Vol. 05, No. 02, pp. 42 – 47 (2024)
ISSN: 2708-1818

JOURNAL OF LIFE AND BIO-SCIENCES RESEARCH

www.jlbsr.org

Phenotypic, Molecular Identification and Virulence Assessment of Cronobacter spp Isolated from Clinical Samples of Children Under Two Years in Mosul City/Iraq

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Abstract

***Cronobacter* spp. is known to be a foodborne causative agent for a variety of diseases in both humans and animals. This study focused on isolating *Cronobacter* species from 150 clinical samples of children under two years (60 from blood and stool samples, 30 from CSF samples) collected from Ibn al Atheer and Al Khanssa Hospitals. The phenotypic identification of bacterial isolates were performed through culture, microscopic, and biochemical examinations and vitek-2 system. The results revealed a mixture of bacteria in 6.5% of the clinical specimens. The identity of isolates was 99% using the vitek-2 system for identifying *Cronobacter sakazakii, Cronobacter malonaticus, Cronobacter muytjensii,* and *Cronobacter pulveris*. Out of 150 specimens there were 8 (5.33 %) of specimens gave positive for *Cronobacter* spp which included: *C. sakazaki* 2/60 blood specimens (3.3%), *C. sakazaki* 2/60 stool specimens (3.3%), *C. malonaticus* 2/60 stool specimens (3.3%), 1/60 *C. muytjensii* and *C. pulveris* 1/60 (3.3%). The eight isolates phenotypically identified as *Cronobacter* were confirmed at the molecular level through 16S rRNA sequencing and submitted to NCBI under the accession numbers (OR825874, OR825875, PP126443, PP126444, PP126445, PP126455). The virulence profile of these isolates showed that 7/8 (87.5%) of *Cronobacter* strains exhibited haemolysin activity, 5/8 strains (62.5%) were able to produce the protease enzyme and 2/8 (25%) of *Cronobacter* strains were positive for lipase and lecithinase. All strains lacked the ability to produce a slime layer. The results also showed the ability of *Cronobacter* strains to produce biofilm by using the tube method and microtiter plate method but with different levels.**

Keywords: *Cronobacter spp, Virulence factors, Molecular Identification.*

Received: April 4th, 2024/ Accepted: September 7th, 2024/Online: October 7th, 2024.

I. INTRODUCTION

Cronobacter spp., belong to the Enterobacteriaceae family. They were first identified in 1961 through the work of Urmenyi and Franklin, who described these organisms as "yellow-pigmented *Enterobacter cloacae*." In 1980, the Japanese microbiologist Riichi Sakazakii reclassified *C. sakazakii* as a distinct species within the genus Enterobacter, primarily based on its unique genotypic and phenotypic characteristics (Pagotto and Abdesselam, 2012). During the period 1980 until 2007, this bacterium was classified in the genus *Enterobacter* but was later reclassified in *Cronobacter* (Iversen *et al*., 2007; Joseph *et al*., 2011). This classification was revised in 2008 and 2012, resulting in the addition of six new species which were recognized worldwide as an emerging foodborne pathogen, considered the sixth most common cause of nosocomial infection and antibiotic-resistant strains being observed more frequently (Forsythe, 2018). In 2002, the International Commission on Microbiological Specifications for Foods (ICMSF) designated *Cronobacter* as a pathogenic organism, causing high risk to human lives and inflicting serious long-term disorders (ICMSF, 2002). The World Health Organization (WHO) designated all *Cronobacter* species as human-harmful bacteria (FAO/WHO, 2008). *Cronobacter* infections can develop in adults, particularly those with major underlying diseases or cancers (Patrick *et al.*, 2014). This species can be isolated from any age group; however it is most common in youngsters under the age of 14 (Patrick *et al.*, 2014; Liu *et al.*, 2013). *Cronobacter* spp. can be isolated from clinical samples including throat swabs, urine, tracheal aspirates, bronchoalveolar lavage, cannulae, and sputum. Members of *Cronobacter* spp. are Gram-negative

doi: 10.38094/jlbsr502119

motile facultative anaerobes, possessing peritrichous flagella (ICMSF, 2002; FAO/WHO, 2008). These organisms may grow on nutrient plates producing glossy and matte colonies. It can also grow on MacConkey agar producing pink colonies due to its ability to ferment lactose (Lai, 2001). These bacteria may create α-glucosidase enzyme, resulting in "Blue-Green" colonies on Eosin Methylene Blue (EMB) and deoxycholate agar. They can also form non-diffusible yellow pigment colonies when grown on TSA medium at 25°C (ICMSF, 2002; Lai, 2001).

