BACTERIAL CONTAMINATION OF BOTTLE MILK IN INFANTS UNDER 6 MONTHS IN CHILDREN'S HOSPITAL, BANGKOK, THAILAND

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Abstract. The bacterial contamination of bottle milk samples obtained randomly from 500 infants under 6 months of age who came to the Out-patient Department of Children's Hospital Bangkok was determined by collecting bottle milk samples prepared at home following interview of their caretakers after obtaining their consent. Bacterial contamination was found in 91.8% (459/500) of bottle milk samples. Among the positive samples, 82.8% (380/459) contained enteric bacteria, another 17.2% were unidentified bacteria. The dominant enteric bacteria isolated from bottle milk were *Klebsiella* spp (56.6%), *Enterobacter* spp (41.3%), *Aeromonas* spp (14.4%), *E. coli* (13.4 %) and *Vibrio cholerae* non O-1 (1.8%). Isolated *E. coli* were further identified as enteropathogenic *E. coli* (7.8%, 4/51) and enterotoxigenic *E. coli* (3.9%, 2/51). About 74% of the contaminated bottle milk contained one type of bacteria, 23.7% had two types and 2.3 % had 3 or more types of bacteria. A level of bacterial contamination greater than the US government limited number (USGLN $2x10^4$ CFU/ml) was found in 86.4% of total examined samples (432/500) [geometric mean (GM) of 2.9 x 10^6 CFU/ml]. About 66% (333/500) of bottle milk samples had coliforms greater than the USGLN ($1x10^2$ CFU/ml) with GM of 1.3 x 10^4 CFU/ml. Therefore, in the preparation of bottle milk, feeding practice should be emphasized in every setting of maternal-child health care and promotion of breast-feeding should be encouraged by the health personnel.

INTRODUCTION

Diarrhea is an important health problem in developing countries including Thailand. It is a leading cause of morbidity and mortality in children under 5 years of age. The isolation rates of enteric pathogens detected from children treated at 10 hospitals in 5 regions of Thailand were enterotoxigenic *Escherichia coli* (ETEC) 10-22%, enteropathogenic *E. coli* (EPEC) 3-30%, *Shigella* spp 3-19%, *Campylobacter jejuni* 8-12% and *Salmonella* spp 1-7% (Sarasombath, 1985). *Salmonella* and *C. jejuni* were commonly isolated from 17% and 15% of the episodes of diarrhea in children under 6 months of age, respectively, while ETEC (8%), *Shigella* (4%) and EPEC (4%) were isolated from children over 6 months of age in Thailand (Varavithya *et al*, 1990).

Nowadays, early weaning has become concomitant with economic development. Bottle-feeding has become vital to the rearing of most babies, especially during the first year of life. A decline in breast-feeding is being experienced in many developing countries (Omondi *et al*, 1990; Muttalib *et al*, 1986). Consequent observations are the increase in morbidity and mortality of bottle-feeding infants from diarrhea. The incidence of bacterial gastroenteritis in breast-fed infants is significantly lower than that in bottle-fed infants less than 3 months of age (Poovorawan *et al*, 1981; Brown *et al*, 1989): Beside, the desirable bottle milk should contain a total bacterial count less than $2x10^4$ organisms/ml of milk and coliform count not more than 100 organisms/ml of milk and other dairy product (Fagerman, 1992). However, the bottle milk and/or the bottle can be contaminated with bacteria by using contaminated water during its preparation or cleansing the bottle. Thereafter, it could be contaminated if it was not properly stored. The number of bacteria in the bottle milk would increase with time if it was left at room temperature. Therefore, the prevalence of enteric bacteria contaminated in bottle milk prepared for infants under 6 months was elucidated in this study, together with types and virulence factors of the enteropathogens.

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MATERIALS AND METHODS

Samples

Five hundred infants under six months old who came to the Outpatient Department of the Children's Hospital, Bangkok, and were bottle fed regardless the purpose of their visits; they were randomly selected and enrolled in this study after obtaining their mother's or caretaker's consent. Questionnaires about the cause of seeking health service, personal and family data were used to record information by interviewing the caretakers. About 15 ml of bottle milk prepared at home were collected into a sterile bottle, kept in an icebox and transferred immediately to the laboratory for microbiological examination. This study was carried out during in 1991-1993.

