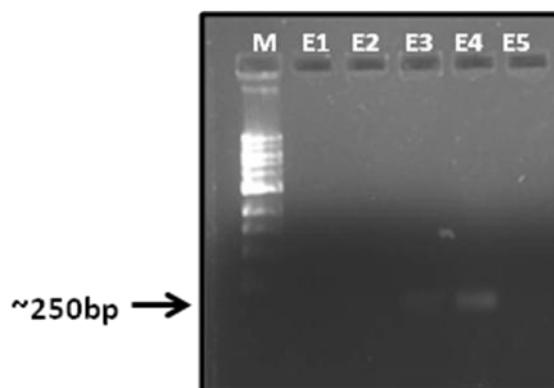


Table.1 Samples used and summary of results obtained from this study

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

All the employed methods differ in their specificity and sensitivity.

This study re-evaluated two separate methods based on rpoB and ITS sequence (Table1. 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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References

- Almeida, C., Azevedo, N. F., Iversen, C., Fanning, S., Keevil, C. W. and Vieira, M. J. 2009. Development and application of a novel peptide nucleic acid probe for the specific detection of *Cronobacter* genomo species (*Enterobacter sakazakii*) in powdered infant formula. *Appl. Environ. Microbiol.*, 75: 2925–2930.
- Anonymous. 2006a. 2006. Milk and Milk Products – Detection of *Enterobacter sakazakii*. Technical Specification ISO/TS 22964. ISO/TS 22964:2006(E) and IDF/RM 210 (E), 1st edn. Geneva: International Organization for Standardization.
- Anonymous. 2006b. *Enterobacter sakazakii* and *Salmonella* in powdered infant formula. Available at: http://www.fao.org/ag/agn/agns/jemra_riskassess-ment_enterobacter_en.asp. Second Risk Assessment Work- shop. Joint FAO/WHO Workshop: Rome, Italy.
- Arseni, A., Malamou-Ladas, E., Koutsia, C., Xanthou, M. and Trika, E. 1987. Outbreak of colonization of neonates with *Enterobacter sakazakii*. *J. Hosp. Infect.*, 9, 143–150.
- Bar-Oz, B., Preminger, A., Peleg, O., Block, C. and Arad, I. 2001. *Enterobacter sakazakii* infection in the newborn. *Acta Paediatr.* 90, 356–358.
- Baumgartner, A., Grand, M., Liniger, M. and Iversen, C. 2009. Detection and frequency of *Cronobacter spp.* (*Enterobacter sakazakii*) in different categories of ready-to-eat foods other than infant formula. *Int. J. Food Microbiol.*, 136, 189–192.
- Bej, A.K. 2003. Molecular based methods for the detection of microbial pathogens in the environment. *J. Microbiol. Methods*, 53, 139–40.
- Besse, N. G., Leclercq, A., Maladen, V., Tyburski, C. and Lombard B. 2006. Evaluation of the International Organization for Standardization—International Dairy Federation draft standard method for detection of *Enterobacter sakazakii* in powdered infant food formulas. *J. AOAC Int.*, 89, 1309–1316.
- Bowen, A. and Braden, C. 2006. Invasive *Enterobacter sakazakii* Disease in Infants. *Emerg. Infect. Dis.*, 12, 1185–1189.
- Chap, J., Jackson, P., Siqueira, R., Gaspar, N., Quintas, C., Park, J., Osaili, T., Shaker, R. 2009. International survey of *Enterobacter sakazakii* and other *Cronobacter spp.* 136: 185-188.
- Derzelle, S. and Dilasser, F. 2006. A robotic DNA purification protocol and real-time PCR for the detection of *Enterobacter sakazakii* in powdered infant formulae. *BMC Microbiol.*, 6, 100.
- Derzelle, S., Dilasser, F., Maladen, V., Soudrie, N., Leclercq, A., Lombard, B. and Lafarge, V. 2007. Comparison of three chromogenic media and evaluation of two molecular-based identification systems for the detection of *Enterobacter sakazakii* from environmental samples from infant formulae factories. *J. Food Prot.*, 70, 1678–1684.