There are different species of *Cronobacter* cause infections that range from mild to dangerous. These comprise osteomyelitis, bacteremia, septicemia, wound infections, and urine infections. Nevertheless, adults who are not immunocompromised can contract serious infections from *Cronobacter* (See *et al*., 2007). Numerous molecular assays based on DNA sequences have been created to identify different species of *Cronobacter* (Smith and Brown, 2023). A different approach to quick and accurate identification is provided by some of these techniques. The identification of Enterobacteriaceae has frequently relied on analysis of conserved regions from the 16S rRNA gene. In a study, 189 isolates of *E. sakazakii* were among the 282 strains of Enterobacteriaceae on which Iversen *et al*. (2006) used the 16S rRNA. Four cluster groups were generated by the *E. sakazakii* strains, according to the analysis of ribosomal DNA genes (Iversen *et al*., 2006). Furthermore, a phylogenetic analysis research utilizing the full-length rDNA sequence was depended by Iversen *et al*., 2007. The findings of this investigation suggested moving *E. sakazakii* group to a different genus (Iversen *et al*., 2007). However, several drawbacks were noticed when using the 16S rRNA method on closely related *Cronobacter* species (Iversen *et al*., 2007). Furthermore, because 16S rRNA showed 99.7% identity to *C. sakazakii* and *C. amalonaticus*, it was unable to differentiate between them; as a result, *C. malonaticus* was classified as a subspecies of *C. sakazakii* (Iversen *et al*., 2007).

It is thought that the true estimate of neonatal *Cronobacter* infections is greater, notwithstanding the WHO's data on the prevalence of these illnesses (Friedemann, 2009). The *Cronobacter*-related newborn diseases in neonatal intensive care units have been epidemiologically connected to contamination of PIF, indicating that the birth canal is not a contributing factor to the infection process (Teramoto *et al*., 2010).

Although recent advancements in understanding the diseases caused by *Cronobacter* have spurred a surge in research, the precise mechanisms of their pathogenicity remain under investigation (Smith and Brown, 2023). Variable virulence capacities of *Cronobacter* isolates have been observed in epidemiological studies in addition to in vitro tissue culture projects (Almajed and Forsythe, 2016). The primary species linked to newborn infections are *C. sakazakii*, *C. malonaticus*, and *C. turicensis*.

Enterotoxins are known to be produced by *Cronobacter* as potential virulence factors. Using an experiment on nursing mice, the effects of *Cronobacter* enterotoxins were found to be fatal. In the same mice, *Cronobacter* was also able to generate proteases that destroyed the tissues at the infection site in addition to producing enterotoxins (Pagotto *et al*., 2003).

This work aimed to identify *Cronobacter* spp. by using traditional microbiological methods besides molecular method in addition to determine some of the virulence factors which is to our knowledge, the first study regarding the isolation, phenotypic, and molecular detection of *Cronobacter* spp. from specimens in the area of study (Mosul city/Iraq).

II. Materials And Methods

A. Collection of samples

A total of 150 samples including: (60) blood specimens from bacteremia patients, (60) stool specimens from patients with diarrhea, and (30) CSF specimens from meningitis patients, were collected from Mosul hospitals (Ibn Al Atheer and Al Khansa hospital) at the period from 1-9-2023 until 1-12-2023. All specimens were collected prior to the initiation of antibiotic medication and transported to the microbiology laboratory at Mosul University, College of Science in a cold box within 1-2 hours.

B. Cultivation of samples

Blood and CSF samples were inoculated in BHI broth and cultured for 24 hours at 37°C before being streaked on MacConkey agar plates. Stool specimens were directly inoculated onto MacConkey agar and incubated for 24 hours at 37 °C, then all colonies were transferred onto TSA medium and incubated for 72 hours at 25°C. Finally, they were cultivated on the Hichrome *Enterobacter sakazakii* agar and incubated at 37°C for 24 hours (Kim *et al*., 2008).

Cronobacter spp. were isolated in accordance with Cappuccino and Welsh (2018) as a second way for diagnosis. The selected isolates were examined under a light microscope to identify *Cronobacter* spp. The samples were then examined using basic biochemical tests such as the catalase, oxidase, triple sugar iron, IMVIC test, urease, DNase test in addition to motility. Final diagnosis was later validated by the Vitek-2 diagnostic system.

C. Molecular identification

Geneaid™ DNA Isolation Kit (Taiwan) was used as recommended by the instructions of the manufacturer for extraction of DNA.