Enumeration of bacteria

Total plate count was determined by using plate count agar (Difco) and spread plate technique. MacConkey agar (MC) and thiosulfate citrate bile-salts sucrose agar (TCBS) were used to presumptively enumerate *E. coli*/coliforms and *Vibrio* spp, respectively. For presumptive enumeration of *Aeromonas* spp, supplemented Dnase agar (Graevenitz and Zinterhofer, 1970) was the medium used in this study. The counting results were recorded as the number of colony forming units (CFU) per ml.

Isolation and identification

MC and Hextoen enteric agar (HE) were used for isolating *Salmonella* and *Shigella*, TCBS agar for isolating *Vibrio* spp and Brucella agar with 5% defibrinated sheep red blood cells (BA) for isolating *Campylobacter*. The milk samples were streaked directly on MC, HE and TCBS agar and then incubated at 37°C overnight. For enrichment, 0.1 ml of milk samples was inoculated into selenite F (SF) broth, alkaline peptone water (APW) + 2% NaCl, and Doyle's broth (Doyle and Roman, 1982). Enrichment media were incubated at 37°C overnight; Doyle's broth was incubated at 37°C under microaerophilic atmosphere in anaerobic jar with GasPak (BBL). After overnight incubation, SF broth was subcultured on MC and HE agar. The culture in APW+2% NaCl was subcultured on TCBS agar. Ten drops of a specimen enriched in Doyle's broth were incubated onto a 0.45 μm sterile millipore membrane filter (Millipore Corp, Bedford, MA, USA) on dried BA plate (Steele and McDermott, 1984). The filter was removed from the plate after 30 minutes and discarded. Streaked-plates were incubated at 37°C overnight and BA plates were incubated at 37°C under microaerophilic atmosphere for 48 hours. The identification of enteric bacteria in this study included coliforms (included *Klebsiella* spp, *Enterobacter* spp, *E. coli,* and *Citrobacter* spp), *Shigella* spp, *Salmonella* spp, *Campylobacter* spp, *Vibrio* spp, *Aeromonas* spp, and *Plesiomonas shigelloides*. Other bacterial colonies found on these media were not identified and were classified as unidentified bacteria. A suspected colony was picked and inoculated onto KIA agar and semisolid-mannitol agar. Further identification of clinically important enteric bacteria followed the methods as described by Farmer *et al* (1985), and Farmer and Kelly (1991). Identification of *Aeromonas* and *Vibrio* followed the method of Graevenitz and Attwegg (1991) and Kelly *et al* 1991, respectively. Identification of *Campylobacter* was performed by the method described by Penner (1991).

Serotyping of enteropathogenic *E. coli* (EPEC)

EPEC serogroups were carried out by slide agglutination test with EPEC polyvalent antiserum type I, II, and III (Difco) as described by Edwards and Ewing (1972). Live *E. coli* isolates, which showed an agglutination reaction with EPEC polyvalent antisera were further examined by slide agglutination with heat killed bacteria. Isolated *E. coli* that showed the positive results in the second agglutination test were confirmed by tube agglutination test. The tube agglutination test was performed at the Thai National Institute of Health, Nonthaburi.

DNA hybridization assays for virulence factors

The virulence factors of pathogenic *E. coli* were determined by DNA hybridization. The EPEC adherence factor (EAF) probe was prepared as described by Nataro *et al* (1985). The DNA probe used to detect EIEC was a 17-kilobase *Eco*RI digestion fragment of the 140-megadalton plasmid of *S. flexneri* 5 cloned into pBR325 (Boileau *et al,* 1984). The Shiga toxin (ST; STI, STII) of enterohemorrhagic *E. coli* (EHEC) specific DNA probes were prepared as previously described by Newland and Neill (1986). All the probes were labeled by nick translation with [α^{32} P] dATP (New England Nuclear Corp, Boston, Mass) and hybridization was performed with *E. coli* DNA fixed onto Whatman no. 541 filters as described previously (Moseley *et al*, 1982). pMAR-22, pRM17, *E. coli* strain 933J and 933W were used as positive controls of EAF, EIEC, and STI and STII of EHEC, respectively. *E. coli* strain Xac was used as a negative control.

Bioassay for enterotoxin

Live *E. coli* culture growing on trypticase soy broth containing 0.6% yeast extract (TSB-Y) was

detected for heat-labile enterotoxin (LT) activity by Y-1 adrenal cell assay (Sack and Sack, 1975). The activity of ST from culture filtrate was assayed by fluid accumulation test in suckling mice (Dean *et al*, 1972). The positive and negative controls of *E. coli* strains were B_2C (LT^+ ST^+) and Xac (LT^-ST^-), respectively.

