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IDENTIFICATION OF *CAMPYLOBACTER SPP*. ISOLATED FROM POWDERED INFANT MILK FORMULA

Short title: *Campylobacter spp.* in powdered infant formula

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ABSTRACT

Campylobacter species are Gram-negative bacilli that are characterized by being catalase-positive, oxidase-positive, motile, microaerophilic bacteria, and non-spore-forming. *Campylobacter* is often isolated from animal sources, as it inhabits the gastrointestinal tract of both wild and domestic animals and birds, especially poultry. Milk may be possibly contaminated by the direct discharge of a mastitis-affected cow or by excrement from diseased or colonized cattle during milking. Powdered infant formula (PIF) is considered a non-sterile product and may be contaminated intrinsically or extrinsically with various bacteria that can cause critical illness in infants. This study was performed to detect and identify *Campylobacter spp.* in powdered infant milk formula by phenotypic and genotypic methods. Ten isolates of *Campylobacter* from eighty-six samples were phenotypically identified and confirmed genotypically by PCR, with a pattern of 11.6%. This *Campylobacter* could potentially be transmitted to children by PIF consumption that has not been adequately handled, prepared or processed.

Key words: *Campylobacter*, PIF, PCR

INTRODUCTION

One of the most frequent causes of bacterial gastroenteritis in people is *Campylobacter spp.* The genus *Campylobacter* includes thirty-nine species, and this number is constantly increasing as new species are discovered (Zhong *et al.,* 2022).

Campylobacter is a spiral or curved "gull-winged" Gram-negative, non-spore-forming, motile rod and microaerophilic bacteria. It ranges between 0.2 and 0.5µm in width and between 0.5 and 0.8 µm in length. Their unique corkscrew-like motion can be explained by the presence of a single flagellum (sometimes multiple flagella) at one or both cell poles. The alteration

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from spiral to coccoid morphology is influenced by nutrient limitation, medium aeration, and the level of free radicals (Barros-Velázquez Jorge *et al.,* 1999).

Campylobacter jejuni, *C. coli*, *C. lari*, and *C. upsaliensis* all grow easily at 37°C in microaerophilic circumstances (5% O_2, 10% CO_2 and 85% N_2), and the majority of these species' strains will also grow at 42°C, demonstrating that all of the clinically relevant *Campylobacter spp*. are thermotolerant in nature (Fouts *et al.,* 2005).

Wild animals, domestic animals, and birds' intestines are heavily colonized by *C. jejuni.* (Luo *et al.,* 2022). Intestinal tracts of animals used for food, particularly birds, are becoming asymptomatically colonized with *Campylobacter spp.* (Colles *et al.,* 2003, Tang *et al.,* 2020 and Luo *et al.,* 2022).

A few hundred bacteria are sufficient to cause intestinal colonization in humans, which leads to campylobacteriosis (Backert, 2020). According to the French National Reference Center for *Campylobacters* and *Helicobacters*, the majority of these that cause human illnesses include the thermotolerant species *Campylobacter jejuni* (*C. jejuni*) and *Campylobacter coli* (*C. coli*) (Sifré *et al.,* 2015), which is followed by a variety of other species, including *C. upsaliensis*, *C. hyointestinalis* and *C. lari* (Ketley, 1997 and Tang *et al.,* 2020).

Campylobacter was proven to be the cause of outbreaks related to the consumption of milk, cheese, and other dairy milk products, with a pattern of 22.2% (EFSA, 2017). Milk may be possibly contaminated by the direct discharge from a mastitis-affected cow or by excrement from diseased or colonized cattle during milking (El-Shaboury *et al.,* 2003).

For young children who are more vulnerable to dehydration and loss of nutrients, such as sodium and protein, as a result of the diarrheal disease, *Campylobacter* infection is considered hazardous (Ahs *et al.,* 2010 and Asuming-Bediako *et al.,* 2019).

Cross contamination and insufficient heat treatment were the most often reported outbreak causes, attributed to pasteurized milk (Mahmood *et al.,* 2009).

Actually, to reduce the danger of developing *Campylobacter*, meals must be heated to a temperature of 70° C. (A. Facciolà *et al.,* 2017). Additionally, after handling contaminated items, appropriate hand washing is important, because a quick rinse or wash may not be enough to entirely eradicate pathogens such as *C. jejuni* (Acuff *et al.,* 1986),

MATERIALS AND METHODS

Ethical statement. According to the World Medical Association's code of ethics (Declaration of Helsinki), the study was approved by the ethical committee of Assiut University's faculty of medicine. The number is 17101914.

