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OCCURRANCE OF *ENTEROBACTER SAKAZAKII* IN EGYPTIAN INFANT FORMULA MILK

(With One Table and 2 Figures)

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تواجد ميكروب الإنتيروباكتر ساكازاكى فى ألبان الأطفال المصرية

عمر حسن رفعت القوصى ، مجدى محمد عثمان

يعتبر ميكروب الإنتيروباكتر ساكازاكى "*Enterobacter sakazakii*" أحد أسباب وفاة حديثى الولادة من الرضع خصوصا ناقصى الوزن وغير مكتملى النمو وذلك لما يحدثه من عدوى متمثلة فى الباكتيريميا والإلتهاب السحائى والتهاب الأمعاء النكروزى. ولما كانت ألبان الأطفال هى المتهم الرئيسى لنقل العدوى فقد أجريت هذه الدراسة لتقرير مدى تواجد هذا الميكروب فى ألبان الأطفال الجافة المتداولة بالصيدليات وكذا الرضعات المحضرة منها بالمستشفيات المصرية بمدينة الإسماعيلية وكذا دراسة تأثير درجة حرارة الحفظ للرضعات المحضرة على نمو الميكروب ودراسة أثر التسخين داخل أفران الميكرويف على الميكروب. هذا وقد تم عزل الميكروب من عينتى ألبان جافة من أصل 26 عينة بنسبة 7.7% و8 عينات رضعات من أصل 38 عينة بنسبة 21.05% بما يعنى ضرورة الاهتمام بمراقبة مصانع إنتاج الألبان الجافة واتباع الطرق السليمة لتحضير الرضعات. كما بينت الدراسة الفارق الكبير فى معدل نمو الميكروب بالرضعات المحفوظة فى درجات حرارة مختلفة. حيث زادت أعداد الميكروب بشكل مطرد فى الرضعات المحفوظة عند 35°م من 3.08لوغ مللى$^{-1}$ إلى 3.75، 4.41، 5.04، 6.82، 8.34 لوغ مللى$^{-1}$ بعد 2، 4، 6، 12، 24 ساعة على الترتيب. وكان معدل زيادة أعداد الميكروب أقل عند حفظ الرضعات تحت درجة 22°م حيث بلغت الأعداد 3.26، 3.60، 4.00، 5.08، 5.90 لوغ مللى$^{-1}$ بعد نفس التوقيتات السالف ذكرها. بينما لم تحدث زيادة تذكر فى أعداد الميكروب لدى حفظ الرضعات عند درجة 4°م خلال النصف الأول من فترة الحفظ وفى نهاية فترة الحفظ (24 ساعة) وصلت أعداد الميكروب إلى 3.3لوغ مللى$^{-1}$. كما ثبت فاعلية التسخين بالميكرويف فى تقليل أعداد الميكروب بشكل ملحوظ بما يعنى إمكانية الاستعانة به مع مراعات تبريد الرضعة والتأكد من ملائمة درجة حرارتها للرضيع قبيل تقديمها له.

SUMMARY

Enterobacter sakazakii has been implicated to be a rare, but life-threatening cause of outbreaks and sporadic cases of neonatal infections,

particularly in premature underweight babies. The prevalence of *E. sakazakii* in powdered and hydrated infant formula milk (IFM) collected from Egyptian pharmacies and hospitals in Ismailia City was investigated using FDA enrichment procedure. A total of 64 samples (26 powdered + 38 hydrated) were tested. The organism was isolated from 2 powdered (7.7%) and 8 hydrated (21.05%) samples. The organism grew rapidly at 35ºC in which its population increased from 3.08 log cfu ml^{-1} at 0 time to 3.75, 4.41, 5.04, 6.82, and 8.34 log cfu ml^{-1} after 2, 4, 6, 12, and 24 hours, respectively. At 22ºC, *E. sakazakii* increased from 3.08 log cfu ml^{-1} at 0 time to 3.26, 3.60, 4.00, 5.08, and 5.90 log cfu ml^{-1} after 2, 4, 6, 12, and 24 hours, respectively. It showed no growth at 4ºC through the first 12 hours and very slow growth along the whole period reaching only 3.3 log cfu ml^{-1} by the end of the 24 hour keeping period. Upon investigating the killing effect of microwave heating, it was proved to be a convenient and fast method to reduce *E. sakazakii* counts in mild, moderate and massively contaminated IFM samples. However, care should be taken to ensure that milk is adequately cooled to the required temperature before being fed to an infant.

