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Phenotypic and Genotypic Characterization of *Cronobacter* isolated from Powdered Infant Formula Retailed in Nigeria

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Abstract

Cronobacter is a genus with emerging pathogens that has been associated with life threatening diseases in neonates, infants and immunocompromised adults. Three *Cronobacter* species were isolated from powdered infant formula retailed in Nigeria. Different methods of phenotypic and genotypic characterization were carried out. All the isolates were identified biochemically by Microscan identification analysis as *Enterobacter sakazakii* (98.87%). The Vitek MALDI-TOF system identified the isolates as *Cronobacter sakazakii.* 16S rRNA sequencing identified the isolates as *C. sakazakii.* In contrast the use of species-specific PCR assays targeting *rpo*B, and *cgc*A, helped to identify two of the three strains as *C. sakazakii* and the last strain was identified as *C. malonaticus.* Multi locus sequence typing (MLST) analysis was used to identify each strain's sequence type and the results identified three new sequence types: 303, 304 and 296. *C. sakazakii* BAA 894 served as a positive control for all the experiments. Biochemical methods and commercial identification systems are not sensitive enough to identify *Cronobacter* strains to the species level. Molecular methods are needed to confirm the species identity of strains.

Keywords: *Cronobacter,* genotyping, phenotyping, powdered infant formula milk

1. Introduction

Cronobacter, (former *Enterobacter sakazakii)* is an emerging, opportunistic pathogen that causes infections such as sepsis, meningitis and necrotizing enterocolitis in neonates and infants, and can sometimes lead to death. It is ubiquitous in various food products such as dairy based products, adult and infant cereals, and spices. It has been associated with the ingestion of contaminated reconstituted powdered infant formula (PIF), and can be found in various environments, in particular PIF production facilities. *Cronobacter* spp have shown high resistance to osmotic stress and this contributes to its persistence in PIF factories, dried products and environments (Osaili & Forsythe, 2009). The frequency of disease caused by *Cronobacter* is very low but the mortality rate has been reported to be as high as 80% with surviving patients often suffering severe neurological sequelae (Alsonosi et al., 2015), hydrocephalus and permanent mental damage. The World Health Organization (WHO) has recognized all *Cronobacter* species as microorganisms pathogenic for human beings of all ages (FAO/WHO, 2008) although this organism is also part of the normal human flora (Holy et al., 2014). Current international microbiological standards require the absence of all *Cronobacter* species in PIF (test volume 10 g) (Jackson et al., 2014). Of all *Cronobacter* species only *C. sakazakii, C. malonaticus* and *C. turicensis* have been linked to infantile infections (Alsonosi et al., 2015).

Cronobacter spp are Gram-negative motile rods of the family Enterobacteriaceae. They were formerly known as the yellow pigmented *Enterobacter sakazakii.* Reclassification based on the results of independent molecular methods and biochemical markers resulted in a new genus with seven species being described: *Cronobacter sakazakii, C. malonaticus, C. muytjensii, C. dublinensis, C. turicensis, C. condimenti* and *C. universalis* (Joseph et al., 2012). Target genes for PCR probe-based methods for *Cronobacter* identification include *cgcA, gyrB,*

ompA, rpoB, gluA, dnaG, zpx, iron acquisition genes, the macromolecular synthesis operon, the 16S rDNA gene, and the 16S-23S intergenic transcribed spacer region (Carter et al., 2013, Grim et al., 2013, Lehner et al., 2012, Stoop et al., 2009). Molecular based techniques such as Multi locus sequence typing (MLST), random amplification of polymorphic DNA, pulsed-field gel electrophoresis (PFGE) have also been successfully applied to the characterization of *Cronobacter* spp (Fields et al., 2011). Pulsed field gel electrophoresis (PFGE) is considered the 'gold standard' method for subtyping foodborne bacteria and the most discriminatory technique for genotyping, and in 2012, a PulseNet protocol was validated for subtyping *Cronobacter* spp. (Brengi et al., 2012).

The aim of this study was to identify *Cronobacter* spp isolated from powdered infant formula products retailed in Nigeria using various phenotypic and genotypic methodologies.

2. Materials and Methods

2.1 Samples Collection

A total of 154 different samples of PIF were purchased from local markets and super markets in different geopolitical zones of Nigeria.

2.2 Bacterial Strains and Cultivation

Cronobacter strains used in this study were isolated from the PIF using the FDA method in combination with the method described by Iversen et al. (2004). Briefly, 25g of powdered infant formula was added to 225ml of buffered peptone water (BPW; pH 7.2+ 0.2). The suspended powdered infant formula was then incubated at 37^0C for 24h without shaking. Four aliquots of 40ml each were removed from the solution and placed into 50ml centrifuge tubes. The tubes were subjected to centrifugation at 3000 x g for 10 min. The supernatants of each tube were discarded and the resultant pellets were suspended in 200µL of phosphate-buffered saline (PBS; pH 7.2+ 0.2). The aliquots were cultured on Druggan-Forsythe-Iversen (DFI) agar. The control strain, *C. sakazakii* ATCC BAA 894 was a gift from the Division of Virulence Assessment, Office of Applied Research and Safety Assessment, Center for Food Safety and Applied Nutrition, U. S. Food and Drug Administration (MD, USA).

2.3 Phenotyping

Cronobacter isolates were phenotyped using the MicroScan WalkAway identification panel (Beckman Coulter Inc., CA, USA) according to the manufacturer's instructions. A sterile applicator stick was used to touch the surface of 4-5 morphologically similar, well isolated colonies from an 18-24 h Brain Heart Infusion (BHI) (Difco, New Jersey) agar plate. This was emulsified in 3 mL of sterile deionized water and the suspension was vortexed for 2-3 s to achieve a final turbidity similar to the 0.5 McFarland Turbidity Standard scale. One-hundred microliters (100 µL) of the standardized suspension was pipetted into 25 mL of inoculum buffer, capped tightly and inverted 8-10 times to mix. An oxidase test was performed using tetramethyl-p-phenylenediamine – dihydrochloride (Sigma-Aldrich, USA) prior to inoculating the panels. The panel was rehydrated and inoculated using the RENOK system with Inoculators-D (Siemens, Frimley, Camberley, UK). A dropper bottle was used to overlay the GLU, URE, H_2S, LYS, ARG, ORN and DCB (Glucose, Urea, Hydrogen sulphide, Lysine, Arginine, Ornithine, and Decarboxylase Base) with 3 drops of sterile mineral oil. The panel was incubated in the Walkaway System for 16-20 h after which the results were read.