Collection of samples: seventy-six PIF samples were collected from Assiut University Children's Hospitals. Samples were collected under a septic condition.

Table 1: Types and numbers of PIF used in this study

Type of PIF	Number
1 - EGY 1	27
2 - EGY 2	8
3 - Bebelac Premature	1
4 - Bebelac 1	7
5 - Bebelac 2	5
6 - Bebelac EC	1
7 - Bebelac LF	7
8 - NAN Premature	1
9- NAN 1	1
10- NAN 2	2
11- NAN LF	3
12- Nestogen 1	5
13- Nestogen 2	1
14- Nactalia 1	1
15- Nactalia 2	2
16- Hero Baby 1	2
17- Hero Baby Nutradefense	1
18- Pediamil 1	1
19- Pediamil LF	1
20- Aptimel 1	1
21- Nutri Baby 1	2
22- Neocate Infant	1
23- Pediasure Complete	2
24- Infatrini	1
25- Nutrica infatrini	1
26-Semilac confort gold	1

Preparation of PIF samples:

For the isolation of *Campylobacter* from PIF, 5 g of PIF sample was mixed with 30 ml of sterile distilled water, as per the general dissolving instructions on milk bottles. The tubes were mixed with vortex and labeled. The PH determination of PIF was done using pH test paper (pH 6–8 range) and sterile NaOH 2N to adjust PH to 7.5 ± 0.2 if necessary. Centrifuge a 50 g portion at 20,000 × *g* for 40 minutes. The supernatant was discarded (the fat layer), while the pellet was dissolved and inoculated (Jan M. Hunt *et al.,* 2001).

The pellet was inoculated in 10 ml of Preston enrichment broth (HIMEDIA-M899) supplemented with Preston selective supplement with *Campylobacter* Selective Supplement-IV (Preston), modified (HIMEDIA-FD158), and defibrinated lysed horse blood. The tubes were incubated using AnaeroPack-Anaero sachets (MGC, Japan) and an anaerobic jar at 41.5 °C for 24 h (ISO 10272-1, 2017).

Then inoculated in *Campylobacter* blood-free selective agar base plates: modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA) using AnaeroPack-Anaero sachets (MGC, Japan) and an anaerobic jar at 41.5 °C for 48 h (ISO 10272-1, 2017).

Culture on solid media:

• mCCDA: *C. jejuni* showed a gray, small, sheen appearance, moist, and have a special odor, while *C. coli* typically had a wet, creamy gray appearance and a slightly raised shiny surface (Sallam, 2007 and Ali A. AL-Edany *et al.,* 2015).

Identification of isolated colonies:

1- Gram-stained smear: (Smith Ann C. *et al.,* 2005).

Films were made from the suspected colonies grown on mCCDA by taking a loopful from the colonies with a drop of distilled water on a clean, properly dry glass slide, then stained with Gram's stain and examined microscopically. *Campylobacter spp*. is Gram-negative, so the suspected result was pink-colored rods that were very slender with curved or spiral-shaped rods with a "gull-winged" appearance.

2- Motility test: (American Society for Microbiology, 2016).

Semisolid media was used to perform this test by using an inoculating needle and the stab technique. Each organism was aseptically transferred to a tube of

the motility medium and incubated for 24-48 h by using AnaeroPack-Anaero sachets (MGC, japan) and an anaerobic jar. Positive results when widespread, hazy growth diffused in the medium, making it a little opaque.

3- Biochemical tests:

a. Catalase test: (Reiner Karen, 2010)

A colony was added to a small spot on a slide. One drop of 3% H_2O_2 was put on it. The evolution of gas indicates catalase activity as a positive result, which is presumptive for *Campylobacter spp*.

b. Oxidase test: (Gupta *et al.,* 2022)

Oxidase activity was tested on oxidase disks. By using a glass loop, a well-grown colony from a fresh culture medium was applied to the reaction on the disk. Violet coloration indicates a positive reaction within 20–60 seconds after getting contact, which is presumptive for *Campylobacter spp*.

c. Hippurate hydrolysis test: (OIE Terrestrial Manual, 2008)

This test was used to differentiate between *C. jejuni* and *C. coli.* A heavy loopful of fresh growth from a suspected colony was suspended in 400 µl of a 1% sodium hippurate solution. The culture was incubated at 37°C for 2 h. Then 200 µl of 3.5% ninhydrin solution was added to the tube's side to make an overlay. The culture was re-incubated at 37°C for 10 minutes. Positive reactions gave a dark purple color, but negative reactions showed a clear or gray color**.** The suspected result with *C. jejuni* was positive, but *C. coli* was negative.

