ORIGINAL ARTICLE

Prevalence and Antibiotic Resistance of Cronobacter Spp. Associated with Powdered Infant Milk Formulas and Dried Milks in Saudi Arabia

Khalid M. Aljarallah*[1]

* For correspondence k.aljarallah@mu.edu.sa

[1]Department of Medical Laboratory Sciences, College of Applied Medical Sciences, Majmaah University, Almajmaah-11952, Kingdom of Saudi Arabia

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Abstract

Introduction: This study examined the presence of the pathogen Cronobacter spp. in powdered infant milk formulas (PIMF), and dried whole and skim milk samples distributed in Saudi Arabia. **Methodology:** Culture-based method, biochemical conformation test and real-time PCR were used for detection. **Results:** Cronobacter spp. was detected in 10% of PIMF and 20% of dried whole milk, but not in dried skim milk samples. Real-time PCR showed that PIMF samples generally contained higher numbers of Cronobacter spp. than dried whole milk samples. Isolates showed resistance to 9 of 10 antibiotics, being more frequently resistant to penicillin and tetracycline Both single and multidrug resistance was observed. **Conclusions:** This study reports the association of antibiotic-resistant Cronobacter spp. with PIMF and dried whole milk products distributed in Saudi Arabia.

Key Words: Cronobacter, antibiotic resistance, infant milk formulas, real-time PCR

الملخص

مقدمة: تناولت هذه الدراسة مدى تواجد بكتيريا الكرونوباكتر Cronobacter spp. (المرضة في عينات المراكب الحليبية لأطفال الرضع اضافة إلى عينات حليب مجفف كامل ومنزوع الدسم والتي يتم تداولها في المملكة العربية السعودية بالإضافة إلى الكشف عن تواجد هذا البكتيريا المرضي في العينات باستخدام عدة طرق شملت الاستزراع والفحص المجهري التأكيدي اضافة إلى فحص الجزيئي باستخدام تفاعل البلمرة المتسلسل الآني (Real time PCR) بالكشف عن تواجد بكتيريا الكرونوباكتر في 10% من عينات المراكب الحليبية لأطفال الرضع و 20 % من عينات الحليب المجفف كامل الدسم بينما خلت من هذه العينات الحليب المجفف منزوع الدسم. بينت تقنية فحص الجزيئي باستخدام تفاعل البلمرة المتسلسل الآني (Real time PCR) أن عينات المراكب الحليبية لأطفال الرضع تحتوي في الغالب أعدادا اكبر من هذا الجنس البكتيري مقارنة بعينات الحليب المجفف منزوع الدسم. إختبارات الحساسية للمضادات الحيوية أظهرت أن البكتيريا المعزولة في هذه الدراسة كانت مقاومة لتسع مضادات حيوية من أصل عشر تم استخدامها , وكانت المقاومة الأكثر تكرارا ووضوحا ضد البنسلين والتراسيكلين . المقاومة الحيوية والمتعددة كانت سمة الحظه . هذه الدراسة تثبت تواجد بكتيريا الكرونوباكتر المقاومة لمضادات الحيوية في مراكب حليبية لأطفال الرضع لذلك في منتجات حليب مجفف كامل الدسم تسوق في المملكة العربية السعودية.

Introduction: Cronobacter spp. (formerly known as Enterobacter sakazakii) is an opportunistic pathogen that has emerged recently in association with sporadic disease cases and outbreaks involving debilitated neonates fed dried infant formulas.[1-3] Infections with Cronobacter spp. may lead to meningitis, necrotising enterocolitis, and bacteraemia that could be associated with a high mortality rate of 40–80%[4] Brain damage or neurological disorders, such as hydrocephalus and quadriplegia, may also manifest in surviving patients[5]. Both infants and elderly individuals were found to be infected by Cronobacter spp.[6] While the pathogen was recovered from various sources, including soil, water, vegetables, and foods, it was most frequently detected in dried milks and powdered infant milk formulas (PIMF).[7-11] Cronobacter spp. has thus been classified as a category A organism that is

clearly associated with causing disease via PIMF.[3] Previous studies have reported the association of Cronobacter spp. with dried milks and PIMF in different Western countries. Other reports also demonstrated its presence in PIMF and dried milk products distributed in the Middle and Far Eastern regions, including Jordan,[12-13] Egypt[10,14] and China.[15] A previous study also showed the contamination of the Saudi Arabian artisanal fermented drink "Sobia," made from wheat and malt flours, with Cronobacter spp.[16] However, no further information is available on the association of Cronobacter spp. with PIMF and powdered milks in Saudi Arabia. The present study was therefore designed to address this aspect, and to characterise the antibiotic resistance of Cronobacter spp. isolates associated with these products.