2.4 Species Identification Using the VITEK®MS MALDI-TOF

Identification of the *Cronobacter* isolates was carried out using VITEK®MS MALDI-TOF (Matrix Assisted Laser Desorption Ionization Time-of-Flight) technology (bioMerieux, France), according to the manufacturer's instructions. Two milliliters of bacterial broth was added to 1.0 ml of lysis buffer (0.6% polyoxyethylene 10 oleoyl ether (Brij 97) in 0.4 M (3-(cyclohexylamino)-1-propane sulfonic acid) (CAPS) filtered through a 0.2-µm-pore-size filter, pH 11.7), vortexed for 5 s, and allowed to incubate for 2 to 4 min at room temperature. The resulting lysate was filtered through a 25-mm 0.45-µm-pore-size filter (catalog no. HPWP02500; Millipore Express PLUS, Billerica, USA), MA and the microbial cells remaining on the filter were removed and washed three times with wash buffer (20 mM Na_3PO_4, 0.05% Brij 97, and 0.45% NaCl), using a 0.2-µm-pore-size filter, pH 7.2); washed three times with deionized water; and removed from the surface by scraping the filter with a micro-swab (Texwipe CleanTips swabs; catalog no. TX754B; Kernersville, NC). Sample processing time was approximately 10–15 min for up to three samples. Microorganisms recovered from the filter were directly applied to VITEK MS target plates and covered with 1 µL of CHCA (α-cyna-4-hydroxycinnamic acid) matrix. If the VITEK MS was unable to identify an isolate on the first attempt, the sample was repeated using double the volume of culture broth and corresponding buffers. A sample was considered to have a valid VITEK MS ID if at least one spot on the target slide gave a confidence level of ≥75% without conflicting identifications from

replicate spots of the same sample. Samples that did not generate an ID on the first attempt were repeated only once.

2.5 DNA Template and PCR Analysis

Polymerase chain reaction (PCR) was performed using 18-24 h culture. DNA templates were prepared by using a sterile inoculating wire to remove a colony and the cells were suspended into 20 µL of sterile distilled water (dH_2O) dispensed into Eppendorf tubes. The reaction mixture (25 µL) was prepared as followed: dH_2O (15.75 µL), Phusion Buffer (5.0 µL; New England BioLabs, MA), Forward primer (1.25 µL; Sigma-Aldrich, USA), Reverse primer (1.25 µL; Sigma-Aldrich, USA), dNTPS (0.5 µL), Phusion DNA polymerase (0.25 µL; New England BioLabs, MA), and Template (1.0 µL).

2.6 PCR Probe Assays of Cronobacter spp

PCR was carried out using the 16S rDNA gene (Sigma-Aldrich, USA), β-subunit of RNA Polymerase gene (*rpo*B) (Sigma-Aldrich, USA), and Diguanylate Cyclase-Encoding gene (*cgc*A) (Sigma-Aldrich, USA). The PCR conditions and primers used are stated on Table 1. PCR amplicons were subjected to agarose gel electrophoresis using 0.8% Tris-borate-EDTA buffer (TBE; Sigma-Aldrich, USA) in a Bio-Rad sub cell GT (Bio-Rad, Belgium) horizontal electrophoresis unit and were photographed with transilluminated UV light using an Alpha Imager system (Alpha Innotech Corp, San Leandro, CA, USA). The PCR products were purified using Wizard SV Gel and PCR clean up system (Promega, USA) and sequenced by GENEWIZ (New Jersey, USA) in a Sanger DNA Sequencer System. The obtained nucleotide sequences were compared with the corresponding sequences of *C. sakazakii* strain ATCC BAA 894 (NCBI accession no. 290339) and with other Enterobacteriaceae using DNASTAR-MegAlign.

Table 1. Primers and PCR conditions used for allelic profiling of *Cronobacter* spp

Target gene	Primer Sequence	PCR conditions	Amplicon size (bp)	Species identification	References
16S rDNA	16SUNI-L 8F- AGAGTTTGATCATGGCTCAG 1492R- GGT TAC CTT GTT ACG ACT T 515F - GTG CCA GCA GCC GCG GTA 1100R - GTT GCG CTC GTT G	*C. sakazakii*: Initial denaturation step at 94^{0}C for 3 min, 30 cycles of 94^{0}C for 60 s, 67^{0}C for 30 s, 72^{0}C for 1 min, with a final extension step at 72^{0}C for 10 min. *C. malonaticus*: Initial denaturation step at 94^{0}C for 3 min, 25 cycles of 94^{0}C for 60 s, 60^{0}C for 30 s, 72^{0}C for 30 s, with a final extension step at 72^{0}C for 10 min	1500	*Cronobacter spp*	Kuhnert et al., 1996
rpoB	*C. sakazakii*– Csakf 5′-ACG CCA AGC CTA TCT CCG CG-3′ Csakr 5′-ACG GTT GGC GTC ATC GTG-3′ *C. malonaticus* - Cmalf 5′-CGT CGT ATC TCT GCT CTC-3′ Cmalr 5′-AGG TTG GTG TTC GCC TGA-3′	*C. sakazakii*: Initial denaturation step at 94^{0}C for 3 min, 30 cycles of 94^{0}C for 60 s, 67^{0}C for 30 s, 72^{0}C for 1 min, with a final extension step at 72^{0}C for 5 min. *C. malonaticus*: Initial denaturation step at 94^{0}C for 3 min, 30 cycles of 94^{0}C for 60 s, 60^{0}C for 30 s, 72^{0}C for 1 min, with a final extension step at 72^{0}C for 10 min	514 251	*C. sakazakii:* *C. malonaticus*	Stoop et al., 2009
cgcA	Cmstu-825F-GGTGGCSGGGTATGACAAAGAC Csak-1317R-GGCGGACGAAGCCTCAGAGAGT Cmal1410R-GGTGACCACACCTTCAGGCAGA	Initial denaturation step at 98^{0}C for 2 min, 30 cycles of 98^{0}C for 60 s, 50^{0}C for 30 s, 72^{0}C for 1 min, with a final extension step at 72^{0}C for 10 min.	492 585	*C. sakazakii:* *C. malonaticus*	Carter et al., 2013

2.7 Pulsed Field Gel Electrophoresis (PFGE)

PFGE analysis of *Cronobacter* isolates was performed as previously described by Ribot et al. (2006) with some modification. The restriction enzyme *Xbal* was used for DNA digestion. Bands were separated using a CHEF-DR III System (BIO-RAD, Belgium) at 14 – 17° C. Electrophoretic conditions of initial switch time 1.8 s to a final switch time of 25 s, at 6 volts/cm for a run time of 17- 18 h were used. Gels were stained for 30 min in 500 mL dH2O containing 25 µl ethidium bromide (10 mg/mL) and visualized under UV light using an Alpha Imager system (Alpha Innotech Corp., San Leandro, CA, USA).

