

Detection and Molecular Characterization of *Cronobacter sakazakii* Isolated from Powdered Infant Formula (PIF) from North Central Region, Nigeria

Simon I. Enem^{1*}, Chidozie O. Ogbu¹, Chinwe E. Okoli¹, Ene Godwin¹, Gabriel K. Omeiza¹, Pascal U. Umeakuana², Wesley D. Nafarnda¹

¹Department of Veterinary Public Health and Preventive Medicine, University of Abuja, Abuja, Nigeria

²Departments of Veterinary Medicine, Faculty of Veterinary Medicine, University of Abuja, Abuja, Nigeria

Email: *simon.enem@uniabuja.edu.ng

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Abstract

Cronobacter sakazakii is an emerging ubiquitous and opportunistic pathogen that currently contaminates a wide spectrum of foods including powdered milk and poses a lethal threat to neonates, the elderly and persons with immune deficiencies. They cause life threatening neonatal meningitis, septicemia, and necrotizing enterocolitis. A total of 360 samples of powdered infant formula were collected from postnatal hospital attendees reconstituting the PIF for their children in the North Central region of Nigeria where cases of infant mortality are very high and presenting as enterocolitis and diarrhea. Pre-enriched samples were cultured in chromogenic *Cronobacter* broth and were then further sub-cultured into a chromogenic *Cronobacter sakazakii* agar. They were positive, exhibiting yellowish cultures typical of *Cronobacter sakazakii*. Biochemical tests of the isolates were also carried out and indicated the presence of *Cronobacter sakazakii*. The isolates were then characterized molecularly using specie specific PCR detection of *Cronobacter sakazakii*. The targeted genes of interest were *ompA* gene and *CPA* gene. The isolates tested showed bands for *ompA* gene on electrophoresis imager and were confirmed as *Cronobacter sakazakii*. In Nigeria, majority of infants are still fed with PIF. There is no existing data on the detection of *Cronobacter sakazakii* previously reported in the North central region of Nigeria hence the need to carry out the present study. The result of the study demonstrated the need for effective prevention and control measures as contamination of PIF with *Cronobacter sakazakii* constituted potential public health risk to neonates and infants.

Keywords

Detection, Molecular Characterization, *Cronobacter sakazakii*, Powdered Infant Formula (PIF)

1. Introduction

Cronobacter sakazakii is considered an emerging ubiquitous and opportunistic pathogen associated with necrotizing enterocolitis, meningitis, septicemia amongst infants and immunocompromised neonates with 40% - 80% mortality rate [1] [2] [3] [4]. In recent times, infection with *Cronobacter sakazakii* has also been reported in all age groups especially those with immunocompromising conditions and the elderly [5] [6].

The organism is ubiquitous and has been isolated from a wide range of foods and beverages such as meat and meat products, milk, cheese, fermented bread, vegetables, grains, herbs and spices [7] [8] [9] [10] [11] and also recovered from the human gastrointestinal tract and thought like as members of the normal fecal flora [12]. While the primary reservoir of *C. sakazakii* is yet to be defined, plant materials are believed to be the likely source [13]. Powdered infant formula has also been implicated as a vehicle of infection in numerous cases [14].

There have been several reports around the world of the recovery of *Cronobacter sakazakii* from PIF [7] [15] [16] and also from various sites and equipment in PIF processing facility [17]. Literature review indicated an epidemiologic linkage between contaminated PIF and case of *Cronobacter sakazakii* infections in infants [1] [15] [18].

Cronobacter sakazakii is gram-negative, rod-shaped, motile and facultative anaerobic bacteria belonging to the family Enterobacteriaceae and was identified as unique species by [19]. The genus *Cronobacter* has been divided into seven species namely *Cronobacter sakazakii*, *Cronobacter malonaticus*, *Cronobacter turicensis*, *Cronobacter mytjensis*, *Cronobacter dublinesis*, *Cronobacter universalis* and *Cronobacter condimenti* [20] [21]. The predominant species associated with neonatal infection is *Cronobacter sakazakii* [22]. Due to the strong ability to resist desiccation environment, *C. sakazakii* strains can persist in PIF for more than one year [23]. There is need to monitor the presence of *C. sakazakii* in commercial PIF [24]. *C. condiment* was identified as the seventh species recently by [20] and *C. universalis* replaced *C. gemospecies* [24].