Methodology

Detection of Cronobacter spp. in powdered infant milk formulas and dried milk samples: A total of 60 samples of powdered infant milk formulas (PIMF) (n=20), dried whole milk (n=20), and dried skim milk (n=20) were randomly collected from local markets in Riyadh, Saudi Arabia. These samples were examined for the presence of Cronobacter spp. using the FDA method[8], as modified by El-Sharoud et al.[10,14,11]. Samples were pre-enriched by mixing a 25 g sample with 225 mL buffered peptone water (BPW) broth (Oxoid, Basingstoke, UK), followed by incubation at 37ºC for 24 h. Aliquots of 10 mL of the pre-enriched samples were then inoculated into 90 mL of the EE broth (Oxoid), followed by incubation at 37ºC for 24 h. A 3 mm loopful (10 µL) of the enterobacteriaceae enrichment (EE) broth culture was finally streaked onto the brilliance Enterobacter sakazakii agar (DFI agar) (Oxoid) followed by incubation at 37ºC for 24 h. Suspected blue-green colonies were then picked up and examined by Gram-staining and for the formation of yellow pigmented colonies on tryptone soy agar (TSA) (Oxoid) at 25ºC for 48–72 h. Potential isolates producing a negative Gram-reaction and yellow pigment on TSA were further examined using the Rapid ID 32 E miniaturised kit (bioMerieux, Marcy l'Etoile, France).

Real-time PCR confirmation of Cronobacter spp.: Real-time PCR was used to confirm Cronobacter spp. isolates recovered from the PIMF and dried milk samples. The real-time PCR protocol[17] of was applied as follows. Cells were pelleted from a 24 h culture of each isolate by centrifugation at 5000 rpm for 10 min. The cell pellet was re-suspended in PrepMan Ultra (Applied Biosystems, Foster City, CA, USA) and heated at 100ºC in a thermal block incubator for 15 min, followed by cooling for 2 min at room temperature, and centrifugation at 5000 rpm for 2 min. Resultant supernatants containing DNA were collected and used for real-time PCR reactions. A PCR reaction mixture of a total volume of 50 µL was

formulated using a 5 µL DNA sample template, 25 µL of TaqMan Universal PCR Master Mix (Applied Biosystems), 5 µL of forward primer, 5 µL of reverse primer, 5 µL of TaqMan probe, and 5 µL of water. The primers and TaqMan probe were designed to target a DNA sequence of 78 bp within the macromolecular synthesis (mms) operon[17] for 2 min, followed by 95 °C for 10 min, and 50 cycles of 95 °C for 15 s and 60 °C for 60 s. PCR reactions were conducted using a StepOne real-time PCR system (Applied Biosystems).

Assessment of the antibiotic resistance of Cronobacter spp.: Cronobacter spp. isolates were examined for their resistance to 10 antibiotics, including ampicillin (10 µg), penicillin G (100 unit), gentamicin (10 µg), tetracycline (30 µg), ciprofloxacin (5 µg), kanamycin (30 µg), streptomycin (10 µg), chloramphenicol (30 µg), nalidixic acid (30 µg), and cefoxitine (30 µg). Antibiotic resistance was tested using the Kirby-Bauer disc-diffusion method. Briefly, a standardised inoculum of a 24 h culture of each isolate was spread onto Muller-Hinton agar (Oxoid), followed by dispensing antibiotic-impregnated discs. After incubation at 37º C for 24 h, the diameter of the inhibition zones around each antibiotic disc was measured and interpreted as resistance, intermediate, or sensitive, according to the criteria of the Clinical and Laboratory Standards Institute.[18]

Results

Presence of Cronobacter spp. in powdered infant milk formulas and dried milk products. The prevalence of Cronobacter spp. in powdered infant milk formulas (PIMF) and dried whole and skim milk samples collected in March 2013 from the local markets in Saudi Arabia was examined.

Table 1: Detection of Cronobacter spp. in powdered infant milk formulas and dried milk samples

Product	No. of samples	No. of presumptive *Cronobacter* spp. positive isolates	No. of bio-chemically confirmed isolates (%)*	No. of real-time PCR confirmed isolates (%)*	Minimum Ct	Maximum Ct	No. of positive samples (%)
Powdered infant milk formulas	20	5	2 (40)	2 (40)	15.8	20	2 (10)
Dried whole milk	20	10	4 (40)	4 (40)	19.8	25	4 (20)
Dried skim milk	20	0	0	0	-		0
Total	60	15	6 (40)	6 (40)			6 (10)

A total of 15 potential isolates of the organism producing typical colonies on the DFI agar, a negative Gram reaction, and a yellow pigment on the TSA were recovered from these samples (Table 1).

However, only 40% of these isolates could be confirmed by biochemical testing using the miniaturised rapid ID 32E kit and a real-time PCR assay targeting the mms operon (Table 1 and Figure 1).

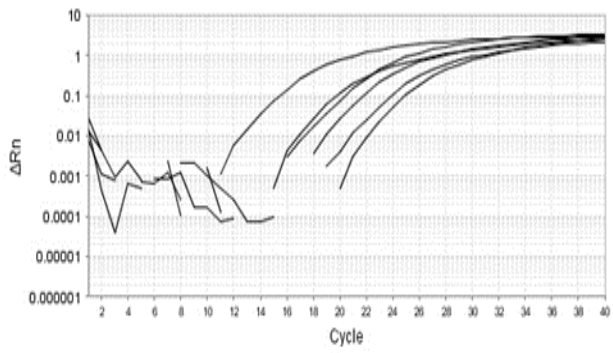


Figure 1: Real-time PCR analysis of Cronobacter spp. Isolates.