2.8 Multi Locus Sequence Typing

MLST was performed as previously described by Baldwin et al. (2009) and the *Cronobacter* PubMLST open access database (http://pubmlst.org/*Cronobacter*/). The seven housekeeping genes amplified were ATP synhase beta chain (*atpD*), elongation factor G (*fusA)*, glutaminyl-tRNA synthetase (*glnS*), glutamate synthase large subunit (*gltB*), DNA gyrase subunit B (*gyrB*), translation initiation factor IF-2 (*infB*) and phosphoenol-pyruvate synthase (*ppsA*) (Sigma-Aldrich, USA).

Table 2. Oligonucleotide nested primer sequences for the amplification and sequencing of genes from *C. sakazakii* and *C. malonaticus*, with gene number and location corresponds to *C. sakazakii* strain ATCC BAA-894 genome (Baldwin et al., 2009)

Gene (Gene label)	Putative Gene Product	Chromosome location (bp)	Gene Size (bp)	Locus Primers (5'→3')	
				Amplification	Sequencing
atpD	ATP synthase β chain	3,689,177 - 3,690,559	1,382	CGACATGAAAGGCGACAT TTAAAGCCACGGATGGTG	CGAAATGACCGACTCCAA GGATGGCGATGATGTCTT
fusA	Elongation factor	3,275,843 - 3,277,957	2,114	GAAACCGTATGGCGTCAG AGAACCGAAGTGCAGACG	GCTGGATGCGGTAATTGA CCCATACCAGCGATGATG
glnS	Glutaminyl-tRNA	660,368 - 662,035	1,667	GCATCTACCCGATGTACG TTGGCACGCTGAACAGAC	GGGTGCTGGATAACATCA CTTGTTGGCTTCTTCACG
gltB	Glutamate synthase	3,538,713 - 3,542,921	4,208	CATCTCGACCATCGCTTC CAGCACTTCCACCAGCTC	GCGAATACCACGCCTACA GCGTATTTCACGGAGGAG
gyrB	DNA gyrase B	3,719,848 - 3,722,262	2,414	TGCACCACATGGTATTCG CACCGGTCACAAACTCGT	CTCGCGGGTCACTGTAAA ACGCCGATACCGTCTTTT
infB	Translation initiation	4,139,051 - 4,141,762	2,711	GAAGAAGCGGTAATGAGC CGATACCACATTCCATGC	TGACCACGGTAAAACCTC GGACCACGACCTTTATCC
ppsA	Phosphoenol-pyruvate	1,218,599 - 1,220,977	2,378	GTCCAACAATGGCTCGTC CAGACTCAGCCAGGTTTG	ACCCTGACGAATTCTACG CAGATCCGGCATGGTATC

2.9 Molecular Characterization of Cronobacter Lipopolysaccharide O-Antigen Gene Clusters Using Serotype Specific PCR Primers

Primers designed based on the *wzx* and *wzy* gene (O antigen) sequences (Sun et al., 2012) were used. Multiplex PCR was performed by mixing all primers in a final volume of 50 µL containing the following components: 1× Taq Reaction buffer; 2.5 mM $MgCl_2$; 400 µM (each) of dATP, dCTP, dGTP, and dTTP; 0.06 to 0.10 µM primer sets listed in Table 3, 2.5 U of *Taq* DNA polymerase and 50 to 100 ng of template DNA. The following PCR conditions were used for amplification: an initial denaturation step at 95°C for 5 min, followed by 30 amplification cycles at 94°C for 30 s, 53°C for 30 s, and 72°C for 1 min, with a final extension step at 72°C for 5 min. Samples (5 µl) of the PCR products were subjected to agarose gel electrophoresis for examination.

Table 3. Primers used for serotyping (Sun et al., 2012)

C. sakazakii serotype	Target gene	Primer	Sequence (5'- 3')	Final conc (uM)	Amplicon size bp
O1	wzy	wl-35646	CCCGCTTGTATGGATGTT	0.10	364
		wl-35647	CTTTGGGAGCGTTAGGTT	0.10	
O2	wzy	wl-37256	ATTGTTTGCGATGGTGAG	0.06	152
		wl-37257	AAAACAATCCAGCAGCAA	0.06	
O3	wzy	wl-37258	CTCTGTTACTCTCCATAGTGTTC	0.10	704
		wl-37259	GATTAGACCACCATAGCCA	0.10	
O4	wzy	wl-39105	ACTATGGTTTGGCTATACTCCT	0.06	890
		wl-39106	ATTCATATCCTGCGTGGC	0.06	
O5	wzy	wl-39873	GATGATTTTGTAAGCGGTCT	0.10	235
		wl-39874	ACCTACTGGCATAGAGGATAA	0.10	
O6	wzy	wl-40041	ATGGTGAAGGGAACGACT	0.06	424
		wl-40042	ATCCCCGTGCTATGAGAC	0.06	
O7	wzx	wl-40039	CATTTCCAGATTATTACCTTTC	0.06	615
		wl-40040	ACACTGGCGATTCTACCC	0.06	

3. Results

Out of the 154 different samples of powdered infant formula analyzed, only 3 (1.95%) were positive for *Cronobacter spp.*

3.1 Biochemical Identification of the Cronobacter Strains Using Phenotypic Assays

MicroScan WalkAway analysis identified all the isolates as *Enterobacter sakazakii* with 98.87% probability. Supplemental Table 1.

3.2 Cronobacter spp. *Identification Using the VITEK®MS MALDI-TOF*

VITEK MS MALDI-TOF (bioMerieux) analysis identified all the isolates as *C. sakazakii*. Supplemental Figures 1-4.

