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Isolation of *Enterobacter sakazakii* from Powdered Foods Locally Consumed in Nigeria

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Abstract: The presence *Enterobacter sakazakii* was investigated from powdered food samples using enrichment procedure of Enterobacteriaceae Enrichment Broth (EEB), Violet Red Bile Glucose Agar (VRBGA) and Tryptic Soy Agar (TSA). A total of 140 food samples were tested. *E. sakazakii* was isolated from 20/70 powdered infant foods, 13/50 powdered milk and 5/20 milk-based products, making a total of 27.1% prevalence. The isolates were tested for their susceptibility to 15 antibiotics. The results showed that streptomycin was the most effective (94.7%), followed by ofloxacin (92.1%), levofloxacin (84.2%), ciprofloxacin (79.0%), pefloxacin (79.0%) and gentamycin (65.8%). The organism was resistant to rifampicin and amoxicillin.

Key words: Food, *Enterobacter sakazakii*, antibiotic, streptomycin, infant milk

INTRODUCTION

Enterobacter sakazakii is a gram negative, facultative anaerobic, straight rod-shaped bacterium. It belongs to the family *Enterobacteriaceae* and genus *Enterobacter* that contains a number of species including *E. agglomerans, E. cloacae, E. aerogenes* and *E. gergoviae*. It is a coliform having dimensions of 3 µm in length and 1 µm in width. The cells are motile by peritrichous flagella and do not form spores (Farmer *et al*., 1980; Farmer and Kelly, 1992).

Enterobacter sakazakii is an opportunistic human pathogen that has been implicated in severe forms of septicemia (Lai, 2001), necrotizing enterocolitis (Van Acker *et al*., 2001) and meningitis (Bar-Oz *et al*., 2001), especially in neonates with mortality rate varying from 40-80% (Muytjens *et al*., 1988). The International Commission for Foods, due to the seriousness of pathologies with *E. sakazakii* has ranked the organism as "severe hazard for restricted populations, life threatening or substantial chronic sequelae or long lasting" (ICMSF, 2002).

E. sakazakii has been isolated from a wide range of foods including ultra high-temperature treated milk (UHT milk), cheese, meat, vegetables, grains, sorghum seeds, rice seeds, herbs, spices, fermented bread, fermented beverage, tofu and sour tea (Gassem, 2002; Leclercq *et al*., 2002; Iversen and Forsythe, 2003). Despite this, studies have confirmed the connection between neonatal *E. sakazakii* infection and infant milk formulas (Muytjens *et al*., 1988; Biering *et al*., 1989; Simmons *et al*., 1989; Nazarowec-White and Farber, 1997b; Van Acker *et al*., 2001).

The US Food and Drug Administration (FDA, 2002) has issued an alert to health care professionals about the risk associated with *E. sakazakii* infections among neonates fed with milk-based infant formula. The alert stated that a major contribution to the avoidance of *E. sakazakii* infections in premature babies and neonates is the prevention of contamination of infant milk formula during production and bottle preparation.

Several outbreaks have occurred in neonatal intensive care units as a result of infections by the organism: in May/ June 1994 in France where 13 neonates were infected and three died, June/ July 1998 in Belgium where 12 neonates developed necrotizing enterocolitis and two twin brothers died and a more serious one in Tennessee in 2001 (CDC, 2002). These outbreaks were traced to contaminated powdered infant formula by the organism (Van Acker *et al*., 2001). But most recently in 2008, a total of five babies were lost in New Mexico due to *E. sakazakii* infection (CDC, 2009). These have alerted the US Centers for Disease Prevention and Control (CDC) on the consumption of powdered infant formula, and have brought the organism to limelight.

E. sakazakii is an emerging food borne pathogen that had been discovered in the developed countries. It is likely that there is a significant under-reporting of infections in all countries. The absence of reports is probably due to a lack of awareness of the problem rather than an absence of illness. Since infant formula is widely used, the presence of *E. sakazakii* in infant formula and its potential effects in infants could as well be a significant public health problem in most countries. This study provides an information on the determination on the incidence of *E. sakazakii* in powdered infant foods and antibiotic susceptibility pattern of the organism.