4- Molecular identification of *Campylobacter* by Multiplex -PCR:

This part was carried out in the Microbiology and Immunology Department Lab, Faculty of Medicine, Assiut University, with multiplex polymerase chain reaction (multiplex PCR). The primers used for the identification of *Campylobacter spp.* by 16S rRNA as a universal gene and the *MapA* gene used for *Campylobacter jejuni* identification (Huang *et al.,* 2009).

Table 2: Oligonucleotide sequences used to identify *Campylobacter spp.* according to (Huang *et al.,* 2009)

Target genes	Primer Sequence (5′→3′)	Amplification Size (bp)
16S rRNA	F: ATCTAATGGCTTAACCATTAAAC R: GGACGGTAACTAGTTTAGTATT	857
MapA	F: CTATTTTATTTTTGAGTGCTTGTG R: GCTTTATTTGCCATTTGTTTTATTA	589

PCR assays

Multiplex PCR was carried out in a 20 µL reaction mixture containing 1 µL from each of both forward and reverse specific primer pairs of both *16s rRNA* and *MapA* genes, 10 µL of PCR master mix (Thermo Fisher Scientific, United States), 4 µL of nuclease-free water, and 2 µL of DNA template.

***16s rRNA* and *MapA* genes**

Initial denaturation at 95°C for 10 minutes; 35 cycles, each consisting of denaturation at 95°C for 30 seconds; annealing at 59°C for 90 seconds; extension at 72°C for 1 minute; and final extension at 72°C for 10 minutes.

Detection of the amplified product

An agarose gel with a 1.5% concentration was used to test the PCR amplicons. Then stained with ethidium bromide, and observed for two hours under ultraviolet (UV) radiation at 80 volts.

Statistical analysis

All results were carried out and analyzed using IBM SPSS 26.0, and categorical variables were described using numbers and percentages (N, %).

Campylobacter spp. was isolated from PIF samples in a percentage of 11.6 % (10 /86). *C. jejuni* and non-*C. jejuni* had a frequency pattern of 50% for each one.

According to age groups of children, the frequency of positive *Campylobacter spp*. isolates from PIF with the group ages from zero to 3 months was 10.5%, while the group ages more than 3 to 6 months was 15.6%, but the age groups >6–9 and >9–12 were zero and 11.1%, respectively, as shown in table (4).

RESULTS

Table 3: Frequency of *Campylobacter spp*. isolated from different types of PIF.

Type of PIF	Total samples No. (86)	*Campylobacter* isolates No. (10)
1- EGY 1	27	4 (14.8%)
2- EGY 2	8	0
3- Bebelac Premature	1	0
4- Bebelac 1	7	2 (28.6%)
5- Bebelac 2	5	1 (20%)
6- Bebelac EC	1	0
7- months Bebelac LF	7	0
8- NAN Premature	1	0
9- NAN 1	1	0
10- NAN 2	2	0
11- NAN LF	3	0
12- Nestogen 1	5	0
13- Nestogen 2	1	0
14- Nactalia 1	1	0
15- Nactalia 2	2	0
16- Hero Baby 1	2	2 (100%)
17- Hero Baby Nutradefense	1	1 (100%)
18- Pediamil 1	1	0
19- Pediamil LF	1	0
20- Aptimel 1	1	0
21- Nutri Baby 1	2	0
22- Neocate Infant	1	0
23- Pediasure Complete	2	0
24- Infatrini	1	0
25- Nutrica infatrini	1	0
26- Semilac confort gold	1	0

Table 4: Frequency of *Campylobacter spp*. in examined PIF isolates according to children's age groups.