3.3 Identification of the Cronobacter Strains Using 16S rDNA Gene Sequencing

Amplification of the 16S rDNA gene from the *Cronobacter* isolates gave PCR products of about 1,500 bp. The PCR products were sequenced and identified using BLAST analysis (http://www.ncbi.nlm.nih.gov/). All amplification products were identified as the 16S rDNA gene of *Cronobacter* and had a 97-98% identity with *Cronobacter* spp.

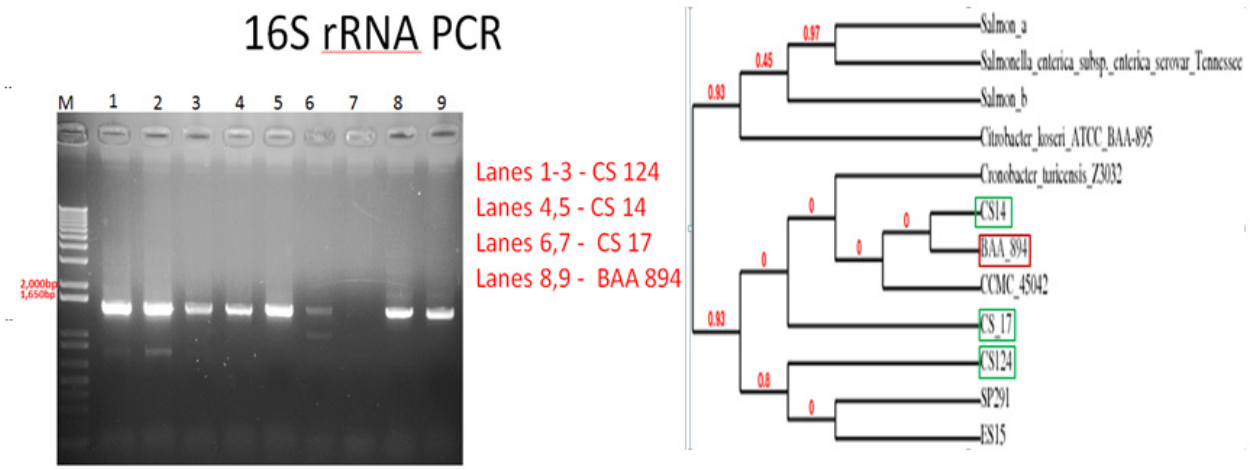

Figure 1. Gel image of the PCR products obtained from the amplification of the 16S rDNA gene from *Cronobacter* samples (CS) isolated from PIF. Lane M, DNA ladder (Invitrogen); lanes 1-3, CS 124; lanes 4 and 5, CS 14; lanes 6 and 7, CS 17; lanes 8 and 9, BAA 894 (positive control). The PCR products were about 1500 bp. Phylogenetic tree derived from the 16S rDNA sequence showing the relationship of CS 14, CS 17 and CS 124 to other *Cronobacter* spp.

3.4 β-subunit of RNA Polymerase Gene (rpoB) Based PCR Identification

Amplification of the *rpoB* gene with *C. sakazakii* primers yielded PCR products of about 514 bp (Figure 2). The four isolates including BAA 894 produced same sized bands suggesting that all the isolates were *C. sakazakii*. *Escherichia coli* which served as a negative control produced no band. When the *C. malonaticus rpo*B primers were used for amplification only CS14 and CS 124 produced bands of 251 bp suggesting that CS 14 and CS 124 are *C. malonaticus*. All other isolates including *E. coli* which served as a negative control, produced no band.

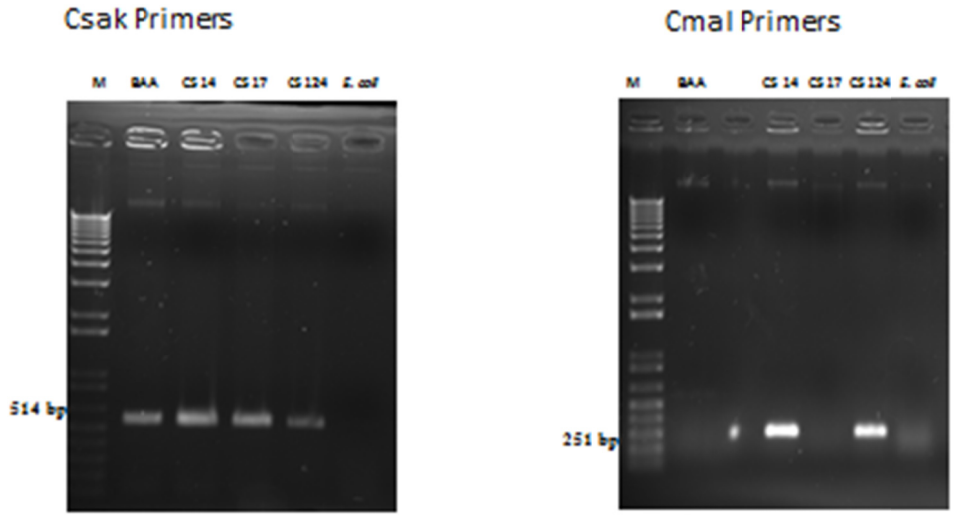

Figure 2. Gel image of the PCR products obtained from the amplification of the *rpoB* gene using *C. sakazakii* and *C. malonaticus rpoB* primers. C.sak: Lane M, DNA ladder (Invitrogen); lane 1, BAA 894; lane 2, CS 14; lane 3, CS 17; lane 4, CS 124 and lane 5, *E. coli*. The PCR products were about 514 bp. C.mal: Lane M, DNA ladder (Invitrogen); lane 1, BAA 894; lane 3, CS 14; lane 4, CS 17; lane 5, CS 124 and lane 6, *E. coli*. The PCR products were about 251 bp. C.mal primer identified CS 124 as *C. malonaticus* instead of *C. sakazakii*

3.5 Diguanylate Cyclase-Encoding Gene (cgcA) Based PCR Identification

Amplification of *cgc*A gene produced two bands that were characteristic of *C. sakazakii* (492 bp) and *C. malonaticus* (585 bp). BAA 894, CS 17 and CS 124 showed a band of approximately 490 bp characteristic of *C. sakazakii* while CS14 showed a fragment around 500-600 bp characteristic of *C. malonaticus* (Figure 3).

