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IDENTIFICATION OF *CRONOBACTER SAKAZAKII* ISOLATED FROM POWDERED INFANT FORMULA AND STOOL OF INFANTS

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ABSTRACT

Cronobacter sakazakii is an emerging opportunistic pathogen contaminating powdered infant formulas causing lethal threats to neonates and immune-deficient infants. It causes life-threatening infections, septicemia, neonatal meningitis, and necrotizing enterocolitis. The aim of this study was to test the commercially available formulas that are intended for consumption by 0-6 months old infants (neonates and immune-compromised infants), for the presence of *Cronobacter* spp., and to determine the presence of *C.sakazakii* in the stool of these infants who consumed these formulas through conventional methods as culturing, biochemical tests and PCR. Fifty PIF samples (different brands) retailed in upper Egypt were collected from Assiut University Children Hospital at the Gastroenterology and Hepatology and Preterm Units, and we checked the presence of *C.sakazakii* in them. Fifty Stool samples were also collected from the infants who were fed the studied PIF samples, to study the presence of *C.sakazakii* in the stool of these infants. The samples underwent three steps of pre-enrichment, enrichment procedures, and subculture onto chromogenic *Enterobacter sakazakii* agar plates. Biochemical tests were afterwards carried out. Finally, molecular characterization using specific PCR was done to detect *Cronobacter sakazakii,* targeting the *ESA_02797 gene* which is found in all *C.sakazakii* strains. The results of this study shed light on the immense need for applying effective prevention and control measures and taking all the precautions needed during the production and preparation of PIF to hinder its contamination with *C.sakazakii* and to prevent the spreading of such fatal infections to infants with low immunity and neonates.

Keywords: *C.sakazakii*, PIF, Percentage, Detection, PCR

INTRODUCTION

Cronobacter sakazakii (C.sakazakii) is attracting considerable attention as a fatal emerging neonatal pathogen, that is associated with many outbreaks of serious life-threatening septicemia, necrotizing enterocolitis, as well as meningitis in infants and neonates globally (Elkhawaga *et al.*, 2020).

C.sakazakii, formerly named *Enterobacter sakazakii*, a non-spore-forming food-borne pathogen that is a peritrichous rod belonging to the family Enterobacteriaceae (*Feeney et al.*, 2015 and Pakbin *et al*., 2022), in 1980 it

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was classified as a new species. The generic assignment for *E. sakazakii* given by DNA-DNA hybridization was not clear, it was 53-54% related to the species in two genera, *Citrobacter* & *Enterobacter*. The species was classified in *Enterobacter* due to being closer to *E. cloacae* phenotypically and genotypically than *C. freundii* (the genera type species) (Iversen *et al*., 2007).

The genus now includes 9 species, *C. sakazakii, C. turicensis, C. malonaticus, C. dublinesis, C. muytjensii, C. condimenti* and *C. universalis*. All of them were linked to clinical infections in immune-compromised adults and infants except for *C. condimenti* (Saad and Amin, 2014).

The pathogen was called the yellow pigmented *Enterobacter cloacae* due to its characteristic yellow pigment production as well as its biochemical reactions. Later, its name was changed to *Enterobacter sakazakii* following the DNA hybridization studies and the antibiotic sensitivity tests. Once more in 2008, it was reclassified by *Iversen* on the basis of sequencing the 16S rRNA gene, ribotyping, and fluorescence labeled-amplified fragment length polymorphism fingerprinting (Gan *et al*., 2021).

Powdered infant formula, a substitute for the milk of mothers and a major nutrition source for infants globally, is industrially produced and formulated using specific amounts of fats, proteins, carbohydrates, minerals, and vitamins. High chances of contamination with pathogens resemble a risk, as it could lead to serious neonatal illnesses (Song *et al*., 2018). The contamination of the reconstituted PIF might occur intrinsically or extrinsically (Jang *et al.*, 2020).

Powdered infant formula is not a sterile product, being a medium that is nutrient-rich, when reconstituted it can support the growth of bacteria, when some favorable growth conditions are available like water, time, and suitable temperature. Thus, once it is rehydrated, the only conditions that can limit bacterial growth and progressing infections are the time of storage and the temperature. *C. sakazakii* possesses the characteristics of high tolerance to desiccation and osmotic stress and can grow at low temperatures as 5.5 °C, which is the temperature of refrigerators at houses (Deeb, 2010).