Keywords: *"Enterobacter sakazakii, powdered/hydrated infant formula milk, storage temperature, microwave heating"*

INTRODUCTION

Enterobacter sakazakii is a motile, non-spore forming, facultative anaerobe, Gram negative rod. It was previously referred to as "yellow-pigmented *Enterobacter cloacae*" until it was designated a unique species by Farmer *et al.* (1980). *E. sakazakii* is an emerging pathogen associated with life-threatening neonatal infections. It was first associated with cases of neonatal meningitis in 1958 (Urmenyi and Franklin, 1961). The organism causes bacteraemia, necrotizing enterocolitis and infant meningitis in premature babies and neonates. Reported case-mortality meningitis rates vary from 40 to 80% among infected infants, with the majority of those who survive *Enterobacter*-associated meningitis (94%) developing an irreversible neurological sequela (Willis and Robinson, 1988). The reservoir for *E. sakazakii* is unknown, however, a growing number of reports suggest a role for powdered milk-based infant formulas as a vehicle for infection (Biering *et al.*, 1989; Simmons *et al.*, 1989; Van Acker *et al.*, 2001; Iversen, and Forsythe, 2003; Gurtler *et al.*, 2005; Shaker *et al.*, 2007). Indeed, *E. sakazakii* has been isolated from environmental sources and from food other than infant formula and milk powder (Kandhai, *et al.*, 2004;

Friedemann, 2007), but why it is associated only with the consumption of infant formulae is still unclear (Conte and Passantino, 2008). Recent taxonomic analyses have determined that *E. sakazakii* comprises a number of genomospecies, and it has been proposed that *E. sakazakii* may be reclassified as a novel genus, "*Cronobacter*". Cawthorn, *et al.* (2008) recommended the use of accurate methods for rapid detection and identification of this group of micro-organisms, since even low cell numbers have been reported to cause disease. In a recent review described the ubiquitous nature of the organism in food other than infant formula, *E. sakazakii* could be isolated from plant food and food ingredients like cereal, fruit and vegetables, legume products, herbs and spices as well as from animal food sources like milk, meat and fish and products made from these foods. The spectrum of *E. sakazakii*-contaminated food covers both raw and processed food. The kind of processing of *E. sakazakii*-contaminated food was not restricted to dry products. Fresh, frozen, ready-to-eat, fermented and cooked food products as well as beverages and water suitable for the preparation of food, were found to be contaminated by *E. sakazakii* (Friedemann, 2007). Concerning its tolerance toward environmental stresses, Lin and Beuchat (2007) demonstrated that *E. sakazakii* can survive for up to 12 months in infant cereals having a wide range of a_w when storage is at temperatures simulating those to which they may be exposed during distribution, at retail, and in the home. Also, Arku *et al.* (2008) investigated the survivability of four strains of *E. sakazakii* upon spray-drying and found that all tested strains had the ability to survive the spray drying process and were detected in the powders with low inoculums (10^2 cfu/g dry wt) and enumerated in all the powders with the high inoculums (10^7 cfu/g dry wt) for at least 12 weeks. They concluded that the controls in place to prevent *E. sakazakii* from getting to the spray drier are essential. Iversen and Forsythe (2004) concluded that hygienic production of powdered IFM and milk production as monitored by control of *Salmonella* and enumeration of Enterobacteriaceae did not control *E. sakazakii*. Indeed, it has been stated that, the effectiveness of prevention depends on the degree of contamination and contamination sites, which are generally unknown (Kandhai, *et al.*, 2004). The present study has three aims; reporting to what extent Egyptian powdered and hydrated infant milk formulae are contaminated with *E. sakazakii*, monitoring the behavior of an isolated *E. sakazakii* organism in hydrated infant milk formula in different incubation temperatures, and investigating the killing effect of microwave heating on the organism.

MATERIALS and METHODS

First: Occurrence of *E. sakazakii* in infant formula milk

A total of 64 infant formula milk (IFM) samples (26 powdered + 38 hydrated) were collected from different Egyptian pharmacies and hospitals in Ismailia City. Collected samples were investigated using the current Food and Drug Administration (FDA, 2002) method which includes a pre-enrichment procedure in buffered peptone water (BPW), enrichment in Enterobacteriaceae enrichment (EE) broth, plating on violet red bile glucose agar (VRBG) and picking of five grown colonies onto trypticase soy agar (TSA) plates, which are incubated at 25°C for 48–72 hours. Yellow-pigmented colonies, typical for *E. sakazakii*, on the TSA plates are confirmed using the API 20E biochemical strips.

Second: Effect of incubation temperature on *E. sakazakii* growth

Hydrated IFM was prepared according to the manufacturer direction inoculated with one of the isolated *E. sakazakii* before being aseptically subdivided into 3×5 bottles. The first set of bottles (No=5) were kept at 4±1ºC, the second set were held at 22±1ºC, while the third set of bottles were held at 35±1ºC. *Enterobacter sakazakii* count of the hydrated IFM was done according to FDA (2002) following preparation (0 time). A separate bottle from each set was examined after 2, 4, 6, 12 and 24 hours of incubation at the specified temperatures for *E. sakazakii* count.