Amplifying the 16S rRNA region of *Cronobacter* spp. was carried out by PCR using the universal primers 27F and 1522R as mentioned (Khaleel *et al*., 2023; Abdulrazzaq and Faisal, 2022). Successful amplicons 16S rRNA PCR products were sent for sequencing to the Psomagene sequencing company (USA). DNA sequences were compared to other 16S rRNA sequences submitted to NCBI using the BLAST website within NCBI. 16S rRNA

sequences were then submitted to GenBank and were provided accession numbers.

D. Phenotypic detection of virulence factors

To detect the ability of strains to produce biofilms, samples were grown on Congo red agar medium and incubated at 37ºC for 24 hours (Mittal *et al.*, 2009). Haemolysin detection was performed by culturing isolates on blood agar and slime layer detection was done according to (Al-Saffar, 2022). Biofilm detection was conducted using the tube method and the microtiter plate method (Christensen *et al*., 1982). The ability of isolates to produce protease, lipase and lecithinase was performed according to (Thomas *et al*., 2014; Faiz *et al*., 2021).

III. RESULTS

All eight Cronobacter spp. bacterial isolates fermented lactose on MacConkey plates and produced pink colonies having a thick mucoid center after 24 hours of incubation at 37°C. On Tryptone soy agar, the selected strains produced colonies that were light yellow to golden yellow pigmented while on Enterobacter Sakazakii agar they produced typical colonies (tiny green to bluish-green colonies) as well as the non-typical colonies that were slightly transparent, violet, and yellow, as shown in Figure (1). Isolates were examined under the microscope, and Cronobacter spp. were seen as Gram-negative rods.

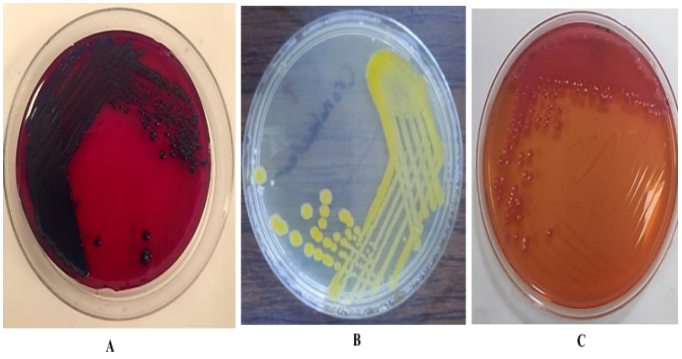

Figure 1. Colony morphology of *Cronobacter* spp on different culture media. (A): Growth on Trypton soy agar; (B): Growth on *Enterobacter sakazakii* agar; (C): Growth on MacConkey agar.

Biochemical identification

Biochemical tests mentioned in materials and methods were used to identify *Cronobacter* spp. isolates. The results are shown in Table 1 below:

Table 1. Biochemical tests used for the primary identification of *Cronobacter* spp

Tests	*Cronobacter pulveris*	*Cronobacter mutjensii*	*Cronoobacter malonaticus*	*Cronobacter sakazakii*
TSI	A/A	A/A	A/A	A/A
Catalase	+	+	+	+
Oxidase	–	–	–	–
Urease	–	–	–	–
Indole	+	+	–	+
Methyl red	–	–	–	–
Voges-Proskauer	+	+	+	+
Citrate	+	–	+	+
Motility	+	+	+	+
Nitrate reduction	–	–	–	–
Growth at 45oC	–	–	+	+

(-) negative result, (+) positive result, A/A: acidic / acidic

VITEK-2 system was used for the diagnosis of *Cronobacter* species, results indicated that all isolates belonged to *Cronobacter* spp with a probabilities ranged from 86.59% to98.45% (86.59%, 90.86%, 93.32%, 97.01%, 97.43%, 98.08%, 98.23% and 98.45%, respectively), after that we used the molecular identification method in order to precisely identify *Cronobacter* spp strains, so, the DNA from primarily identified *Cronobacter* isolates were extracted and strains were diagnosed via 16S rRNA sequencing technique, all of eight isolates were successfully amplified, amplicon of universal primer with a size of 1495bp, as shown in Figure 2.

The DNA sequencing of the amplified fragments for the isolates was performed with sequencing at (Psomagene company, Maryland, USA) and the results showed that they belonged to *Cronobacter* strains and were then submitted to gene bank under the accession numbers (OR825874, OR825875, PP126443, PP126444, PP126445, PP126455) as shown in BLAST NCBI website.