In March 2020, neonatal sepsis cases caused by the emerging lethal pathogen *C. sakazakii* were reported for the first time in Egypt. *C. sakazakii* was detected in the water, herbs, and the contaminated PIF (Elkhawaga *et al*., 2020).

Infants that were able to survive the infection with *C. sakazakii* often suffer delayed neurological symptoms like brain abscesses, delayed development of the brain, or hydrocephalus. For that reason, the International Commission on Microbiological Specification for Foods (ICMSF) decided to classify *C. sakazakii* as one of the severe hazards for restricted populations and has considered it life-threatening having substantial chronic sequelae throughout long durations (Feeney *et al.*, 2015 and Gan *et al.*, 2021).

Researchers focused on isolating *Cronobacter sakazakii* from dairy products as PIF, and from baby foods as herbs and cereals. PIF was epidemiologically linked to infections in infants caused by *C. sakazakii.* Hence the immune system of infants is immature; researchers tried preventing the contamination of baby foods by irradiation and the addition of probiotic bacteria and tried to control the existing infection in foods by plant essential oil (Abdelhameed, 2017).

The whole genome of all six *C. sakazakii* strains was analyzed revealing twenty eight different virulence genes present (Holý *et al.*, 2020).

Cronobacter species, like most of the enteric pathogens that interact with humans, has a preferable contact site that it targets which is the mucous membranes or the human

mucosa, to easily follow a well-known bacterial infection stratagem that comprises of: (A) Colonizing the mucosal site (intestinal, urinary tract, or respiratory epithelia). (B) Circumvention, subversion, and host defenses exploitation (invasion of the epithelial cells, then internalization and survival within the phagocytic cells, providing a niche for the pathogen with less competition from other organisms, and new rich nutrients). (C) Spreading systemically & multiplying. (D) Host damage (due to host immune system pro-inflammatory modulation, or through expressing exoproteins like toxins,). The flagellum of *Cronobacter* species induces inflammatory cytokines like IL-8, IL-10, and TNF-α (Jang, Gopinath, *et al.,* 2020).

The general infection's fatality rates range from 42: 80%, and 15: 25% for the neonatal meningitis and septicemia cases, respectively. The highest incidence and severity is in infants, and in outbreaks in intensive care units of neonates (NICU) (Holý *et al.*, 2020).

The causes of high mortality& fatality rates are still poorly understood, and this list of the pathogen's virulence factors is not yet complete:

- Outer membrane proteins (OMPs) (Kim *et al.,* 2010).
- Enterotoxins (Ling *et al.,* 2021).
- Utilization of Sialic acid (Joseph *et al.,* 2013).
- Iron acquisition gene system (Singh *et al.,* 2015).
- Copper and silver resistance cation efflux system (Kucerova *et al.,* 2010).
- Formation of biofilms (Abdullah, 2017).

Aim of the work:

1) Determination of the prevalence of *Cronobacter sakazakii* in PIF commercially available in the city of Assiut & its prevalence in stool of infants suffering from gastroenteritis fed on these rehydrated PIFs collected from Assiut University Infant Hospital.
2) Determining if there is a link between the presence of *C. sakazakii* in contaminated PIF & its presence in the stool of infants fed on it.

MATERIALS AND METHODS

Study design and duration and setting:
A hospital-based cross-sectional observational single-center study was carried on from June 2020 to April 2022 at Assiut University Children Hospitals [Gastroenterology and Hepatology and Preterm units (Incubators)] and Microbiology & Immunology Department, Faculty of Medicine, Assiut University.

Samples:

- A total number of 50 powdered infant formula (PIF) samples were collected from formulas used for feeding infants.

- Stool samples were collected from infants suffering from acute diarrhea, and from non-diarrheic infants at infant incubators and at the Gastroenterology and Hepatology unit of Assiut University Children Hospital. A total of 50 samples were collected (40 samples from diarrheic infants & 10 samples from non-diarrheic infants).

Preparation of samples:

- Sterilization of the surface covers of PIF cans was done with 70% ethanol, then cans were aseptically opened inside a laminar flow cabinet, and the samples were aseptically taken from each product can.