Clinical presentations of *Cronobacter* infections in infants include NEC, bacteremia and meningitis, with case fatality rates ranging between 40% and 80% being reported [8] [25]. Infections in older infants have also been noted [25].

Several molecular means, including PCR assays that targeted the gene responsible for α -glucosidase activity (*gluA*), 16S rRNA gene sequencing and internal transcriber spacer (ITS) sequence between 16S and 23S rRNA, have been shown to be more reliable method for detection of *Cronobacter species* [26]. The outer mem-

brane protein A (*ompA*) of *C. sakazakii* plays an important role in invading human intestinal epithelial cells and brain microvascular endothelial cells [27] [28]. The sequence analysis of *ompA* gene has been applied to identify and type this pathogen for purposes of pathogenicity [27] [29]. Furthermore, *rpoB* allele sequence is also included in the international *PubMLST* database [24]. Therefore, a comprehensive comparative analysis of *C. sakazakii* strains isolated from PIF using MLST, O-antigen serotyping, *ompA* scheme, and *rpoB* scheme is warranted [24].

2. Materials and Methods

2.1. Study Area

Nigeria is divided into six geo-political zones. The study was carried out in the North Central zone comprising of six states namely Niger, Kogi, Nassarawa, Benue, Kaduna and Plateau states and Abuja FCT.

2.2. Study Design

The work was a cross sectional epidemiological study and a multi-staged sampling method was adopted. In the first place, a simple random sampling by balloting was used to select 3 out of the states in the North Central zone of Nigeria namely Abuja FCT, Nasarawa and Kogi states. Simple random sampling was also used to select 2 out of the selected major hospitals in each of the states. Systematic sampling method was then used to select one out of every five mothers coming for postnatal hospital examination.

2.3. Sample Collection and Isolation

Samples were collected under aseptic conditions and transported to Peak Laboratory Limited, Gwagwalada, Abuja where the initial analysis was carried out. A total of 360 samples of powdered infant formula were collected from nursing mothers attending postnatal hospital examination in the three of the six North Central states of Nigeria (Nasarawa—142; Abuja—98; Kogi—120).

A modified version of ISO/TS 22964 [30] method was used in the isolation of bacteria. An enrichment media of buffered peptone water (BPW) supplemented with 8 mg/litre vancomycin 10 mg/litre cefsulodin and 0.05 mg/litre cefixine (BPW-VCC) was prepared to suppress the growth of gram positive organisms, *Aeromonas* and *Proteus* spp. [31]. A total of 25 g of sample was mixed with the pre-enrichment medium of buffered peptone water (BPW) and incubated at 37° for about 20 h. This was followed by a transfer of 0.1 ml of the pre-enriched culture to *Cronobacter* screening broth (Oxoid, UK) [32] and incubated at 42° for 24 h. The positive samples which showed yellowish-white colouration were then streaked on chromogenic *Cronobacter sakazakii* agar and incubated at 37°C for 24 h. The isolates that showed yellowish colouration were further subjected to the following biochemical tests—indole production, catalase test, oxidase production, methyl red, Voges-Proskauer and acid production with nucitol.

2.4. DNA Extraction

Presumptive *Cronobacter* isolates were later incubated overnight at 37°C in Luria Bertani broth. Genomic DNA was extracted using a Genomic DNA isolation kit (Zymos, USA) according to the manufacturer's instructions. Concentration and purity of DNA samples were estimated by Nanodrop 2000 (Thermo Scientific, USA).

2.5. Molecular Characterization

The molecular characterization of the positive isolates was carried out at the West African Centre for Cell Biology of Infectious Pathogens (WACCBIP), University of Ghana. The DNAs from the positive isolates were first extracted using Zymos test kits according to manufacturer's specifications. Amplification of 16S rRNA specific for *Cronobacter species* was carried out using conventional PCR.