Third: Killing effect of microwave heating on *E. sakazakii*

Hydrated IFM was inoculated with *E. sakazakii* with 3 different microbial population densities (simulating mild, moderate and massive contaminated formulae). Each preparation of the 3 inoculated IFM was further subdivided aseptically into 4×100 ml bottled portions. Each bottle was examined for *E. sakazakii* count before and after heating in a household microwave oven (KOG-134K, 1000 Watt, 2450 megahertz) for 20, 40, 60 and 80 seconds. The temperature following each heating process was monitored in a pilot bottle containing the same quantity of IFM.

RESULTS

Table 1: Occurrence of *E. sakazakii* in powdered and hydrated IFM

Powdered samples			Hydrated samples		
No	+ve	%	No	+ve	%
26	2	7.69	38	8	21.05

No: Number of examined IFM samples

+ve: Number of positive samples (containing *E. sakazakii*)

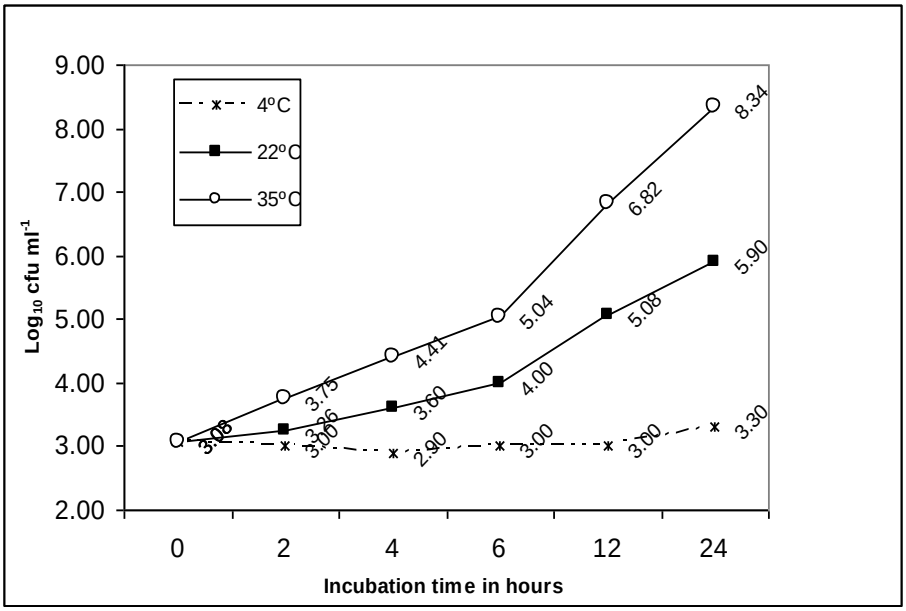

Fig. 1: Effect of storage temperature on the growth of *E. sakazakii*

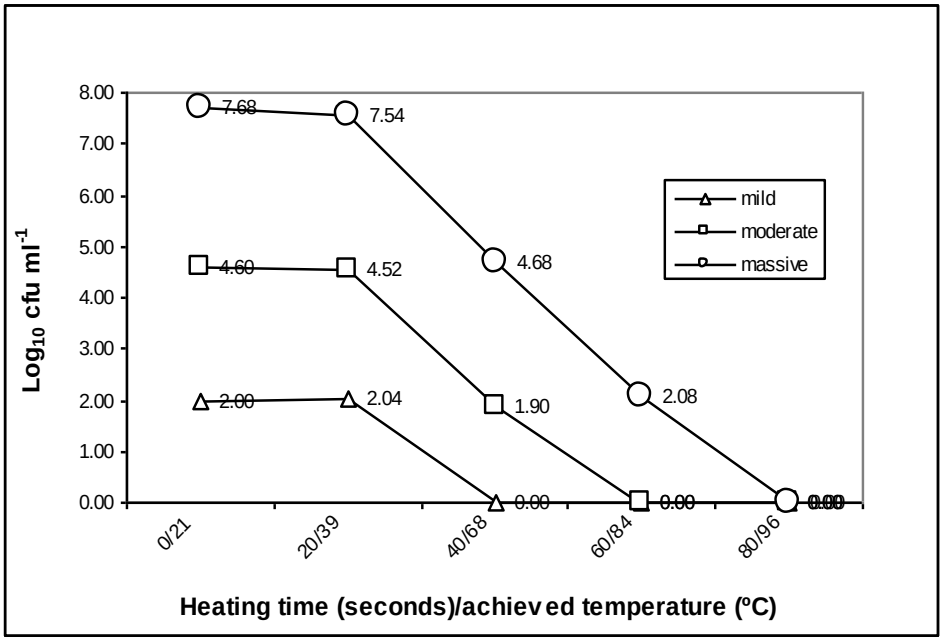

Fig. 2: Killing effect of microwave heating on *E. sakazakii*

DISCUSSION

First: Occurrence of *E. sakazakii* in infant formula milk

The present study gives an idea about the prevalence of *E. sakazakii* in powdered and hydrated infant formula milk collected from Egyptian pharmacies and hospitals in Ismailia City. As outlined in the presented Table 1, only two out of the 26 powdered IFM samples representing 7.69% were contaminated by *E. sakazakii*. Hydrated samples showed more incidence in which 21.05% (8/38 samples) were positive. Such higher incidence in hydrated samples reflects the contribution of other environmental cross contaminating sources during IFM preparation. Temperature abuse of hydrated samples may also constitute another cause for the higher positive percentage. These results appear to be not far from those reported by Nazarowec-White and Farber (1997) and Shaker *et al.* (2007). Food and Agriculture Organization (FAO) categorized *Enterobacter sakazakii* among microorganisms of great concern with powdered infant formula due to the clear evidence of a causal association between their presence in powdered infant formula and illness in infants. Also, according to Regulation (EC) No. 2073/2005 on the microbiological criteria for foodstuffs, *E. sakazakii* is considered a microorganism of greatest concern in infant formulae and follow-on formulae. It is included within "safety criteria". However, Friedemann (2007) mentioned that, Although *E. sakazakii*-contaminated food do not have general public health significance, measures for prevention should consider its presence in food, food ingredients, their processing and preparation as possible source of contamination, colonization or infection. Hence, such obtained results should alert concerned authorities to be more careful in supervising IFM producing factories as well as assuring the right way of preparing and keeping hydrated IFM by concerned hospital stuffs.

Second: Effect of incubation temperature on *E. sakazakii* growth