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MATERIALS AND METHODS

Collection of samples: A total of 140 different commercial food samples from different manufacturers were purchased from retail stores across Benin City, Nigeria. The samples composed of 70 powdered infant foods (recommended for from birth to one year old infants), 50 powdered milk and 20 milk-based products. The samples were labeled appropriately and approved by National Agency for Food and Drug Administration and Control (NAFDAC).

Detection and Isolation of *Enterobacter sakazakii*: The method of Food and Drug Administration (2002) for the isolation of the pathogen was used. For the pre-enrichment step, 10 g of each powder was aseptically dissolved in 50 ml of sterile water pre-warmed to 40°C, to make a solution in the 60ml screw-capped bottles. From the bottle, 10-fold dilution was made using sterile test tubes. The test tubes were screwed. The tubes were incubated at 36°C for 24 h in an incubator.

For the enrichment step, 90 ml of Enterobacteriaceae Enrichment Broth (EEB) was prepared into the 100 ml screw-capped bottles. The incubated tubes (10 ml each) were aseptically inoculated into the bottles containing 90ml of EEB. A bottle was left uninoculated to act as a control. The bottles were incubated at 36°C for 24 h in an incubator.

The selection step was carried out by aseptically streaked a loop full of the incubated bottles into a duplicate plates of Violet red bile glucose agar. The plates were then incubated at 36°C for 24 h in an incubator. From the cultured plates, purple colonies were picked using sterile inoculating loop and sub-cultured into Tryptic Soy Agar (TSA) plates in duplicates by streaking. The plates were incubated at 25°C for 72 h in an incubator.

The isolated colonies were further detected by biochemical methods.

Antibiotic susceptibility test: Antibiotic susceptibility test was performed using Kirby-Bauer disc diffusion method. The medium used for this test is Tryptic soy agar. Prepared plates containing the medium were dried in hot air oven for about five minutes. A sterile inoculating loop was used to pick isolated colonies and emulsified into 5 ml of sterile nutrient broth. After proper homogenization, the broth was poured into the agar surface to make a lawn of growth. The surface of the agar plate was allowed to dry for 5 min. A sterile forceps was used to place the antibiotic discs unto the inoculated plates. The antibiotic discs were in two sets and each set was used for isolation. The antibiotics contained in the discs were: streptomycin (30 µg), norfloxacin (10 µg), chloramphenicol (30 µg), ciprofloxacin (10 µg), levofloxacin (20 µg), gentamycin (10 µg), rifampicin (20 µg), amoxicillin (20 µg), ofloxacin (10 µg), pefloxacin (10 µg), augmentin (30 µg), cephalexin (10 µg), nalidixic acid (30 µg), trimethoprim-sulfamethoxazole (30 µg), ampicillin (30 µg).

The plates were incubated at 37°C for 24 h in an incubator. After incubation, the diameter of the zone of inhibition was measured in millimetres using a ruler. All results were recorded appropriately and interpreted using the National Committee for Clinical Laboratory Standards (NCCLS) interpretation chart (NCCLS, 2002).

RESULTS

A total of 140 food samples were tested for the presence of *E. sakazakii*, 38 samples were found positive. The positive strains of *E. sakazakii* formed yellow colonies on Tryptic soy agar after 48 h of incubation at 25°C and satisfied the biochemical screening.

Table 1 indicates the number of *E. sakazakii* isolates obtained from each food brand. The organism was detected in all the food brands except in Bournvita. Among the powdered infant foods, SMA was the most prevalent, while Frisocrem and Nutrend were the least prevalent for the organism. Peak choco had the highest prevalence among the milk-based products, with Bournvita had zero prevalence.