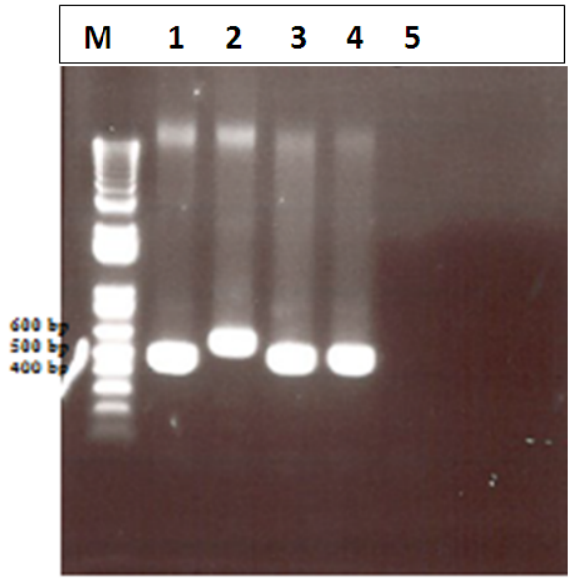

Figure 3. Gel image of the PCR products obtained from the amplification of the *cgc*A gene. Lane 1, BAA 894; lane 2, CS 14; lane 3, CS 17; lane 4, CS 124 and lane 5, sterile distilled water (negative control). *C. sakazakii* produced a band of about 492 bp while *C. malonaticus* produced a band of 585 bp. These results suggest that sample CS 14 (lane 2) corresponds to *C. malonaticus* and samples CS 17 (lane 3) and CS 124 (lane 4) correspond to *C. sakazakii*

3.6 Pulsed Field Gel Electrophoresis (PFGE)

Genomic DNA from *Cronobacter* isolates were analyzed by PFGE using *Xba*I as restriction enzyme. Eleven (11) to 16 fragments ranging from 40 to 1000 kbp were captured. All the pulsotypes obtained from each isolate were different from one another (Figure 4).

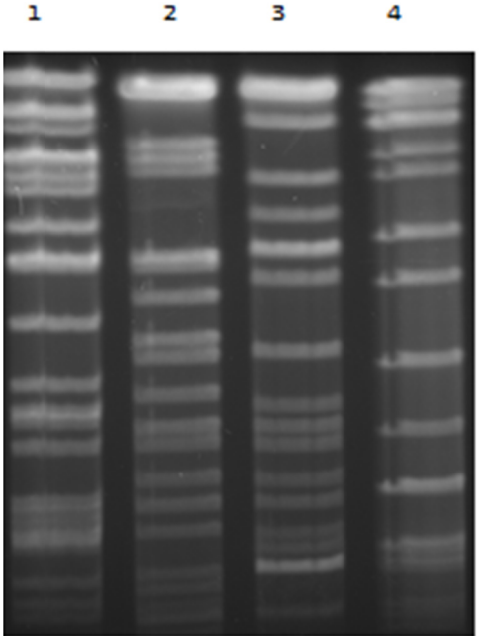

Figure 4. *Xba*I-pulsed-field gel electrophoresis (PFGE) patterns obtained from *Cronobacter* isolates. Lane 1, CS 14; lane 2, CS 17; lane 3, BAA 894, and lane 4, CS 124. Distinct PFGE profiles correspond to epidemiologically unrelated sources

3.7 Multi Locus Sequence Typing

Sequence Types (STs) and allelic profiles numbers were assigned to the isolates in accordance to the scheme initially established for *C. sakazakii* and *C. malonaticus* by Baldwin et al. (2009). The seven housekeeping genes were successfully sequenced from each isolate. MLST analysis showed that all three strains possessed new sequence type allelic variants and were different from those available on the *Cronobacter* PubMLST database (Table 4). CS17 and CS124 shared the allelic profile of *atp*D while all the three isolates shared the allelic profile of *pps*A.

Table 4. Multi locus sequence typing for *Cronobacter* isolates using the seven housekeeping genes

	atpD	*fusA*	*glnS*	*gltB*	*gyrB*	*inf*B	*ppsA*	Sequence Type
CS14 C. mal	10	13	64	75	72	14	1	303
CS 17 C. sak	3	17	13	57	58	63	1	304
CS 124 C. sak	3	15	28	22	5	38	1	296

3.8 Molecular Characterization of Cronobacter Lipopolysaccharide O-Antigen Gene Clusters Using Serotype Specific PCR Primers

Serotyping assays based on PCR specific to O-antigen genes have become acceptable methods for typing many Gram-negative bacteria. At the present work, this method was successfully applied to identify *Cronobacter* serotypes. PCR analysis using the primers described by Sun *et al*. (2012) identified sample CS 14 as *C. sakazakii* serotype O:6, which is also the same as *C. malonaticus* serotype O:2 according to Yan et al. (2015).

PCR-products obtained from the analysis of samples CS 17 and CS 124 correspond to *C. sakazakii* serotype O:2 and O:4, respectively. As expected, the O-antigen serotyping scheme obtained for the control strain of *C. sakazakii* ATCC BAA 894 agrees with that reported for the O1 serotype (Figure 5).

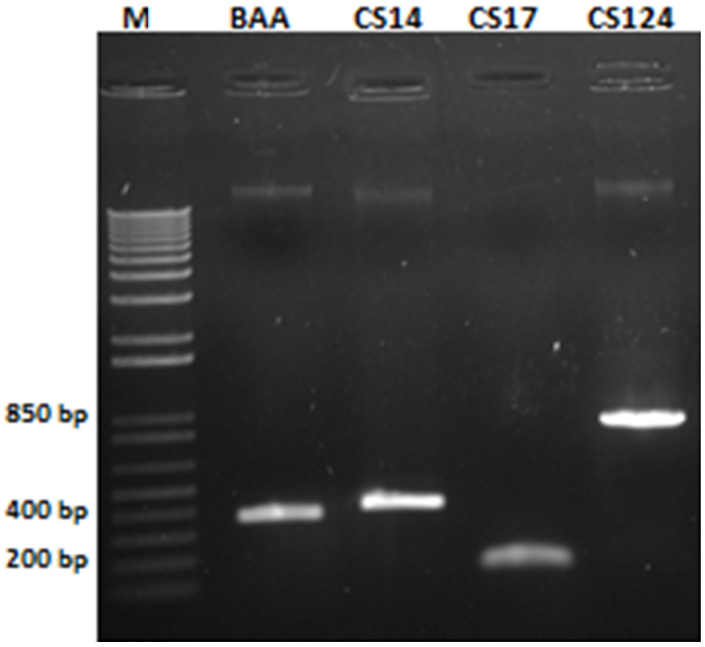

Figure 5. Gel electrophoresis showing the O-antigen binding patterns for all C. sakazakii isolates. Bands of different sizes define dstinct serotypes. Lane 1, BAA 894; lane 2, CS 14; lane 3, CS 17; lane 4, CS 124. *C. sakazakii* O:1 (364 bp); *C. sakazakii* O:2 (152 bp); *C. sakazakii* O:4 (840 bp); *C. malonaticus* O:2 (424 bp)