- We suspended ten milligrams of stool in 90 ml buffered peptone water, then incubated it for 1 hour at 37°C, before inoculating each sample onto plates of chromogenic Brilliance *Enterobacter sakazakii* agar (Chandrasekaran *et al.,* 2018).

Isolation and identification of *C. sakazakii* (Yan *et al.*, 2012, Amer *et al.*, 2020, and Mardaneh, 2021):

The procedure of detection of *C. sakazakii* and its isolation is done through 3 successive steps of pre-enrichment in buffered peptone water broth (BPW), then enrichment in selective Enterobacteriaceae Enrichment Broth (EEB), and finally plating on selective chromogenic media**.** The suspected colonies were then picked up and then subcultured for further microscopic and biochemical identification.

- **Pre-enrichment, Enrichment and culturing procedures:**

Following the FDA protocol, a flask containing sterile distilled water was prepared (pre-warmed to 45°C), then PIF was added and mixed till completely dissolved, then the flask was incubated at 35 ± 2°C for 18-24 hours.

Ten-fold serial dilution was done through adding 10 ml of the dissolved PIF to 90 ml of EE broth medium (*Enterobacteriaceae* enrichment), then the diluted solution was used as the base solution to make an additional dilution through transferring 10 ml of it to 90 ml of EE broth, and we repeated this three times to reach final concentrations of 10^{-1}, 10^{-2}, and 10^{-3}.
The diluted solutions were then incubated at 35 ± 2°C for 18- 24 hours.

A loop full of each sample that is incubated in EE broth was streaked by plating out on plates of Brilliance Enterobacter sakazakii agar, then incubated for another 18-24 hours at 35 ± 2°C. The green colonies that were presumptive for *C.sakazakii* (Figure.1) were picked and pure subcultures were performed on MacConkey agar, TSA, and blood agar, and then were incubated overnight at room temperature (25°C) (Table 1).

Gram-stained films were performed from the presumptively positive cultures and the smears were examined.

Biochemical Tests such as Catalase test, Oxidase test, H2S production test, Citrate utilization, and Urease Test were done to further confirm the isolates (Abdeltawab *et al.*, 2019).

- **Preservation:**

The purified isolates were saved in LB broth supplemented with 20% glycerol at -20°C.

Molecular detection of *Cronobacter sakazakii* using PCR (Qiming *et al.*, 2015):
Amplifying the *ESA_02797 gene* using a pair of primers that are (Fw: GGCAGCATGTCATTATCGG, Rv: CATCAGTGGCATTCGGTCTA) which amplify a fragment sized 152bp, to allow the specific detection of all *Cronobacter sakazakii* strains.

DNA extraction (Fayyad and Dwaish, 2016):
It was done by rapid boiling, with some modifications: first, the bacterial cultures were centrifuged (5000 rpm/10 min.) to be concentrated to obtain heavy growth, and were placed in 1.5 ml Eppendorf tubes that contained 300 microliters of distilled water, vortex of the samples for a few seconds, and then the tubes were placed in a water bath at 95˚C for 30 minutes, followed by 10 minutes of centrifugation at 5000 rpm, then we transferred the supernatant to a new sterile Eppendorf tube and stored it at -80˚C until used.

Preparation of PCR reactions:
A 20-µL reaction mixture was prepared to carry out the PCR in, consisting of 10µL of the PCR Master Mix (2X). The solutions of the template DNA were added, and the master mix was prepared according to Table (2).

DNA amplification: a TECHNE thermocycler was used, and the thermocycler program was as follows:

- Initial denaturation for 4 minutes at 95°C.
- 30 cycles each consisting of:
  - Denaturation for 30 seconds at 94°C.
  - Primer annealing for 30 seconds at 60°C.
  - Primer extension for 1 minute at 72°C.
- Final extension for 5 minutes at 72°C.

Post-amplification Detection by Gel Electrophoresis of the PCR Product:

- The obtained PCR products from each reaction (20µl), in addition to a Ladder marker 100-500bp underwent electrophoresis onto submerged agarose gel that is of 1% concentration containing Ethidium bromide in 1x concentration TBE buffer (2.5mM EDTA; 89 mM Tris-borate).