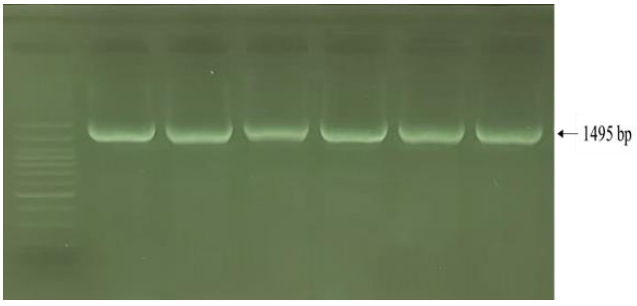

Figure 2. PCR Ampilification product of 16S rRNA region running on 1.5%agarose for a selection of *Cronobacter* isolates. First lane on the left shows the 100bp DNA ladder.

The results showed that 8/150 (5.3%) samples were positive for *Cronobacter* spp. 4(6.6%) isolates of Stool and 4(6.6%) isolates of blood were isolated and identified as *Cronobacter* spp by using different identification methods. *Cronobacter* spp. was not obtained from CSF samples as shown in Table 2. According to the previous identification methods, the results showed that out of 150 clinical specimens, *C. sakazakii* has been identified in 4 cases (2.6%) distributed as *C. sakazaki* 2/60 blood specimens (3.3%), *C.sakazaki* 2/60 stool specimens (3.3%), *C. malonaticus* 2/60 stool specimens (3%), and 1/60 *C. muytjensii* (1.6%) and *C. pulveris* 1/60 (1.6%) theme from blood samples (Table 2).

Table 2. Number and isolation ratio of *Cronobacter* spp. strains isolated from different clinical cases.

Clinical specimens	Number of specimens	Number of Cronobacter isolates (%)	Cronobacter species
Stool	60	4 (6.6%)	*Cronobacter sakazakii* (2) *Cronoobacter malonaticus* (2)
Blood	60	4 (6.6%)	*Cronobacter sakazakii* (2) *Cronobacter mutjensii* (1) *Cronobacter pulveris* (1)
Cerebrospinal fluid from meningitis	30	0 (0%)	
Total	150	8 (5.3%)	*Cronobacter sakazakii* (2) *Cronoobacter malonaticus* (2) *Cronobacter sakazakii* (2) *Cronobacter mutjensii* (1) *Cronobacter pulveris* (1)

The ability of *Cronobacter* spp isolates to hydrolyze blood was confirmed by streaking the isolates on blood agar, results showed that 7/8 isolates produced β-haemolysis on blood agar after 72 hours of incubation at 37°C. Only one isolate belonging to *Cronobacter sakazaki* was not able to hydrolyze blood. The results for protease activity using blood agar showed that two *C. malonaticus* strains, one *C. sakazaki* strain, *Cronobacter muytjensii*, and *Cronobacter pulveris* were positive protease producers showing a clear proteolysis phenotype.

The results showed that 7 (87.5%) isolates under study showed a strong ability to form the biofilm. five (62.5%) isolates were highly biofilm producers, 2(25%) isolates were moderate producers as shown in Table (3). Furthermore, the enzymatic activity observed in this study, where 25% of isolates were able to produce lipase and lecithinase enzymes, highlights the pathogenic potential of Cronobacter spp.

IV. Discussion

The current investigation, which was conducted among hospitalized children under the age of two in Mosul city/Iraq, found that the prevalence of *C. sakazakii* was 6.6% in stool and blood. A local investigation of *C. sakazakii* by (Al-Joubert *et al*., 2014) verified the presence of these bacteria (11.9%) in environmental and clinical samples, while another study demonstrated the presence of *C. sakazakii* in the stool of patients with diarrhea by 3% (Mahindroo *et al*., 2016). In 2006, The World Health Organization (WHO) confirmed that the annual infection rate in the United States among infants under normal weight was 8.7 per 100,000 children. There is currently no effective global control system in place to combat this pathogen. However, between 1961 and 2008, the World Health Organization recorded 120 occurrences of *Cronobacter* infection among newborns and children under the age of three (FAO/WHO, 2008). Although just 120 instances have been recorded worldwide, the prevalence of these microbes is growing (Teramoto *et al*., 2010). A study confirmed that infection with these bacteria threatens the lives of newborn newborns (infants 4 weeks old) (Mullane *et al*., 2008). The death rate ranges between 40 and 80 percent (Friedeman, 2007). *C. sakazakii* causes meningitis in babies, which is lethal within hours or days of birth (Henry and Fouladkhah, 2019). *C. sakazakii* affects 13% of neonates and infection in children under the age of two years is caused by their low weight, weak immune system, and consumption of dried baby milk, which results in intestinal necrosis (5-10%) and meningitis (80-40%) (Fiore *et al*., 2008).

Table 3. Virulence factors determination in *Cronobacter* spp strains understudy.