4. Discussion

The risk of *Cronobacter* infection to neonates and immunocompromised individuals is very high. The use of correct methods for identification of this bacterium will provide accurate results on the contamination of *Cronobacter* in food products and help in understanding the epidemiology of infections.

MicroScan WalkAway analysis (Beckman Coulter) is designed for use in determining antimicrobial agent susceptibility and/or identification of an organism to the species level. All the isolates were identified by the MicroScan as *Enterobacter sakazakii* (98.87% probability). *Cronobacter sakazakii* was formerly referred as *E. sakazakii,* and most likely the MicroScan WalkAway system database used in this study needs to be updated with the correct *Cronobacter* taxonomy.

Matrix Assisted Laser Desorption Ionization Time-of-Flight analysis (VITEK®MS MALDI-TOF) (bioMerieux) is a rapid and cost-effective system that is replacing conventional phenotypic methods for routine identification of bacteria. Microbial identification is based on the comparison of a protein spectrum generated from intact whole bacterial cells to a database of species-specific reference protein profiles using a particular algorithm (Dubois et al., 2012). All three of the isolates were identified by the MALDI-TOF system as *C. sakazakii* but CS14 was later identified as *C. malonaticus* using the *cgcA* and *rpoB* species-specific PCR assays. *C. sakazakii* and *C. malonaticus* are very closely related with 99% gene sequence similarity (Li et al., 2012). This could be the reason why CS14 was identified as *C. sakazakii* instead of *C. malonaticus* by the MALDI-TOF system. Jamal et al. (2014) reported 99.9% (n= 806) correct identification by VITEK®MS MALDI-TOF to the genus level and 99.0% to the species level of 507 Gram negative bacilli, 16 Gram negative cocci, 267 Gram positive cocci and 16 Gram positive bacilli, made up of 39 genera and 70 species.

The 16S rDNA gene is widely used as a phylogenetic target as it is a highly conserved gene, ubiquitous in all organisms and contains variable and hypervariable sequence regions (Kuhnert et al., 1996). A BLAST comparison of the 16S rDNA nucleotide sequences of the isolates showed a sequence similarity of about 97% to 98% with *Cronobacter* species. The phylogenetic tree generated with the 16S rDNA sequences showed all of the isolates grouping within *Cronobacter* species clusters. The 16S rDNA gene sequence analysis however, has limitations for

discrimination between very closely related organisms such as *C. sakazakii* and *C. malonaticus* because of minimal sequence diversity or the presence of multiple copies of 16S rDNA gene loci (Carter et al., 2013) CS 14 which was previously characterized as *C. malonaticus* using the *cgcA* and *rpoB* species-specific PCR assays was closely clustered to *C. sakazakii* ATCC BAA 894. This is highly misleading, thus showing the limitation of the 16s rDNA gene.

The *rpoB* gene sequence analysis has been proposed as a method for inferring relationships among very closely related species (Adekambi et al., 2009). Li et al. (2012) reported that phylogenetic analysis based on partial *rpoB* gene sequence analysis cannot distinguish between *C. sakazakii* and *C. malonaticus* even though it can differentiate these two species from other *Cronobacter* species. All isolates including BAA 894 showed similar band patterns using the *C. sakazakii rpo*B primers while CS 14 and CS 124 showed a similar pattern using the *C. malonaticus rpo*B primers. This result confirms the reported by Li et al. (2012) because CS 124 was identified as *C. sakazakii* using the multiplex *cgc*A primers.

Cyclic diguanylate (c-di-GMP) is a bacterial second messenger signal transduction molecule recognized for its involvement in the regulation of a number of complex physiological processes, including bacterial virulence, biofilm formation, and persistence (long-term survival) (Sondermann et al., 2012). Carter *et al*., (2013) reported the use of *Cronobacter* multiplex *cgc*A PCR assay to identify *Cronobacter* strains in a single reaction. This PCR assay was found to be 100% specific (n=305) and 100% sensitive (n=20). The multiplex *cgc*A primers were used to identify sample CS14 as *C. malonaticus,* and CS17 and CS124 as *C. sakazakii.* The control strain BAA 894 was correctly identified as *C. sakazakii.*

Applying discriminatory molecular subtyping methods to characterize foodborne pathogens facilitates the detection of outbreaks, sources of infection, and transmission pathways (Fields et al., 2011). Epidemiologically related isolates share the same profile (Kuhnert et al., 1996). All *Cronobacter* isolates and the control strain BAA 894 demonstrated distinct PFGE profiles, indicating different sources.

The set of seven housekeeping genes in MLST has greater sequence diversity than the 16S rRNA gene and has been applied to identify many bacteria (Urwin & Maiden, 2003). New sequence types were created for each of the isolates based on the allelic combination of the seven loci. None of the *fus*A profiles were shared between any of the isolates, thus proving that all the isolates are different. This locus has been recommended for use with two PCR primer sets to define species of *Cronobacter* without the ambiguity of 16S rRNA gene sequence analysis. MLST has proven to be an effective and robust typing scheme for the *Cronobacter* genus and has exhibited a high level of discrimination between the isolates (Joseph et al., 2012). The new sequence types (303, 304 and 296) are not clustered with any other known *Cronobacter* pathovars but further work will be needed to analyze the genes that are present in these strains.