PCR using a hot start Taq polymerase for the identification of *Cronobacter sakazakii* by the amplification of *cpaA* gene and the virulence associated genes of the outer membrane protein A (*ompA*) was carried out. The following two species specific primer sets for *Cronobacter sakazakii* were commercially procured (Inqaba Biotec, South Africa) and used for the PCR (Table 1). For a 100 µM stock solution of the Forward and Reverse *CPA* primers, 588.31 µl and 543.34 µl of water were added respectively. Also for the Forward and Reverse *ompA* primers, 554.58 µl and 516.66 µl of water were added respectively. The PCR cycling conditions consisted of an initial denaturing step at 95°C for 8 min and a second denaturing at 95°C for 30 s, followed by 30 cycles of 58°C for 30 s, extension at 72°C for 30 s, followed by a final 7 min extension step at 72°C. Another 4°C was added for the end of PCR and for keeping the samples till removal and storage. The PCR results were subjected to gel electrophoresis using 1.5% agarose gels and were visualized with ethidium bromide (EtBr) using a computer imager.

3. Results

Of the 360 samples analyzed, 16 tested positive after cultural examination as presented in Table 2.

The positive samples were further confirmed to be *Cronobacter specie* using biochemical tests (Table 3).

The two genes of interest (GoI) typed in this study were *CPA* and *ompA* genes. Only the *ompA* gene showed clear bands on agarose gel (Figure 1).

An image of the Agarose gel result post electrophoresis following amplification

Table 1. Specific primers for identification of *Cronobacter sakazakii*.

Primer name	Primer sequence (5'-3')	Barcode	Length
<i>ompA</i>	F (<i>ompA</i>) TCAAAGCTCAGGGCGTACAG	S3CE9	20 bases
	R (<i>ompA</i>) ACCCTGGTTGTAAGCGTCAG	S3CEA	20 bases
<i>CPA</i>	F (<i>cpA</i>) GACAACCCTGAGTTCTGGTAA	S3CE7	22 bases
	R (<i>cpA</i>) ATGCGTATTTCTGCTGGTAA	S3CE8	20 bases

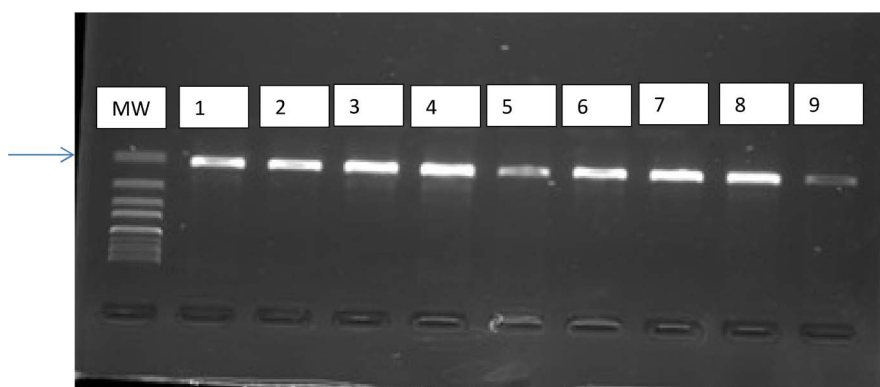
Table 2. Cultural isolation of Cronobacter species.

Sample location	No of samples collected	No positive	% positive
Nasarawa	142	8	5.6
Abuja	98	3	3.1
Kogi	120	5	4.2
Total	360	16	4.4

Table 3. Biochemical identification of Cronobacter species.

Isolate no & Source state	Indole	Biochemical tests				
		MR	VP	Inocitol	Oxidase	Catalase
Nasarawa						
N5	-	-	+	+	-	+
N28	-	-	+	+	-	+
N37	-	-	+	+	-	+
N76	-	-	+	+	-	+
N78	-	-	+	+	-	+
N82	-	-	+	+	-	+
N112	-	-	+	+	-	+
N126	-	-	+	+	-	+
Abuja						
A15	-	-	+	+	-	+
A27	-	-	+	+	-	+
A63	-	-	+	+	-	+
Kogi						
K9	-	-	+	+	-	+
K22	-	-	+	+	-	+
K46	-	-	+	+	-	+
K51	-	-	+	+	-	+
K85	-	-	+	+	-	+

Key: Indole production, MR—Methyl red, VP—Voges Proskauer, acid production with Inositol, Oxidase and Catalase tests.