The result in Table 2 showed that 38(27.1%) of the total 140 powdered foods were positive for *E. sakazakii*, with powdered infant foods having the highest frequency of 33.9%, followed by powdered milk (32.7%) and milk-based products (31.4%) having the least.

On testing the 38 isolates from the samples using 15 antibiotics in antibiotic susceptibility discs, almost all the isolates were sensitive to streptomycin (94.7%), followed by ofloxacin (92.1%), levofloxacin (84.2%), ciprofloxacin (79.0%), pefloxacin (79.0%) and gentamycin (65.8%) in the decreasing order (Table 3).

Cephalexin (42.1%), norfloxacin (31.6%), amoxicillin-clavulanate (31.6%) was poorly sensitive; while nalidixic acid (10.5%), trimethoprim-sulfamethoxazole (10.5%), chloramphenicol (7.9%), ampicillin (5.3%) were resistant. But rifampicin (0%) and amoxicillin (0%) were strongly resistant.

DISCUSSION

Enterobacter sakazakii is an emerging food-borne pathogen that had been linked with infantile meningitis, septicemia and necrotizing colitis transmitted through the consumption of contaminated powdered infant foods and other milk products (Lai, 2001; Van Acker *et al*., 2001; Bar-Oz *et al*., 2001).

This study was set up to investigate the incidence of this organism in powdered foods and its products. *E. sakazakii* was isolated from the three food types-powdered infant food, powdered milk and milk-based products, in 27.1% of samples. Among the three food types, powdered infant food had the highest frequency.

Table 1: *Enterobacter sakazakii* isolated from powdered foods

Food samples/brand	No. of samples	SP (+) with *E. sakazakii*	SN (-) with *E. sakazakii*
Powdered infant food			
SMA	10	4	6
NAN	10	3	7
Peak 123	10	3	7
Nutrend	10	2	8
Soya	10	3	7
Cerelac	10	3	7
Frisocrem	10	2	8
Powdered milk			
Peak	10	3	7
Cowbell	10	3	7
Nunu	8	2	6
Coast	8	1	7
Blue boat	7	2	5
Jago	7	2	5
Milk-based product			
Milo	4	1	3
Ovaltine	4	1	3
Peak choco	4	2	2
Cowbell choco	4	1	3
Bournvita	4	0	4

SP = Samples Positive (+) with *E. sakazakii*
SN = Samples Negative (-) with *E. sakazakii*

Table 2: Total (percentage) contamination of the sample type

Sample type	Total number	No. (%) of sample contaminated
Powdered infant food	70	20 (33.9)
Powdered milk	50	3 (32.7)
Milk-based product	20	5 (31.4)
Total	140	38 (27.1)

These results correlated with the works of (Muytjens *et al*., 1988; Biering *et al*., 1989; Simmons *et al*., 1989; Noriega *et al*., 1990; Nazarowec-White and Farber, 1997b; Iversen and Forsythe, 2003; Shaker *et al*., 2007), who had found a direct relationship between infant formula and *E. sakazakii*. Muytjens *et al*. (1988) tested 141 samples of powdered infant milk formula manufactured in different countries. They found that *E. sakazakii* and other *Enterobacteriaceae* were isolated from 14.1 and 52.2% of the total samples respectively. Nazarowec-White and Farber (1997b) surveyed the presence of *E. sakazakii* in 120 dried infant milk samples (five manufacturers) obtained from Canadian retail market and reported that the prevalence of this bacterium ranged between 0 and 12% of the samples/manufacturer. Iversen and Forsythe (2004) isolated *E. sakazakii* from 24% of 82 powdered infant milk formulas.

Many studies have focused on the infant formula as the main source of this serious pathogen (Postupa and Aldova, 1984; Muytjens *et al*., 1988; Nazarowec-White and Farber, 1997b; Van Acker *et al*., 2001; Block *et al*., 2002). Despite the fact that formulas are exposed to heat treatment during processing, *E. sakazakii* was still isolated from these products. Post-processing contamination of the infant formula from food production environments may be responsible for the presence of this pathogen in infant formula since standard pasteurization practices are effective for the inactivation of the organism (Iversen *et al*., 2004).