Age groups	*Campylobacter* isolates No.	Negative No.	Total No. (86)	*Campylobacter* strain	
				C.jejuni (5)	Non-*C.jejun* (5)
0 – 3	2 (10.5%)	17 (89.5%)	19	2 (100%)	0 (0%)
>3 – 6 months	7 (15.6%)	38 (84.4%)	45	3 (42.9%)	4 (57.1%)
>6 – 9 months	0 (0%)	13 (100%)	13	0 (0%)	0 (0%)
>9 – 12 months	1 (11.1%)	8 (88.9%)	9	0 (0%)	1 (100%)

On Culture media

As shown in figure (1), *Campylobacter spp*. were isolated on mCCDA as creamy to grayish-colored, moistened, with or without a metallic luster, with a slightly raised, shiny surface, and having the tendency to spread across the plate

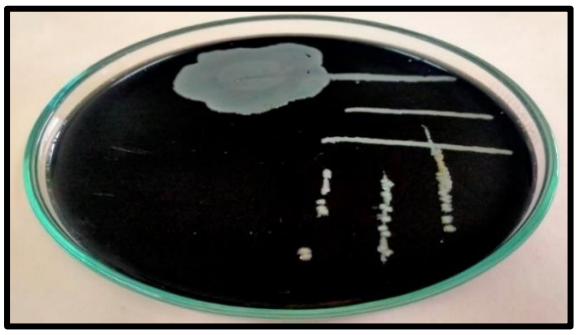

Figure (1): *Campylobacter spp*. on mCCDA

Identification of the isolated *Campylobacter* colonies by Gram stain, motility test, biochemical tests and genotypic identification by PCR

1- Gram stain:

Film from colonies under the microscope revealed Gram-negative, very slender, curved, or spiral-shaped rods with a "gull-winged" appearance. Tendency to form coccoid and elongated shapes as shown in figure (2)

2- motility test:

Campylobacter spp. showed widespread, hazy growth diffused in the medium, making it a little opaque, indicating positive results for the motility test, as shown in Figure (3).

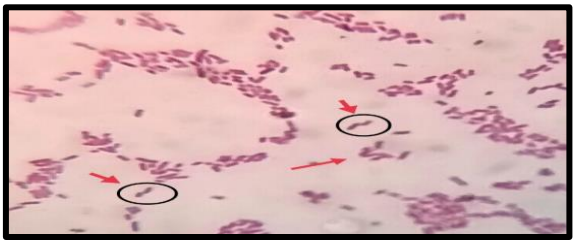

Figure (2): *Campylobacter spp*. with Gram stain

Figure (3): *Campylobacter spp*. is motile in semi solid agar
(A): Negative
(B): Positive

3- Biochemical testes:

a. Catalase test:

Campylobacter spp. showed the evolution of gas bubbles, indicating positive results for the catalase test, as shown in Figure (4).

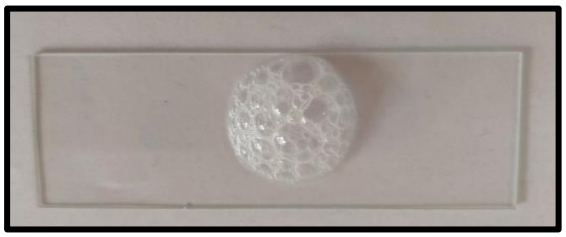

Figure (4): *Campylobacter spp*. is catalase positive

b. Oxidase test:

Campylobacter spp. showed blue coloration of the oxidase disk, indicating positive results for the oxidase test, as shown in Figure (5).

Figure (5): *Campylobacter spp*. is oxidase positive

c. Hippurate hydrolysis test:

Positive reactions gave dark purple, but negative reactions showed a clear or gray color, indicating *C. jejuni*, as shown in Figure (6)**.**

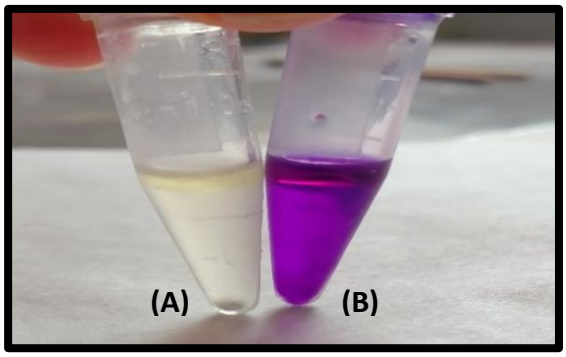

Figure (6): *C. jejuni* by Hippurate hydrolysis test

(A): Negative Hippurate hydrolysis test (*C. jejuni*)

(B): Positive hippurate hydrolysis test (non-*C. jejuni*)

4- Genotypic identification of *Campylobacter spp*

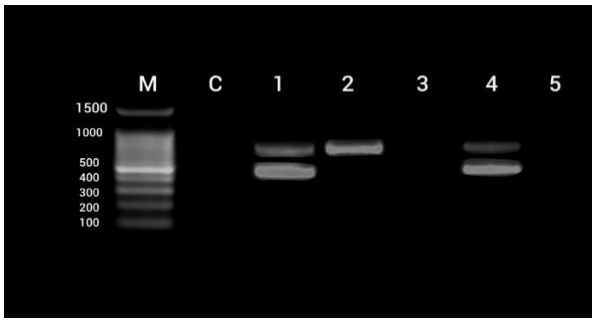

Figure (7): Agarose electrophoresis gel for the *16s* and *MapA* genes positive ***Campylobacter* isolates**. M: Marker C: Control