Bacterial species	Haemolysin production	Protease	Lipase	Slime layer	Lecithinase	Biofilm production	
						Tube method	Microtiter plate
C. malonaticus	+	+	–	–	–	+++	+++
C. malonaticus	+	+	–	–	–	+++	+++
C. sakazakii	+	–	–	–	+	+++	+++
C. sakazakii	+	–	–	–	–	++	+++
C.muytjensii	+	+	+	–	+	–	+++
C. pulveris	+	+	–	–	–	++	+++
C. sakazakii	+	+	–	–	–	+++	++
C. sakazakii	–	–	+	–	–	+++	+

According to Healy *et al*. (2010), *Cronobacter* can cause meningitis, intestinal necrosis, and bacteremia, as well as transmission from mother to child following cesarean birth (Zogay *et al*., 2003). While (Hunter and Bean, 2013) indicated that the infection could arise through the vagina during normal delivery and that *Cronobacter* could be involved. The results of virulence factors determination are showed in the Table (3). From these results we concluded presence difference between our *Cronobacter* spp in their abilities to produce the phenotypic virulence factors.

Cronobacter spp. ability to lyse blood and proteins was further validated by the presence of many haemolysin and protease-associated genes. Six potential genes linked with haemolytic activity were found in the genomes of all examined *Cronobacter* spp strains (Joseph *et al*., 2012). Other virulence factors found in *C. malonaticus* strains include bacterial haemolysins and protease. These activities are significant virulence factors in bacterial diseases (Alsonosi, 2017).

Bacterial haemolysin is a toxin that can lyse red blood cells (RBCs). It has three different effects: β haemolysis, α-haemolysis, and γ-haemolysis (Goebel *et al*., 1988). While bacterial proteases, such as the zinc-containing metalloprotease encoded by *zpx*, have the potential to degrade structural and functional proteins in host tissue, *Cronobacter* zinc metalloprotease has been shown to cause cell damage by inducing rounding of the Chinese hamster ovary. All studied strains successfully lysed horse blood RBCs, indicating type β-haemolysis activity. In addition, the bacteria destroyed the milk protein employed in the investigation.

The isolation of *Cronobacter* spp isolates from clinical samples was investigated using Congo red agar. The test yielded a favorable result for dark brown colonies with dry crystalline density, but a negative result for pink colonies. The investigation found that all *Cronobacter* spp. isolates were unable to generate the slime layer. This result disagreed with Alsonosi (2017) who showed that all isolates were active for slime layer production.

Two methods were employed to examine the bacterial ability to create biofilms. The tube method was used to assess the ability of bacterial isolates from clinical samples to produce biofilms. The presence of adhering cells on the test tube walls and bottom indicated a positive outcome.

The ability to form the biofilm was varied among isolates. Pathogenic bacteria generate biofilms that help them survive environmental challenges such as drying and antimicrobial treatments. Many variables could contribute to biofilm formation on the surface of medical equipment or even infected tissue (Annous *et al*., 2009). The results showed that 2 isolates (25%) under study showed the ability to produce lipase and lecithinase enzyme by destruction the egg yolk agar and six isolates did not produce this enzyme.

In a similar study conducted in Iraq, Al-Hilali *et al*. (2015) reported that a significant proportion of *Cronobacter* isolates from clinical samples exhibited strong biofilm-forming abilities. Their research indicated that biofilm formation enhances the bacteria's ability to survive on medical equipment surfaces and within host tissues, contributing to their persistence in hospital environments and their role in nosocomial infections. The presence of biofilm on medical equipment can lead to chronic infections and increased resistance to cleaning and sterilization procedures, posing a significant challenge for healthcare settings.

The ability to produce these enzymes, which can degrade egg yolk agar, indicates that these bacteria possess mechanisms to break down host tissues and evade immune responses. This enzymatic activity was also observed by Mahdi *et al*. (2016) in their study on *Cronobacter* isolates in Iraq, where a subset of the isolates produced lipase and lecithinase, contributing to their virulence.

V. Conclusion

This study provides valuable insights into the prevalence and characteristics of *Cronobacter* spp. isolated from clinical samples of children under two years of age. four *Cronobacter* species have been identified. *C. sakazakii*, *C. malonaticus*, *C. muytjensii*, and *C. pulveris*. most *of Cronobacter* spp. exhibited different virulence enzymes with varied ranges. These findings underscore the clinical relevance of *Cronobacter* spp. as an emerging pathogens in young children, highlighting the importance of accurate identification and characterization of these bacteria. The study's results contribute to a deeper understanding of the pathogenic potential and diversity of *Cronobacter* spp., which can inform preventive measures and therapeutic strategies in managing infections caused by these bacteria.

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