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Quality of Powdered Substitutes for Breast Milk with Regard to Members of the Family *Enterobacteriaceae*

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Members of the family *Enterobacteriaceae* were cultured from 52.5% of 141 milk substitute infant formulas which were obtained in 35 countries. The concentration did not exceed a level of 1 CFU/g in any product. The species which were isolated most frequently were *Enterobacter agglomerans*, *Enterobacter cloacae*, *Enterobacter sakazakii*, and *Klebsiella pneumoniae*. If infections due to these organisms occur, it can be useful to include a check of the hygienic precautions which are taken during the preparation and storage of the formula. Milk powders without members of the *Enterobacteriaceae* might offer extra protection to the newborn if some multiplication does occur in the formula.

Enterobacter sakazakii can be a cause of neonatal meningitis and sepsis (5, 24). The source and mode of transmission of this organism are unknown. A mode of transmission other than passage through the birth canal is likely at least for some patients who were delivered by cesarean section or were found not to be colonized after delivery (16, 24). This rarely seen microorganism has been isolated several times from prepared formula in a hospital kitchen during an outbreak. Although it was not cultured from the formula powder itself, this might have been due to an unequal distribution in the powder or its presence at such a low concentration that it escaped detection by conventional methods. To test this supposition, large amounts of powdered substitutes for breast milk, obtained from various countries, were analyzed for the presence of members of the family *Enterobacteriaceae*.

MATERIALS AND METHODS

Sampling. Employees of the University of Nijmegen and others were asked to buy two or three different powdered substitutes for breast milk abroad. Only in a few instances (all Hungarian and Israeli products and some Japanese products) were the products supplied by the manufacturers. The countries and the corresponding numbers of specimens tested were as follows: Argentina, 5; Australia, 3; Austria, 1; Belgium, 7; Brazil, 2; Canada, 6; Chile, 4; People's Republic of China, 1; Czechoslovakia, 5; Denmark, 3; Finland, 1; France, 2; Federal Republic of Germany, 8; Gabon, 4; German Democratic Republic, 6; Hungary, 8; Ireland, 4; India, 5; Israel, 1; Italy, 4; Japan, 8; The Netherlands, 19; New Zealand, 1; Norway, 2; Poland, 1; Portugal, 3; USSR, 3; Spain, 2; Switzerland, 2; Tunisia, 2; Turkey, 2; Uruguay, 1; United Kingdom, 2; United States, 12; and Yugoslavia, 1. The names of the products are available upon request.

Sample analysis. (i) Decontamination. The lid margins of the tins and the spoons used for withdrawal of the samples were flamed with 93% (wt/vol) ethanol. The surfaces of the other packages were disinfected with 70% (wt/vol) ethanol before the samples of the milk powders were withdrawn.

(ii) Enumeration of members of the *Enterobacteriaceae*. Initially, 900, 90, and 9 ml of buffered peptone water (pH 7.2) (Oxoid Ltd., London, England) were added slowly at a temperature of 45°C to 100, 10, and 1 g, respectively, of milk powder in glass containers, which were swirled by hand until the powder was dissolved. All tests were performed in triplicate (or quintuplicate if the amount of material was sufficient). If the content of a package was not sufficient, the quantities were proportionally smaller. The glass containers were incubated in a water bath at 45°C for 5 min to dissolve the powder. After overnight incubation at 36°C, 10 ml of the culture was withdrawn from each flask and added to 90 ml of EE broth (buffered glucose, brilliant green, bile broth; catalog no. CM317; Oxoid). After overnight incubation, 1 ml of the broth was mixed with 20 ml of fluid violet red-bile-glucose agar (Oxoid) in a 9-cm-diameter petri dish in duplicate. After solidification of the agar the cultures were incubated overnight at 36°C. Growth on the agar was subcultured on sheep blood agar and eosin-methylene blue agar (catalog no. 1342; E. Merck AG, Darmstadt, Federal Republic of Germany) according to Levine. The concentration of members of the *Enterobacteriaceae* was estimated by the most-probable-number technique. The strains were identified with the API 20E system (API System S.A., La Balme les Grottes, France). Additional tests were used to identify *E. sakazakii*: the production of yellow colonies on nutrient agar (Difco Laboratories, Detroit, Mich.) after 48 h at 25°C, the production of extracellular DNase on toluidine blue agar after 6 days at 36°C, and a positive α-glucosidase reaction after 4 h (15).