We supplied the submarine gel with a 100 volts direct current for 1 hour through a power supply. Then we visualized the gel and photographed it using the gel documentation system.

RESULTS

- *C. sakazakii* was isolated from a total of 24/50 PIF samples with a percentage of 48% positive PIF samples for *C. sakazakii*. It was isolated in a percentage of 43.75% from PIF samples intended for feeding infants aged 1-3 months, and in a percentage of 55.55% from PIF samples intended for infants aged 4-6 months (Table 3).

- *C. sakazakii* was isolated from 25/50 of the total stool samples with a percentage of 50% positive stool samples. It was isolated from 53.125% of the stool samples of infants aged 1-3 months, and from 44.44% of the stool samples from infants aged 4-6 months (Table 4).

- The results of the stool samples collected from infants with acute diarrhea were positive in a percentage of 50%. And according to the ages of infants, *C. sakazakii* was isolated from 31.58% of the diarrheic stool samples from infants aged 1-3 months, and in a percentage of 18.42% from the diarrheic stool samples from infants aged 4-6 months (Figure 2).

- The study included 12 stool samples from non-diarrheic infants. The samples were positive in a percentage of 50% (Figure 3).

The correlation between the results of PIF and Stool samples:

We found that 62% of the infants had similar results for their PIF and stool samples, as 30% of the infants in the study showed positive results for both their samples, and 32% of the infants in our study showed negative PIF and stool samples.

While 38% of the studied infants showed different results in their PIF and Stool samples (one of their samples showed positive results while the other sample appeared to be negative) (Figure 4).

The PCR results are illustrated in Figure (5).

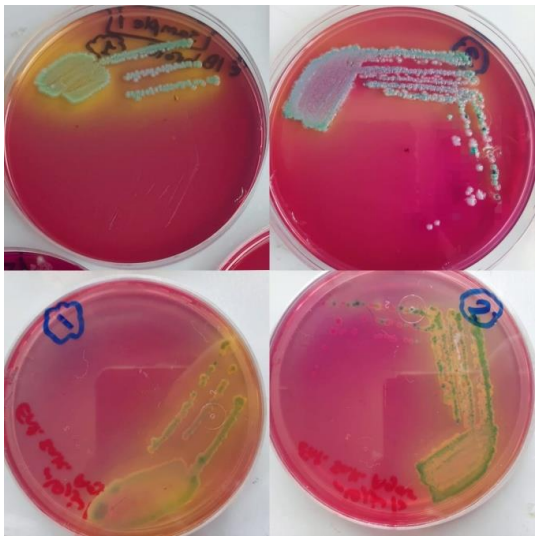

Figure (1): *C.sakazakii* on *Enterobacter sakazakii* agar showing green colonies (dark green/ yellowish green).

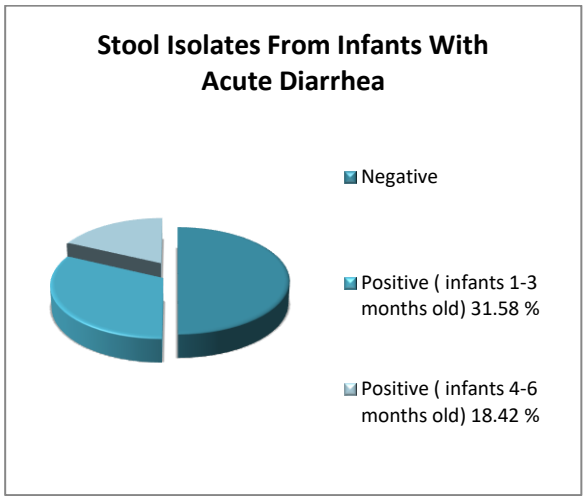

Figure (2): The percentage of positive stool samples in infants suffering from acute diarrhea in different age groups.