Nazarowec-White and Farber (1997a) stated that *E. sakazakii* can gain access to the powder from the environment or from the addition of the ingredients at the powder stage, especially using the Dry-mix process of production. Iversen and Forsythe (2003) reported that the presence of *E. sakazakii* in powdered infant milk formula depends on the process conditions and nature of the product. However, the prevalence of the organism following the drying stage and survival in powdered foods for a long time may be due (in part) to the organism's ability to resist desiccation and osmotic stress (Arku *et al*., 2008).

Unlike commercially available ready-to-feed liquid infant formula, which is sterile, powdered infant formula (including dried bovine milk and milk products) is not a sterile product (FDA, 2002). Powdered infant formula has been known to be contaminated, on occasion, with bacterial pathogens, including *Bacillus* species, *Clostridium* species, *Staphylococcus* species and *Enterobacteriaceae*, notably *Cronobacter* (Forsythe, 2005). Therefore, hygienic measures and practices must be used during the manufacture of formula to minimize entry of contaminants into the process.

For the case of the milk-based product, the organism was not detected in Bournvita, *E. sakazakii* could not be ruled from being a possible pathogen of this product, putting into consideration that each sample represented itself only. This result is unusual as the organism has been isolated from a wide range of foods including chocolate, spices, rice seeds, cereals, potato flour and pasta (Shaker *et al*., 2007). Also, it could that the organism is unequally distributed in the sample or its presence escaped detection (Muytjens *et al*., 1988).

From the results obtained from the antibiotic sensitivity test (Table 3), streptomycin (94.7%) seems to be a better drug for treatment. Others include Ofloxacin (92.1%), levofloxacin (79.0%), pefloxacin (79.0%) and gentamycin (65.8%). Streptomycin and gentamycin are aminoglycosides; while Ofloxacin, levofloxacin, ciprofloxacin and pefloxacin are quinolones.

These results were consistent with the findings by Stock and Wiedemann (2002) that *E. sakazakii* is susceptible to tetracyclines, aminoglycosides, quinolones, antifolates and chloramphenicol. Aminoglycosides and quinolones inhibit protein synthesis and DNA replication respectively in the organism (McKane and Kandel, 1996).

Enterobacter sakazakii like other *Enterobacter* species have acquired resistance by inactivating broad spectrum beta-lactam antibiotics due to the production of beta-lactamases (Drudy *et al*., 2006). Other mechanisms

Table 3: Number (percentage) of *E. sakazakii* isolates sensitive to antibiotics

Sample brands	No. of isolates tested	Antibiotics							
		S	NB	CH	CPX	LEV	RD	CN	AML
SMA	4	4(100)	1(25)	0(0)	3(75)	3(75)	0(0)	3(75)	0(0)
NAN	3	3(100)	1(33.3)	1(33.3)	3(100)	3(100)	0(0)	2(66.7)	0(0)
Peak 123	3	3(100)	0(0)	0(0)	2(66.7)	3(100)	0(0)	2(66.7)	0(0)
Nutrend	2	2(100)	1(50)	1(50)	2(100)	2(100)	0(0)	1(50)	0(0)
Soya	3	2(66.7)	1(33.3)	0(0)	1(33.3)	3(100)	0(0)	1(33.3)	0(0)
Cerelac	3	2(66.7)	2(66.7)	0(0)	1(33.3)	2(66.7)	0(0)	2(66.7)	0(0)
Frisocrem	2	2(100)	1(50)	0(0)	1(50)	1(50)	0(0)	2(100)	0(0)
Peak	3	3(100)	1(33.3)	0(0)	3(100)	1(33.3)	0(0)	1(33.3)	0(0)
Cowbell	3	3(100)	1(33.3)	0(0)	2(66.7)	3(100)	0(0)	3(100)	0(0)
Nunu	2	2(100)	0(0)	0(0)	2(100)	1(50)	0(0)	2(100)	0(0)
Coast	1	1(100)	1(100)	1(100)	1(100)	1(100)	0(0)	0(0)	0(0)
Blue boat	2	2(100)	1(50)	0(0)	2(100)	2(100)	0(0)	2(100)	0(0)
Jago	2	2(100)	0(0)	0(0)	2(100)	2(100)	0(0)	1(50)	0(0)
MBP	5	5(100)	1(20)	0(0)	5(100)	5(100)	0(0)	3(60)	0(0)
Total (%)	38	94.7	31.6	7.9	79.0	84.2	0.0	65.8	0.0