RESULTS

A total of 141 different powdered substitutes for breast milk, obtained in 35 countries, were examined for the presence of members of the *Enterobacteriaceae*. These organisms were cultured from 52.5% of the products, originating from 28 countries (Table 1). The species which were isolated most frequently and the numbers of isolates were as follows: *Enterobacter agglomerans*, 35; *Enterobacter cloacae*, 30; *E. sakazakii*, 20; and *Klebsiella pneumoniae*, 13. The remaining 31 isolates belonged to 14 other species, including the following: *Citrobacter freundii*, 5; *Escherichia coli*, 4; *Klebsiella oxytoca*, 4; *Citrobacter diversus*, 3; and *Hafnia alvei*, 1. *E. sakazakii* was cultured from products available in Australia, Belgium, Canada, Denmark, France, the Federal Republic of Germany, the German Democratic Republic, India, The Netherlands, New Zealand, the USSR,

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TABLE 1. Concentrations of members of the *Enterobacteriaceae* in powdered substitutes for breast milk

Country	Package (g)	Concn (CFU/100 g)					
		E. agglomerans	*E. cloacae*	*E. sakazakii*	*K. pneumoniae*	*Citrobacter* spp.	Other spp.
Argentina	450	0.74	0.36				
Australia	400		0.36	0.36			
	400		0.36				0.36[a]
Belgium	1,000		91.78	1.27			
	150		31.10				1.0[b]
	150	1.0	7.7				1.0[c]
	1,000		54.22				
Brazil	454	0.36					
Canada	450	0.3	0.36				
	450		0.36				
	450		0.92	0.36	4.27		
Chile	450	1.47			0.36		
	450		0.36				
People's Republic of China	454	0.36					
Czechoslovakia	100						0.36[a]
	100						4.6[d]
	350					0.36[e]	
	350		0.36				0.36[b]
	100						4.6[d]
Denmark	1,000			2.7			
France	300			0.51			
Finland	390		0.92				
Federal Republic of Germany	1,000			0.4	0.19	0.19[f]	
	300			0.42			
	1,000	0.19	2.16	0.44	0.19	0.19[f]	
German Democratic Republic	250	0.60	2.45	0.60	0.60	0.60[e]	0.50[b], 1.23[g]
	400		0.74		7.49		
	400		0.74		46.22		
	250	0.6	0.6	0.6			
	500						0.36[h], 0.36[i]
	500	0.36					0.36[h]
	500		0.92		0.3		0.92[j]
Hungary	500	0.74	2.31				1.47[j], 0.3[k]
	500	0.36					
	500				0.36		0.36[j]
Ireland	453	0.36	0.36				
India	500		0.3				
	500	0.74					
	500	0.36	0.36			0.36[d]	
	500			0.92		0.36[d]	
	500	0.36					
Italy	300	0.51					0.51[l]
Japan	1,200	0.19					
	450		0.36				
	450	0.36					0.36[b]
The Netherlands	900	0.19					
	110	1.2					
	110	1.0					
	1,800	0.36					
	750	2.31					
	220		0.6			0.6[e]	
	500	0.36					0.3[d]
	500	0.36		0.36			
	450		9.33	0.36			0.36[m]
	900	0.44					
New Zealand	500			0.36			
Norway	500	0.36					
Portugal	1,000						0.19[h], 0.19[m]
	1,000	0.44	0.19		0.44		
	453	14.94					
USSR	300			66.0	6.1		1.6[d]
	300		13.32	6.1			0.51[b]
	300		0.5	1.3	1.6		0.5[j], 0.5[n], 1.6[o]

Continued on following page

TABLE 1—*Continued*

Country	Package (g)	Concn (CFU/100 g)					
		E. agglomerans	*E. cloacae*	*E. sakazakii*	*K. pneumoniae*	*Citrobacter* spp.	Other spp.
Tunisia	500	2.31					
Turkey	450	0.7					0.36[p]
Uruguay	500	0.36		0.36			
United Kingdom	450					0.36[f]	
United States	454	0.7					0.36[o]
	454						0.36[m]
	454		0.92				
	454	0.36		0.36			
	454				0.36		
	454	0.92					
	397			0.92			