Figure 1 shows real-time PCR curves resulting from the analysis of the Cronobacter spp. isolates recovered from the PIMF and whole milk powder samples. Ct number is inversely related to the amount of amplicon in the reaction (target) whereas the lower the Ct, the greater the amount of amplicon[9]. This was reflected here where the number of isolates in the powdered infant milk formulas and dried whole milk were 2 and 4 which correspondent to the Ct values 17.9 and 22.4 respectively.

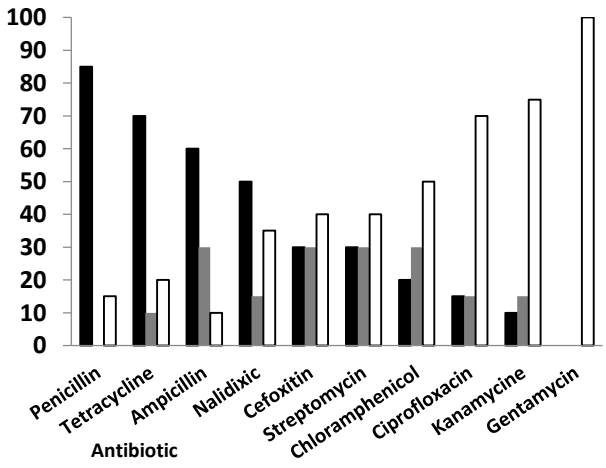


Figure 2: Response of Cronobacter spp. Isolates Isolates showed variable rates of resistance (■), intermediate response (■), and sensitivity (□) to the examined antibiotics.

Antibiotic resistance of Cronobacter spp. Isolates: Among the examined products, 10% and 20% of PIMF and dried whole milk samples were found to be contaminated with Cronobacter spp., respectively (Table 1). Dried skim milk samples were found free of the organism.

The susceptibility of Cronobacter spp. isolates recovered from PIMF and dried whole milk samples to the 10 antibiotics was examined. With the exception of gentamycin, Cronobacter spp. isolates showed variable resistance to the examined antibiotics (Figure 2).

Discussion

The highest resistance was found against penicillin, followed by tetracycline, with a resistance rate of 85% and 70% of the isolates, respectively. Resistance to other antibiotics ranged from 10% to 60% of the isolates. These results were consistent with those of Farmer et al.[19], who reported resistance to penicillin in all examined Cronobacter spp. cultures isolated from clinical samples.

Kilonzo-Nthenge et al.[20] also showed that the highest antibiotic resistance rates in Cronobacter spp. isolates recovered from domestic kitchens were found against penicillin followed by tetracycline. The authors have also found the isolates to be susceptible to gentamycin. However, they reported a higher resistance to ciprofloxacin of 57% of the isolates compared to the present study (15%), reflecting the effect of

the isolate source on its antibiotic resistance pattern.

Cronobacter spp. isolates recovered from PIMF and dried whole milk samples examined in this study showed both single antibiotic resistance and multidrug resistance to more than two antibiotics. Multidrug resistance was observed against penicillin, tetracycline, and streptomycin. Previous studies demonstrated multidrug resistance to various combinations of antibiotics in Cronobacter spp. isolates cultured from an adult patient with a wound infection and from domestic kitchens.[20-21]

Despite the assumption that Cronobacter spp. infections can be eradicated using antibiotics, the present study confirms the results of other previous reports showing that the pathogen may be able to resist several effective antibiotics. This raises concern regarding the use of antibiotics to treat these infections that could possibly lead to serious illnesses, including meningitis. Drug resistance in foodborne bacteria has been frequently linked to the abuse of antibiotics in feeding animals.[22] In addition to complicating medical treatments, antibiotic-resistant foodborne pathogens could serve as a reservoir spreading genetic elements of antibiotic resistance to other bacteria in the human gut.[23]

These results indicated the importance of employing biochemical and molecular identification testing for reliable detection of Cronobacter spp. This is consistent with previous reports demonstrating the value of incorporating the rapid ID 32E system and real-time PCR analysis in the detection protocols of Cronobacter spp. in PIMF and other related products.[24-25]

Conclusion

In conclusion, the present study demonstrates the association of the emerging pathogen Cronobacter spp. with PIMF and whole dried milk in Saudi Arabia. It also reports variability in the prevalence rates of the pathogen in these products compared to the rates in other regions. The study highlights the ability of Cronobacter spp. isolates to develop both single and multidrug resistance, which raises concern regarding the effectiveness of available antibiotics to combat serious illnesses associated with Cronobacter spp. infection. This requires further research in order to develop more effective antibiotics and/or alternative therapeutic strategies.

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