- Farmer, J. J. III, Asbury, M. A., Hickman, F. W., Brenner, D. J. 1980. *Enterobacter sakazakii*: a new species of *Enterobacteriaceae* isolated from clinical species. *Int. J. Syst and Evol. Bacter.*, 30, 569-584.
- Friedemann, M. 2009. Epidemiology of invasive neonatal *Cronobacter* (*Enterobacter sakazakii*) infections. *Eur. J. Clin. Microbiol. Infect. Dis.*, 28, 1297-1304.
- Gurtler, J. B., Kornacki, J. L. and Beuchat, L. R. 2005. *Enterobacter sakazakii*: a coliform of increased concern to infant health. *Int. J. Food Microbiol.*, 104, 1-34.
- Hassan, A. A., Akineden, O., Kress, C., Estuningsih, S., Schneider, E. and Usleber, E. 2007. Characterization of the gene encoding the 16S rRNA of *Enterobacter sakazakii* and development of a species-specific PCR method. *Int. J. Food Microbiol.*, 116, 214-220.
- Healy, B., Cooney, S., O'Brien, S., Iversen, C., Whyte, P., Nally, J., Callanan, J. J., Fanning, S. 2010. *Cronobacter* (*Enterobacter sakazakii*): an opportunistic food borne pathogen. *Food borne Pathog Dis.*, 7, 339-350.
- Iversen, C. and Forsythe, S.J. 2004. Isolation of *Enterobacter sakazakii* and other *Enterobacteriaceae* from powdered infant formula milk and related products. *Food Microbiol.*, 21: 771-776.
- Iversen, C., Druggan, P., Forsythe, S. 2004. A selective differential medium for *Enterobacter sakazakii*, a preliminary study. *Int. J. Food Microbiol.*, 96, 133-139.
- Iversen, C., Druggan, P., Forsythe, S. 2004. A selective differential medium for *Enterobacter sakazakii*, a preliminary study. *Int. J. Food Microbiol.*, 96, 133-139.
- Iversen, C., Lehner, A., Mullane, N., Bidlas, E., Cleenwerck, I., Marugg, J., Fanning, S., Stephan, R., Joosten, H. 2007a. The taxonomy of *Enterobacter sakazakii*: proposal of a new genus *Cronobacter* gen. nov. and descriptions of *Cronobacter sakazakii* comb. nov. *Cronobacter sakazakii* subsp. *sakazakii*, comb. nov., *Cronobacter sakazakii* subsp. *malonaticus* subsp. nov., *Cronobacter turicensis* sp. nov., *Cronobacter muytjensii* sp. nov., *Cronobacter dublinensis* sp. nov. and *Cronobacter* genomospecies 1. *BMC Evol. Biol.*, 7:64.
- Iversen, C., Mullane, N., Mc Cardell, B., Tall, B. D., Lehner, A., Fanning, S., Stephan, R., Joosten, H., 2008. *Cronobacter* gen. nov., a new genus to accommodate the biogroups of *Enterobacter sakazakii*, and proposal of *Cronobacter sakazakii* gen. nov. comb. nov., *C. malonaticus* sp. nov., *C. turicensis* sp. nov., *C. muytjensii* sp. nov., *C. dublinensis* sp. nov., *Cronobacter* genomospecies 1, and of three subspecies, *C. dublinensis* sp. nov. subsp. *dublinensis* subsp. nov., *C. dublinensis* sp. nov. subsp. *lausannensis* subsp. nov., and *C. dublinensis* sp. nov. subsp. *lactaridi* subsp. nov., *Int. J. Systematic and Evol. Microbiol.*, 58: 1442-1447.
- Jaradat, Z. W., Ababneh, Q. O., Saadoun, I. M., Samara, N. A. and Rashdan, A. M. 2009. Isolation of *Cronobacter* spp. (formerly *Enterobacter sakazakii*) from infant food, herbs and environmental samples and the subsequent identification and confirmation of the isolates using biochemical, chromogenic assays, PCR and 16S rRNA sequencing. *Microbiol.*, 9, 225.