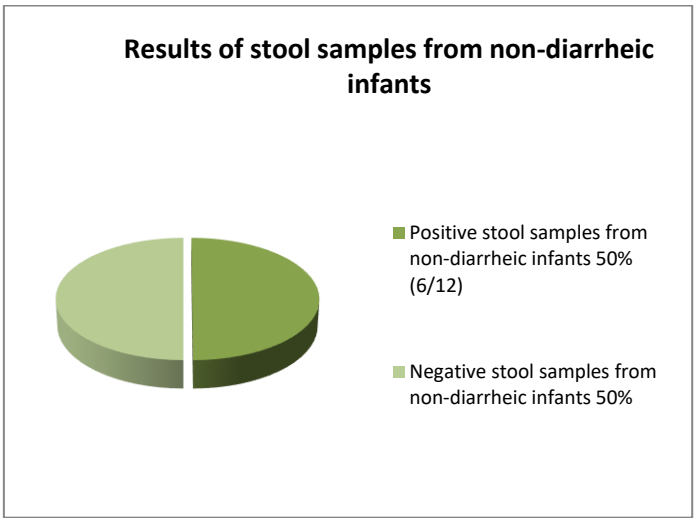

Figure (3): The percentage of positive stool samples collected from non-diarrheic infants.

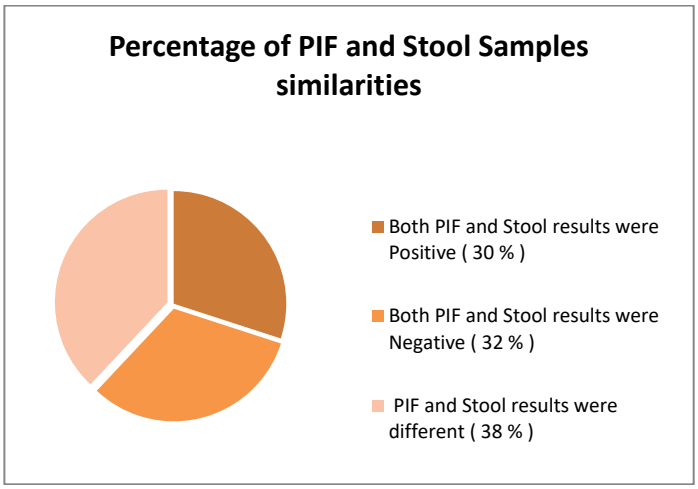

Figure (4): The correlation between the results of PIF and Stool samples of the same infant in the study.

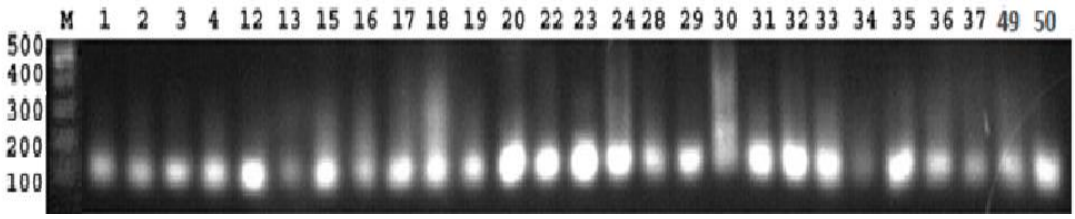

Figure (5): Agarose gel electrophoresis for the ESA_02797 gene positive isolates from milk samples.

- Lanes (1, 2, 3, 4, 12, 15, 16, 17, 18, 19, 20, 22, 23, 24, 28, 29, 31, 32, 33, 35, 36, 37, 49 and 50) show bands for *ESA_02797 gene* 152bp.
- Lanes (13, 30 and 34) show negative samples.
- Lane (M) shows 100-500bp DNA Molecular Weight Marker.

Table 1: The characters, microscopic morphology and biochemical reactions of *C. sakazakii.*

Characters of *C. sakazakii* on solid culture media			
on *Enterobacter sakazakii* agar	on TSA	on Blood Agar	On MacConkey's agar
Dark green, yellowish green or bluish green small rounded colonies.	Yellow small rounded colonies.	Non hemolytic yellow circular raised colonies.	Pink lactose fermenting colonies.
Microscopic morphology and biochemical reactions of *C.sakazakii*			
Gram's stain	Citrate utilization test	Catalase test	Urease test
Gram negative pink rod shaped bacilli.	Blue color.	Gas bubbles evolution.	Yellow negative tubes.

Table 2: PCR components and their quantities.