- Lanes (1, 2 and 4) show bands for *16s RNA* gene 857 bp indicating *Campylobacter* positive*,*
- Lanes (1 and 4) bands for *MapA gene* 589 bp indicate *Campylobacter jejuni* species.
- Lanes (3 and 5) show no bands indicating negative *Campylobacter*.
- Lane M: DNA ladder (100 – 1500 bp)
- Lane C: Negative control sample (Distilled Water)

DISCUSSION

According to the current investigation, 11.6% of *Campylobacter spp*. isolates were found in PIF. About 50% of PIF-positive samples were *C. jejuni,* and 50% were non-*C. jejuni*. Mahmood *et al.* (2009) in Pakistan investigated that 4.1% of skim milk powder encompassed *Campylobacter spp*., of which 80% were *C. jejuni* and 20% were *C. coli*. According to a study conducted by Andrzejewska *et al.* (2019) in Northern Poland, *Campylobacter spp*. it was present in 11.8% of the raw milk samples obtained from private suppliers, which was similar to our outcome. In Ethiopia, it was 16% *Campylobacter* isolates, which all were *C. jejuni* in raw milk and 9% in pasteurized milk by Admasie *et al.* (2023). On the other hand, in Eastern Cape Province, South Africa, it was higher than this study by 26.38% in raw

milk samples by Igwaran and Okoh, (2020). In Hatay, Turkey, by Elmalı and Can, (2019), *Campylobacter spp*. frequency was lower by 5.2% in raw milk samples. This variety in results may be due to environmental factors, the year that samples were collected, and the geographical location.

This was the first investigation that studied PIF as a potential source of campylobacteriosis infection in children, which may be due to:

1- PIF couldn't be sterile, according to Losio *et al.* (2018).
2- Mother cross-contamination during handling and preparation of PIF.

CONCLUSION

Powdered infant formula (PIF) was considered a source of *Campylobacter* infection as it can survive osmotic and high-temperature conditions up to 70 °C, so we recommend reconstitution of PIF with hot water to avoid any possible *Campylobacter* contamination.

Even a quick hand wash or rinse may not remove *C. jejuni* absolutely after handling contaminated foods, so we recommend washing hands properly by mothers during handling and preparation of PIF, as it is very important to prevent *Campylobacter* infection in their children.

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تعريف الكامبيلوباكتر المعزولة من ألبان الأطفال الرضع المجففة

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أنواع الكامبيلوباكتر هي عصيات سلبية الجرام تتميز بكونها إيجابية الكاتلاز، وإيجابية الأكسيداز، ومتحركة، وتنمو فى بيئة هوائيه ٥٪ اكسجين ، وغير جرثومية .غالبًا ما يتم عزل بكتيريا كامبيلوباكتر من مصادر حيوانية لأنها تسكن الجهاز الهضمي لكل من الحيوانات والطيور البرية والمنزلية، وخاصة الدواجن. قد يكون الحليب ملوثًا عن طريق الافرازات المباشرة للبقرة المصابة بالتهاب الضرع أو عن طريق براز الماشية المريضة أو المستعمرة أثناء الحلب .تعتبر ألبان الأطفال الرضع المجففة منتجًا غير معقم وقد يكون ملوثًا جوهريًا أو خارجيًا ببكتيريا مختلفة يمكن أن تسبب مرضًا خطيرًا عند الرضع .أجريت هذه الدراسة للكشف والتعرف على بكتيريا كامبيلوباكتر في ألبان الأطفال الرضع المجففة بالتوصيف المظهرى والوراثى .تم تشخيص عشر عزلات من بكتيريا كامبيلوباكتر من ستة وثمانين عينة ظاهرياً وتأكيدها وراثياً بواسطة تفاعل البوليميراز المتسلسل، بنمط ١١,٦٪. ولذلك من المحتمل أن تنتقل بكتيريا كامبيلوباكتر إلى الأطفال عن طريق استهلاك ألبان الأطفال الرضع المجففة التي لم يتم التعامل معها أو تحضيرها أو معالجتها بشكل كافٍ.