[a] *Yersinia intermedia.*
[b] *Enterobacter intermedium.*
[c] *Escherichia vulneris.*
[d] *E. coli.*
[e] *C. freundii.*
[f] *C. diversus.*
[g] *Enterobacter amnigenus.*
[h] *Escherichia hermannii.*
[i] *Buttiauxella agrestis.*
[j] *K. oxytoca.*
[k] *Yersinia frederiksenii.*
[l] *H. alvei.*
[m] *Escherichia adecarboxylata.*
[n] *Cedecea* sp.
[o] *Rahnella aquatilis.*
[p] *Serratia plymuthica.*

Uruguay, and the United States. The concentration of 78% of the species was 1 CFU/100 g or less. The highest concentration observed was 91.78 CFU/100 g (*E. cloacae*). The concentration of members of the *Enterobacteriaceae* did not exceed a level of 1 CFU/g in any product tested.

DISCUSSION

E. sakazakii was cultured from powdered substitutes for breast milk available in 13 countries worldwide. It was third in frequency to *E. agglomerans* and *E. cloacae*. Farmer and co-workers (5) reported that one of the original isolates in the National Collection of Type Cultures was from an unopened can of dried milk. It has also been reported that four strains of *E. sakazakii* were cultured from powdered milk and two strains were cultured from powdered infant formula in Czechoslovakia (18).

Infant formula is not always sold in the country where it is manufactured; e.g., formula bought in Gabon was manufactured in The Netherlands. It is also possible that some or all ingredients are imported. However, it may be assumed that the contamination by members of the *Enterobacteriaceae* took place after the last heat treatment (pasteurization) of the product. There was no correlation between the date of expiration and the concentration of members of the *Enterobacteriaceae*. However, it is unlikely that the keeping qualities of all products were the same, due to the differences in production. The date of manufacture of most products was unknown. The concentrations of members of the *Enterobacteriaceae*, including *E. sakazakii*, did not exceed a level of 1 CFU/g in the products tested. There was no particular part of the world associated with contaminated or noncontaminated products. In an extensive U.S. survey which included milk substitute infant formulas, the mean value for coliform organisms was less than three per gram (22). Regulations that govern the production of infant formula in different countries are not very divergent with regard to members of the *Enterobacteriaceae* or coliforms (26). Some countries, including Argentina (90/g), Australia (10/g), Brazil (90/g), the People's Republic of China (40/100 g), Finland (1/0.1 g), Spain (1/0.1 g), Switzerland (10/g), and Yugoslavia (1/0.01 g), permit a higher concentration of coliforms than the more usual 1/g. Members of the *Enterobacteriaceae* were cultured from 6 of 16 products manufactured in these countries. These results are comparable to the overall results. The Food and Agricultural Organization of the United Nations has recommended bacterial counts for coliform organisms in good-quality powdered infant formulas of less than 3 CFU/g (7). All formulas met this requirement. Most species isolated from the powdered substitutes for breast milk have also been mentioned as causes of bacterial meningitis (6), including *E. agglomerans* (1, 4), *E. cloacae* (16, 19), *E. sakazakii* (5, 14, 16, 24), *K. pneumoniae* (11), *Citrobacter* spp. (8–10, 17, 20, 23, 25), *E. coli* (4), and *H. alvei* (13). *Salmonella* spp., which can be a cause of outbreaks associated with milk products (2, 3, 12, 21, 27), were not isolated.

It is not known whether the presence of members of the *Enterobacteriaceae* in prepared formula at the low concentrations determined in this study are associated with occasional cases of neonatal meningitis. However, milk powders without members of the *Enterobacteriaceae* might offer extra protection to the newborn and especially to the premature baby if some multiplication during the preparation or storage in contaminated milk food does occur. If neonatal infections due to *Enterobacter* spp. (including *E. sakazakii*) or *Klebsiella* spp. are observed, a check of the hygienic precautions taken during these periods should be a part of the control measures.

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