Components	**Amount of one PCR reaction**
dH_2O	8.5 µl
2X master mix	10 µl
Forward primer	0.25 µl
Reverse primer	0.25 µl
Templet DNA	1.0 µl
Total volume	**20.0 µl**

Table 3: The percentage of the positive results of PIF samples intended for feeding different age groups:

Age Group	**No. of studied PIF Samples**	**No. of Positive PIF Samples**	**Percentage %**
< 3 months	32	14	43.75%
4-6 months	18	10	55.55%
Total	50	24	48%

Table 4: The percentage of positive results in Stool samples.

Age Group	**No. of studied Stool Samples**	**No. of Positive Stool Samples**	**Percentage of Positive %**
< 3 months	32	17	53.125%
4-6 months	18	8	44.44%
Total	50	25	50%

DISCUSSION

Our study isolated *C. sakazakii* from 43.75% of the PIF samples intended for feeding infants aged 1-3 months. And the pathogen's percentage in PIF samples intended for infants aged 4-6 months was 55.55%. Making 48% of the total studied PIF samples positive.

Similarly, Elsheikh *et al.* (2021) found that the prevalence of *Cronobacter sakazakii* in preterm showed a significant increase than in full-term.

These results were very different from the range of prevalence reported by many other studies. Enem *et al*. (2020) declared that the prevalence of *C.sakazakii* in their study that was isolated from PIF samples ranged between 5.6% and 3.1% between the different locations where their study took place.

Another study conducted by Mardaneh and Soltan Dallal, (2017) showed different prevalence rates of 7.2% of the samples positive for *C.sakazakii*. While others isolated *C.sakazakii* strains from the studied PIF samples in a percentage of 6.86% *(Pakbin et al.*, 2022).

Jung and Park in 2006 found that 20% of their studied PIF samples in the Republic of Korea were contaminated with Cronobacter. And another study that

included 100 PIF samples showed a very low prevalence of 1% (Awadallah *et al*., 2018).

The variation of the prevalence rates may be directly proportional to the different levels of perception of personal hygiene, and the educational level of the caregivers and the PIF handlers, however, there are some reports that indicate that the contamination of PIF with *C. sakazakii* is very common, as the formulas are the most common vehicle for *C.sakazakii* known in neonatal infections (Strysko *et al.,* 2020), and the contamination by Enterobacteriaceae members is inevitable if poor hygienic practices were used in the manufacturing of PIF (Güner *et al.,* 2011).

In our study, *C. sakazakii* was found in stool samples collected from infants aged 1-3 months fed on the studied PIF samples in a percentage of 53.125% and was isolated from stool samples of infants aged 4-6 months in a percentage of 44.44%, making the total percentage of positive stool samples in the study 50%.

Results of another study found 1271 stool samples out of 2304 samples (55.2%) containing Cronobacter rRNA gene sequences (Chandrasekaran S *et al.,* 2018). And another previous study showed different results finding a low prevalence in infant stool samples, as low as 4% (Awadallah *et al.,* 2018).

Upon studying the prevalence in the stool of infants with the clinical presentation of acute diarrhea that might be caused by ingesting PIF infected with C. sakazakii, the total percentage of positive stool samples in our study was 31.58% in diarrheic infants aged from 1-3 months, and 18.42% in diarrheic infants aging from 4-6 months.

Our study also included 12 stool samples from non-diarrheic infants, and *C. sakazakii* was isolated from them at a percentage of 50% (6 stool samples were positive out of 12 samples).

The study held by Elsheikh *et al.* in (2021) showed close results as the prevalence of *C.sakazakii* they found in preterm infants was 28%, and in full-term infants it was 32%.

In our study we evaluated the correlation between ingesting contaminated PIF with *C. sakazakii* and the prevalence of *C. sakazakii* infections in PIF-fed infants, and found that the percentage of Infants in the study showing similar positive results for both their PIF and stool samples was 30%, and the percentage of Infants who showed similar negative results for both their PIF and stool samples in the study was 32%, while the percentage of infants in the study with different results in their PIF and Stool samples was 38%. Thus, we found that we can't ensure that the PIF samples were the only source that resulted in the presence of the pathogen in the GIT and the stool of the tested infants, as some infants had the pathogen in their stool while their analyzed PIF samples were not contaminated and vice versa.