Sample brands	No. of isolates tested	Antibiotics						
		OFX	PEF	AU	CEP	NA	SXT	PN
SMA	4	4(100)	4(100)	1(25)	1(25)	0(0)	1(25)	0(0)
NAN	3	3(100)	2(66.7)	1(33.3)	1(33.3)	0(0)	0(0)	0(0)
Peak 123	3	3(100)	1(33.3)	2(66.7)	0(0)	0(0)	0(0)	1(33.3)
Nutrend	2	2(100)	1(50)	1(50)	1(50)	0(0)	0(0)	0(0)
Soya	3	3(100)	3(100)	1(33.3)	2(66.7)	1(33.3)	0(0)	1(33.3)
Cerelac	3	2(66.7)	2(66.7)	0(0)	1(33.3)	0(0)	0(0)	0(0)
Frisocrem	2	2(100)	2(100)	0(0)	2(100)	0(0)	1(50)	0(0)
Peak	3	2(66.7)	2(66.7)	0(0)	2(66.7)	1(33.3)	1(33.3)	0(0)
Cowbell	3	3(100)	2(66.7)	0(0)	2(66.7)	0(0)	0(0)	0(0)
Nunu	2	2(100)	1(50)	0(0)	1(50)	1(50)	0(0)	0(0)
Coast	1	1(00)	1(100)	0(0)	0(0)	0(0)	1(100)	0(0)
Blue boat	2	2(100)	2(100)	1(50)	1(50)	0(0)	0(0)	0(0)
Jago	2	2(100)	2(100)	1(50)	1(50)	0(0)	0(0)	0(0)
MBP	5	4(80)	5(100)	4(80)	1(20)	1(20)	0(0)	0(0)
Total (%)	38	92.1	79.0	31.6	42.1	10.5	10.5	5.3

S = Streptomycin, CN = Norfloxacin, CH = Chloramphenicol, CPX = Ciprofloxacin, LEV = Levofloxacin, RD = Rifampicin, CN = Gentamycin, AML = Amoxicillin, OFX = Ofloxacin, PEF = Pefloxacin, AU = Amoxicillin-clavulanate, CEP = Cephalexin, NA = Nalidixic Acid, SXT = Trimethoprim-sulfamethoxazole, PN = Ampicillin. NZI = No Zone of Inhibition

include: decreased cell permeability, active efflux, modification of drug receptor site, synthesis of resistant metabolic pathway and acquisition of plasmids and transposons (Chao *et al*., 2007). These could be the reasons for the strong resistance against cephalexin (42.1%), amoxicillin-clavulanate (31.6%), ampicillin (5.3%) and amoxicillin (0%) as observed in this research. Other studies have also shown rifampicin, macrolides and fusidic acid to be resistant (Stock and Wiedemann, 2002).

Conclusion: *E. sakazakii* is an emerging pathogen, often transmitted through the consumption of powdered infant foods and its products. It is responsible for series of infections with potential fatal outcomes in infants. The findings from this study showed that *E. sakazakii* was detected in powdered infant foods, powdered milk and milk-based products. The organism was found to be resistant to some antibiotics; the quinolones, in combination with an aminoglycosides would be a better choice drug for treatment of infections caused by this organism.

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