The O-antigen is a highly variable component of the lipo-polysaccharide of Gram-negative bacteria and is used for the development of both serological and molecular typing methods (Jarvis et al., 2013). *C. sakazakii* O-antigen gene clusters of all seven serotypes are located on the chromosome between the housekeeping genes *galF* and *gnd.* All the isolates in this study proved to be of different serogroups. Sun et al. (2012) reported the high-level identity (99.3%) of *C. sakazakii* O6 and *C. malonaticus* O2, implying the recent lateral transfer of the respective O-antigen gene cluster between these two species. However, Yan *et al*. (2015) reported that the strains originally used to design the Sun-based serotype primers may have been misidentified as *C. sakazakii.*, which subsequently led to an incorrect identification of the corresponding serotypes. So, *C. sakazakii* O6 serotype should be corrected to *C. malonaticus* O2. Interestingly, *C. sakazakii* O2, to which CS 17 belongs, was isolated between 2010 and 2011 from infant clinical cases of *Cronobacter* infections in the USA (FDA/CFSAN, 2011).

5. Conclusion

The emerging pathogenic genus *Cronobacter* was identified as a contaminant in powdered infant formula retailed in Nigeria. The use of PCR assays, biochemical identification tests, and gene sequenced-based methods gave a reliable identification and profiling of all the *Cronobacter* isolates thus limiting the misidentification of false positive and negative results. Our results suggest that more severe measures must to be taken in order to improve the quality control on powdered infant formula production in order to protect neonates and infants from diseases caused by *Cronobacter* spp., which sometimes can be fatal.