The presence of *C.sakazakii* in the stool of these infants proven to have clear uncontaminated PIF samples analyzed might be due to having ingested a previous contaminated PIF can prior to our analysis, or that these infants might have caught the pathogen from the hands of caregivers or other contaminated sources that might have reached the infant's mouth, which is linked to unhygienic practices of the caregivers in uneducated families and in rural areas (Cho *et al.,* 2019).

This conclusion differed from what Chandrasekaran S. *et al.* (2018) found, as they linked the presence of *C. sakazakii* in the stool of infants, to the discontinuation of feeding PIF to these infants, and they found that the proportion of specimens containing > 4.0% of reads mapping to *C.*

sakazakii fell to 0.9% from 4.3% after the PIF was discontinued.

CONCLUSION

- Neonatal infections were attributed to the consumption of rehydrated PIF because *C. sakazakii* can survive osmotic, desiccation stress & temperature extremes. It can also colonize the pieces of equipment used to prepare and administer milk formulas. Appropriate cleaning and sterilization procedures and good storage conditions can help prevent such infections.
- *Cronobacter sakazakii* was isolated from PIF samples at a percentage of 48% and was isolated from stool samples in a percentage of 50%**.**
- The percentage of similarities in the number of positive PIF and Stool samples was 30%.
- The percentage of infants showing negative results for both PIF and stool samples in the study was 32%.
- The percentage of infants showing different results in their PIF, and stool samples was 38%.
- According to our research, we can't ensure that consuming the contaminated PIF samples was the only cause that resulted in the presence of the pathogen in the GIT and the stool of the infected infants.

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التعرف للكورونوباكتر ساكازاكي المعزولة من الألبان المجففة وبراز الرضع

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بكتيريا كورونوباكتر ساكازاكي هي أحد أخطر مسببات الأمراض لحديثي الولادة التي ارتبطت بتهديد حياة الرضع لكونها تسبب تفشي تسمم الدم، التهاب الأمعاء والقولون الناخر، والتهاب السحايا للرضع، مع ارتفاع معدل الوفيات. ٨٠ و٤٠٪ بين حديثي الولادة والمبتسرين
حليب الأطفال المجفف هو بديل صناعي لحليب الأم، وهو مصدر رئيسي للبروتينات والدهون والكربوهيدرات والفيتامينات والمعادن. ويُعتقد أن مسحوق حليب الأطفال هو مصدر عدوى الكرونوباكتر ساكازاكي في حديثي الولادة والأطفال ، لأن تلوث مساحيق الالبان يمكن أن يحدث داخليًا اوخارجيًا.

هدفت هذه الدراسة إلى اختبار تركيبات ألبان الرضع المجففة المتاحة تجاريًا والمخصصة للاستهلاك من قبل الرضع الذين تتراوح أعمارهم بين ٠-٦ أشهر، لذلك تم جمع عدد ٥٠ تركيبة بودرة للرضع من التركيبات المستخدمة للرضع في مستشفى الأطفال بجامعة أسيوط، وجمع ٥٠ عينة براز من الأطفال الذين يعانون من الإسهال الحاد، ومن الأطفال حديثي الولادة في حضانات الأطفال ووحدة أمراض الجهاز الهضمي والكبد بمستشفى الأطفال بجامعة أسيوط. وقد كشفت دراستنا الحالية أن كورونوباكتر ساكازاكي تم عزله من مساحيق حليب الأطفال المجففة بنسبة ٤٨٪ من عينات مساحيق التركيبات موجبة, وأظهرت عينات البراز التي تم جمعها ٢٥ عينة موجبة من أصل٥٠ عينة تم جمعها بنسبة إجمالية قدرها ٥٠ ٪ عينات موجبة ملوثة بـكورونوباكتر ساكازاكي.

لتأكيد العزلات وفحص وجود كورونوباكتر ساكازاكي تم استخدام تفاعل البلمرة المتسلسل تم استخدام زوج من البادئات للسماح بتضخيم جين .ESA_02797

وعند تحليل أوجه التشابه بين نتائج عينات مساحيق الالبان وعينات البراز وجد أن 30٪ من الأطفال الذين تناولوا عينات مساحيق تركيبات حليب الأطفال الملوثة ببكتيريا كورونوباكتر ساكازاكي أظهروا عزلات إيجابية في عينات البراز الخاصة بهم أيضًا .