- Jarvis, K.G., Grim, C.J., Franco, A. A., Gopinath, G., Sathyamoorthy, V. and Hu, L. 2011. Molecular characterization of *Cronobacter* lipopolysaccharide O-antigen gene clusters and development of serotype-specific PCR assays. *Appl. Environ. Microbiol.* 77, 4017–4026.
- Mollet, C., Drancourt, M. and Raoult, D., 1997. *rpoB* sequence analysis as a novel basis for bacterial identification. *Mol. Microbiol.*, 26, 1005–1011.
- Mullane, N. R., Drudy, D., Whyte, P., O'Mahony, M., Scannell, A. G. M., Wall, P. G. 2006. *Enterobacter sakazakii*: biological properties and significance in dried infant milk formula (IMF) powder. *Int. J. Dairy Technol.*, 59, 102-11.
- Mullane, N. R., Murray, J., Drudy, D., Prentice, N., Whyte, P., Wall, P.G., Parton, A. and Fanning, S. 2006. Detection of *Enterobacter sakazakii* in dried infant milk formula by cationic magnetic bead capture. *Appl. Environ. Microbiol.*, 72, 6325–6330.
- Mullane, N.R., Healy, B., Meade, J., Whyte, P., Wall, P. G. and Fanning, S. 2008c. Dissemination of *Cronobacter* spp. (*Enterobacter sakazakii*) in a powdered milk protein manufacturing facility. *Appl. Environ. Microbiol.*, 74, 5913–5917.
- Mullane, N.R., Iversen, C., Healy, B., Walsh, C., Whyte, P., Wall, P.G., Quinn, T. and Fanning, S. 2007a. *Enterobacter sakazakii* an emerging bacterial pathogen with implications for infant health. *Minerva Pediatr.*, 59, 137–148.
- Mullane, N.R., O'Gaora, P., Nally, J. E., Iversen, C., Whyte, P., Wall, P. G. and Fanning, S. 2008a. Molecular analysis of the *Enterobacter sakazakii* O-antigen gene locus. *Appl. Environ. Microbiol.*, 12, 3783–3794.
- Mullane, N.R., Ryan, M., Iversen, C., Murphy, M., O'Gaora, P., Quinn, T., Whyte, P., Wall, P. G. 2008b. Development of multiple-locus variable number tandem repeat analysis for the molecular subtyping of *Enterobacter sakazakii*. *Appl. Environ. Microbiol.*, 74, 1223–1231.
- Mullane, N.R., Whyte, P., Wall, P. G., Quinn, T. and Fanning, S. 2007b. Application of pulsed-field gel electrophoresis to characterise and trace the prevalence of *Enterobacter sakazakii* in an infant formula processing facility. *Int. J. Food Microbiol.*, 116, 73–81.
- Muytjens, H. L., Van der Ros-van de Repe, J. And Van Druten, H. M. 1984. Enzymatic profiles of *Enterobacter sakazakii* and related species with special reference to the α -glucosidase reaction and reproducibility of the test system. *J. Clin. Microbiol.* 20, 684-686.
- Muytjens, H.L., Roelfos, W. H. And Jaspar, G.H.J. 1988. Quality of powdered substitutes for breast milk with regard to members of family *Enterobacteriaceae*. *J. Clin. Microbiol.* 26: 743–746.
- Nair, M. K.M. and Venkitanarayanan, K.S. 2006. Cloning and Sequencing of the *ompA* Gene of *Enterobacter sakazakii* and development of an *ompA*-targeted PCR for rapid detection of *Enterobacter sakazakii* in infant formula. *Appl. Environ. Microbiol.*, 72: 2539-2546.
- Oh, S.W. and Kang, D. H. 2004. Fluorogenic selective and differential medium for isolation of *Enterobacter sakazakii*. *Appl. Environ. Microbiol.* 70, 5692-5694.
- Rashidata, E.A., Olugbo, A. O., Ifeanyib, S. S., Adetounb, F. W., Adedayob, O., and Moses, B. 2013. Isolation and

- PCR Detection of *Cronobacter sakazakii* from Powdered Infant Formulae retailed in Nigeria. *American J. Food and Nutri.*, 3: 182-187.
- Restaino, L., Frampton, E. W., Lionberg, W. C., Becker, R. J. 2006. A chromogenic plating medium for the isolation and identification of *Enterobacter sakazakii* from foods, food ingredients and environmental sources. *J. Food Protect.*, 69, 315-322.
- Seo, K.H. and Brackett, R.E. 2005. Rapid, specific detection of *Enterobacter sakazakii* in infant formula using a real-time PCR assay. *J. Food Prot.*, 68, 59-63.
- Steigerwalt, A.G., Fanning, G.R., Fife-Asbury, M. A., Brenner, D. J. 1976. DNA relatedness among species of *Enterobacter* and *Serratia*. *Can. J. Microbiol.*, 22, 121-137.
- Stoop, B., Lehner, A., Iversen, C., Fanning, S., Stephan, R. 2009. Development and evaluation of rpoB based PCR systems to differentiate the six proposed species within the genus *Cronobacter*. *Int. J. Food Microbiol.*, 136, 165-168.
- Wang, M., Cao, B., Gao, Q., Sun, Y., Liu, P., Feng, L., Wang, L. 2009. Detection of *Enterobacter sakazakii* and other pathogens associated with infant formula powder by use of a DNA Microarray. *J. Clin. Microbiol.*, 10, 3178-84.

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