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Appendix A

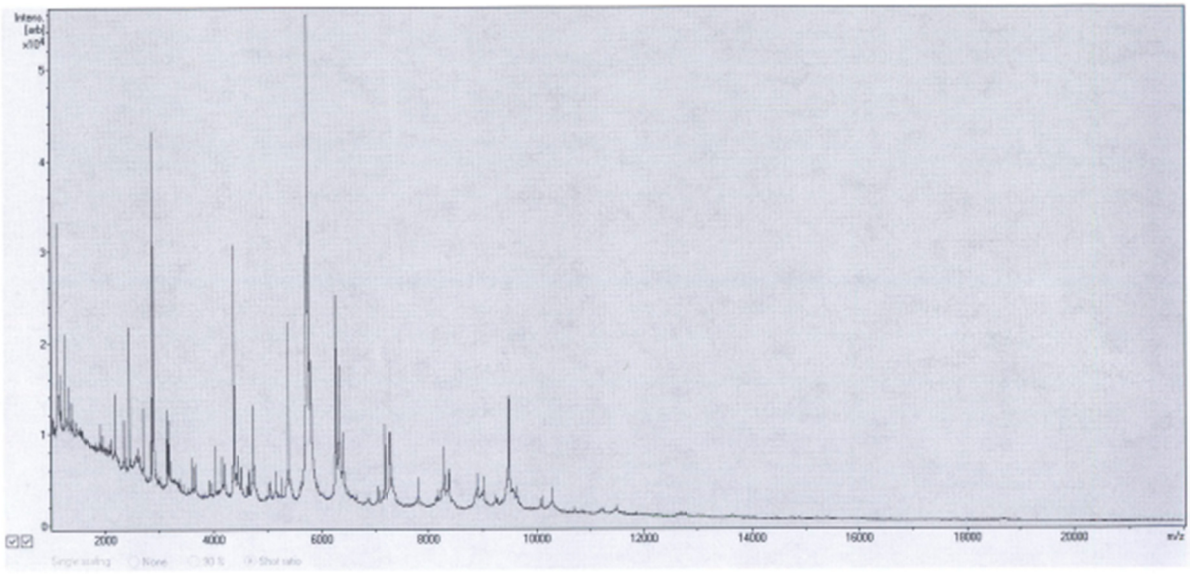

Figure A1. Mass Spectrometry of VITEK MALDI-TOF for CS 17

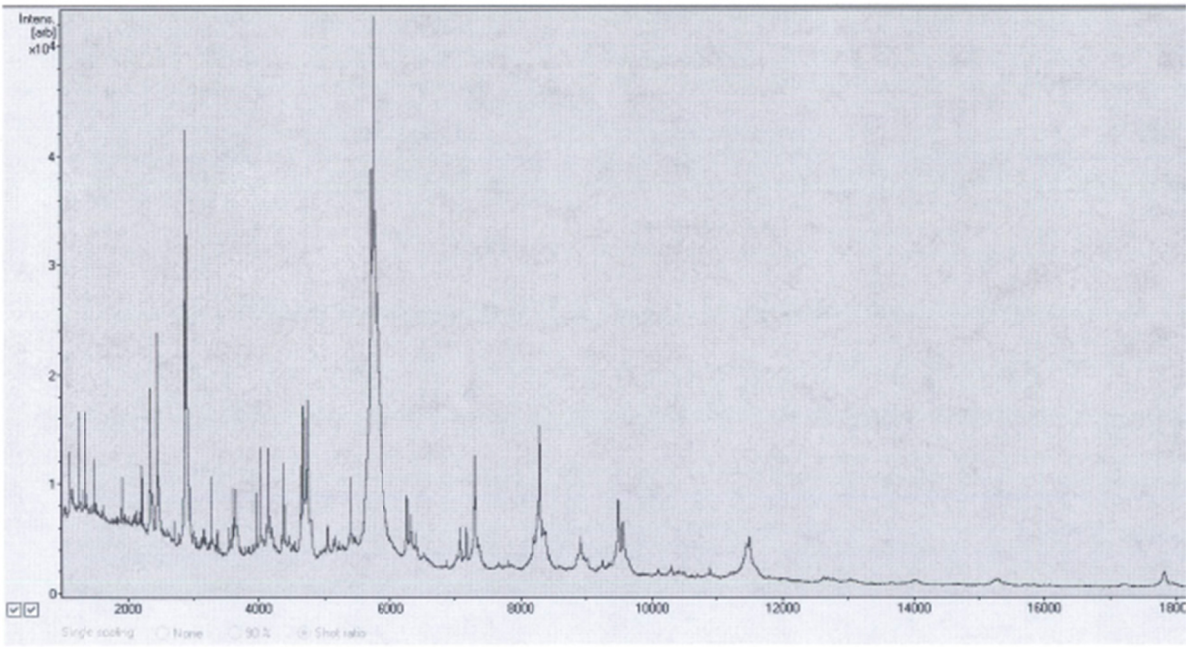

Figure A2. Mass Spectrometry of VITEK MALDI-TOF for CS 14

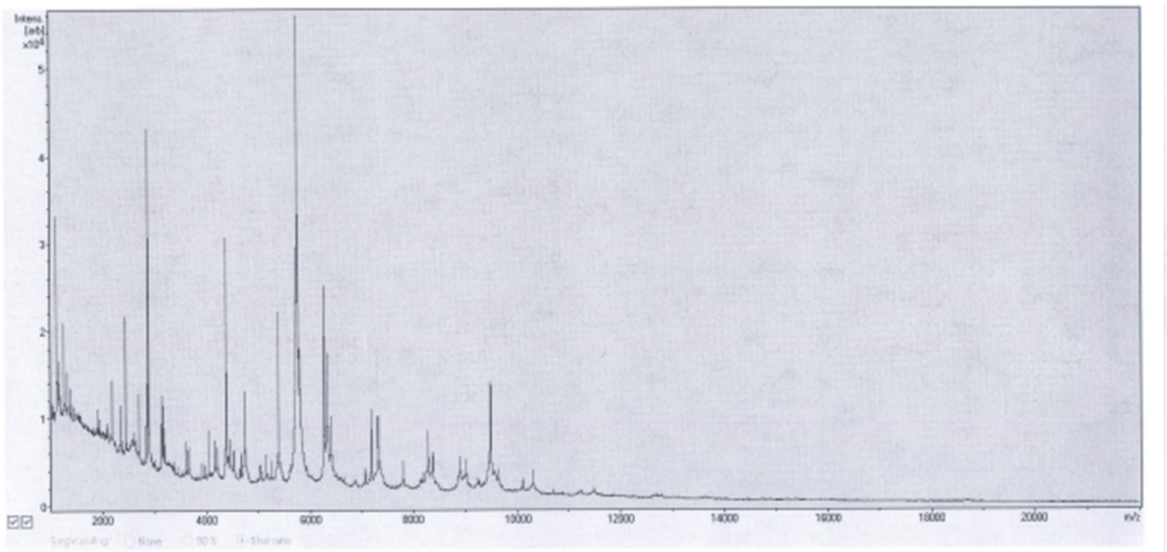

Figure A3. Mass Spectrometry of VITEK MALDI-TOF for CS 124

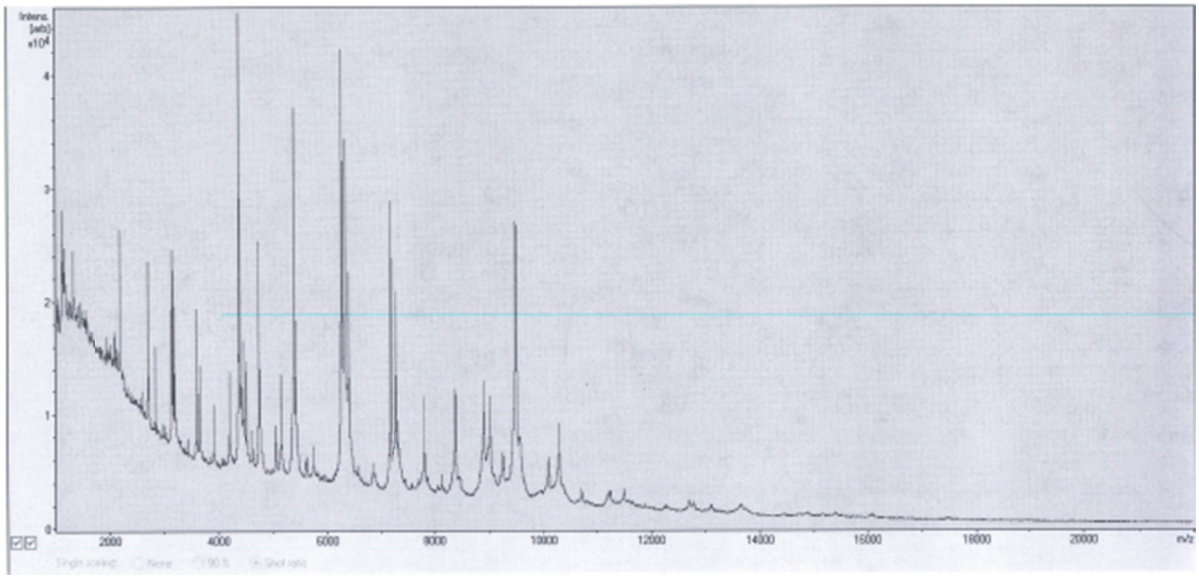

Fig A4. Mass Spectrometry of VITEK MALDI-TOF for BAA 894

Table A**1.** Biochemical characterization of isolates based on Microscan walkaway analysis.

Biochemicals	BAA 894	CS 14	CS 17	CS 124
Gram Reaction	-	-	-	-
Glucose-GLU	+	+	+	+
Sucrose-SUC	+	+	+	+
Sorbitol-SOR	-	-	-	-
Raffinose-RAF	+	+	+	+
Rhamnose-RHA	-	-	-	-
Arabinose-ARA	+	+	+	+
Inositol-INO	+	+	+	+
Adonitol-ADO	-	-	-	-
Mellibiose-MEL	+	+	+	+
Urea-URE	-	-	-	-
H_2S	-	-	-	-
Indole-IND	-	-	-	-
Lysine-LYS	-	-	-	-
ArginineARG	+	+	+	+
Ornithine-ORN	+	+	+	+
Tryptophan Deaminase-TDA	-	-	-	-
Esculin Hydrolysis-ESC	+	+	+	+
Vogues-Proskauer-VP	+	+	+	+
Citrate-CIT	+	+	+	+
Malonate-MAL	-	+	-	-
Galactosidase-ONPG	+	+	+	+

Colistin-Cl_4	-	-	-	-
Cephalothin-CF_8	+	+	+	+
Oxidase-OXI	-	-	-	-
Acetamide-ACE	-	-	-	-
Cetrimide-CET	-	-	-	-
Nitrofurantoin-Fd_{64}	-	-	-	-
Kanamycin-K_4	-	-	-	-
Nitrate-NIT	+	+	+	+
Oxidation-Fermentation-OF/G	+	+	+	+
Penicillin-P_4	+	+	+	+
Tartrate-TAR	-	-	-	-
Tobramycin-TO_4	-	-	-	-
CAT	+	+	+	+
Identification	*E. sakazakii*	*E. sakazakii*	*E. sakazakii*	*E. sakazakii*