**Figure 1.** PCR result.

of *ompA* gene. Wells were loaded with PCR products from test samples 1 - 10 and positive control. EtBr was added to the gel before electrophoresis to a final concentration of 0.5 µg/l followed by separation at 100 v for 1 hr.

4. Discussion

Cronobacter infection poses a very high risk to neonates and immunocompromised individuals and proper identification of the organism will provide good understanding of the epidemiology of the infection. Contamination of PIF due to *Cronobacter sakazakii* has been given serious attention in the developed world [16] [33]. In the developing world and Nigeria in particular, scanty work has been carried out on Cronobacter species [34] [35] [36] hence it needs further investigation. No reported study on *C. sakazakii* has been carried out in the North Central region of Nigeria where the incidence of meningitis and colitis remains very prevalent.

In the present study, out of the 360 samples analyzed, 16 were positive indicating an average prevalence rate of 4.4% in the North Central zone of Nigeria. The prevalence rate fell within the range reported in previous studies [37] [24]. The prevalence in this study could be closely compared to the work of [38] (4.3%, 23 out of 530). The highest prevalence was recorded in Nasarawa state (5.6%) followed by Kogi state with 4.1% prevalence and then Abuja showing the least prevalence of 3.1%. The variation in the prevalence rate may be directly proportional to the level of perception of personal hygiene. Abuja is more cosmopolitan inhabiting relatively more educated and civilized members of the public.

The presumptive positive cultures after isolation were equally subjected to biochemical tests for initial confirmation. They all responded to the key biochemical tests for identification of *C. sakazakii*. The isolates were Catalase positive and Oxidase negative. They were negative to Indole and Methyl Red production and positive for Voges Praskaur. They were all able to produce acid from inositol. The result is in agreement with the reports by [19] [39] [40]. Inositol fermentation has been proposed recently as a marker of pathogenicity for Cronobacter based on the presence of the inositol monophosphatase gene (*suhB*) in some pathogenic strains. However, the inositol utilization operon *GR29* was found in Cronobacter strains isolated from the environment, whilst also being absent in genomes of pathogenic strains [41]. Therefore, its role in virulence is unclear at present [42].

One of the genes of interest in this study *OmpA* reported to be involved in the basolateral invasion of the brain by *C. sakazakii* [43] was identified. The outer-membrane protein A (*OmpA*) is probably the best-characterized virulence marker of Cronobacter [44] [43]. *OmpA* appears to play a major role in Cronobacter invasion. In vitro, it was found that *OmpA* plays a critical role in the invasion of human intestinal epithelial cells (*INT-407*), *Caco-2* cells and *HBMECs* [27] [45] [28] [43]. Invasion studies proved that *OmpA* is crucial for

C. sakazakii invasion of *INT-407* and *Caco-2* cells [42].

The second gene of interest tested was *Cronobacter* plasminogen activator (*CPA*) gene. There was no clear bands post electrophoresis following amplification with *CPA* gene. Further research through plasmid sequencing is suggested. According to [42], *CPA* along with other genes and associated putative adhesions locus are harboured on a family of *RepFIB*-related plasmids (*pESA3* and *pCTU1*) suggesting that these are common virulence plasmids. The report indicated that 98% of the 229 tested *Cronobacter* strains possessed these plasmids. However, the report stated that the presence of *CPA* gene depends on species, demonstrating strong correlation with the presence of virulence traits, plasmid type and species.

5. Conclusion

The result of the study demonstrated the need for effective prevention and control measures as *Cronobacter sakazakii* constituted potential public health risks to neonates and infants. Powdered Infant Formula (PIF) is not sterile products and should be treated as a possible food safety issue for high risk population such as infants and neonates due to the presence of *C. sakazakii*.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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