Since delayed consumption of reconstituted infant milk formulae in conjunction with temperature abuse are considered to be fairly common, the growth pattern of *E. sakazakii* was followed up at some specified temperatures. The used temperatures were 4ºC (representing refrigerated storage), 22ºC (representing room temperature), and 35ºC (representing bottle warmer temperature). The results presented in Fig. 1 showed rapid microbial growth at 35ºC in which the organism population increased from 3.08 log cfu ml^{-1} at 0 time to 3.75, 4.41, 5.04, 6.82, 8.34 log cfu ml^{-1} after 2, 4, 6, 12 and 24 hours, respectively. At 22ºC, *E. sakazakii* grew moderately and increased from 3.08 log cfu ml^{-1}

at 0 time to 3.26, 3.60, 4.00, 5.08, and 5.90 log cfu ml^{-1} after 2, 4, 6, 12 and 24 hours, respectively. On the other hand, the organism showed no growth at 4ºC through the first half of the keeping period and very slow growth along the second half as it reached only 3.3 log cfu ml^{-1} by its end. Likely results have been recorded by Richards *et al.* (2005) and Gurtler and Beuchat (2007). These results indicate that hydrated IFM should be consumed immediately after preparation or held at 4°C for no longer than 12 hours.

Third: Killing effect of microwave heating on *E. sakazakii*

The killing activity of microwaves of 2450 MHz frequency and 1000 Watt power on *E. sakazakii* inoculated at 3 different microbial population densities (simulating mild, moderate and massive contaminated formulae) was investigated Fig 2. The organism population in mild-contaminated samples (2.0 log cfu ml^{-1}) couldn't be isolated after exposing to microwave heating for ≥40 seconds. Moderately-contaminated samples (4.6 log cfu ml^{-1}) showed somewhat similar pattern of microbial count reduction. The organism population decreased to 4.52 and 1.9 log cfu ml^{-1} after heating for 20 and 40 seconds, respectively. It couldn't be isolated from samples heated for longer periods. Samples with massive *E. sakazakii* counts (7.68 log cfu ml^{-1}) showed positive growth after 60 seconds period of heating exposure (2.08 log cfu ml^{-1}). Upon heating for 80 seconds the temperature achieved 96ºC in which no survivors of *E. sakazakii* could be traced. These results agreed with those recorded by (Kindle *et al.*, 1996). The effect of microwave heat treatment depends on the quantity of the product and the geometry of the vessel used. So it is logic to mention that in this experiment we used the same type of bottles and the same quantities (100 ml) of IFM samples. It is concluded that, inasmuch as biological experiments up till now showed no evidence for the hazards of microwave heat treatment of milk (Sieber *et al.*, 1996), it can be relay upon as a convenient and fast method to reduce microbial contamination of IFM. However, great care should be taken to ensure that milk is adequately cooled to the required temperature before it is fed to an infant.

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