Data analysis

Isolation rates of enteric bacterial contamination in bottle milk samples were calculated by percentage. All bacterial count values were transformed to base 10 logarithms; average values of bacterial counts were antilog of geometric means.

RESULTS

Types and isolation rates of enteric bacteria

The bacteria were isolated from 459 (91.8%) of 500 bottle milk samples; in 41 samples (8.2%) no bacteria were found. Among the positive samples, 82.8% (380/459) contained enteric bacteria, and 17.2% contained unidentified bacteria.

The type and isolation rates of enteric bacteria isolated from bottle milk samples are shown in Table 1. *Klebsiella* spp was isolated from 215 (56.6%) of 380 bottle milk samples containing enteric bacteria, followed by *Enterobacter* spp (41.3%), *Aeromonas* spp (14.4%), *E. coli* (13.4%) and *Vibrio* spp (1.8%). Among the 380 milk samples positive for enteric bacteria, 74.2% (282/380) were contaminated with a single type of enteric bacteria, 23.7% of the samples contaminated with two types and 2.3 % contaminated with 3 or more types of enteric bacteria.

Table 1
Types and isolation rates of enteric bacteria isolated from 380 bottle milk samples.

Type of enteric bacteria	No.(%) of positive samples[a]
Klebsiella spp	215 (56.6)
K. pnuemoniae	188 (87.4)
K. ozaenae	26 (12.1)
K. terrigena	1 (0.5)
Enterobacter spp	157 (41.3)
E. cloacae	110 (70.1)
E. agglomerans	29 (18.5)
E. aerogenes	11 (7.0)
E. gergoviae	4 (2.5)
E. amnigenus biogroup 1	1 (0.6)
E. intermedium	1 (0.6)
E. sakazakii	1 (0.6)
Aeromonas spp	55 (14.4)
A. caviae	34 (61.8)
A. hydrophila	16 (29.1)
A. sobria	5 (9.1)
Escherichia coli	51 (13.4)
E. coli	45 (88.2)
Enteropathogenic *E. coli*	4 (7.8)
Enterotoxigenic *E. coli*	2 (3.9)
Vibrio spp	7 (1.8)
V. cholerae non O-1	7 (100)

[a]There were 98 samples contaminated with more than one type of enteric bacteria.

Enumeration and serotyping of bacteria isolated from bottle milk samples

The total plate count of bottle milk samples ranged from $4.0x10^2$ to $8.7x10^9$ CFU/ml. According to the US government limited total bacteria count number (USGLN) for milk and dairy products ($<2x10^4$ CFU/ml), only 5.4% (27/500) of bottle milk samples were acceptable. The unacceptable milk samples were as high as 94.1% (432/459) of the positive samples or 86.4% of the total samples. Moreover, 66.6% (333/500) of total samples or 87.6% (333/380) of the enteric positive samples had coliform counts greater than the US acceptable coliform count number ($1x10^2$) (Fagerman, 1992). The presumptive *Aeromonas* spp, *E. coli* , ETEC and EPEC counts and their geometric means are shown in Table 2.

All *E. coli* strains examined in this study were negative for virulence factors tested, namely EAF, EIEC, ST I and ST II probes. Only 3.9% (2/51) of all *E. coli* isolated from bottle milk samples were ETEC LT^+ ST^+. EPEC serotypes were found only 7.8% (4/51) of bottle milk samples. There were O125:K70 (2 isolates), O126:K71 (1 isolate) and O128:K67 (1 isolate).

DISCUSSION

The result of this study revealed that as many as 86.4% (432/500) and 66.6% (333/500) of bottle milk samples were contaminated with total bacteria and coliform counts above the USGLN recommended level, respectively. The GM of coliforms was 1.3 x 10^4 CFU/ml. This data were quite high compared to other studies (Surjono *et al*,1980; Black *et al*,1989). The studied population was confined to the infants seeking health care in the hospital. The majority of the infants, 86.6% were sick and 82% of their illnesses were infectious in nature. The sick infants

Table 2
Positive samples for bacterial contamination of total 500 samples and geometric means (GM) of total plate count and enteric bacteria count.

Enumeration	Total No. of positive samples (%)	Samples (%) with total count > USGLN[a]	GM (CFU/ml)	Range of bacterial count (CFU/ml)
Total plate count	459 (91.8)	432 (86.4)	2.9×10^6	4.0×10^2 - 8.7×10^9
Coliform count	360 (72)	333 (66.6)	1.3×10^4	20 - 5.6×10^7
Aeromonas spp[b]	55 (11)	-	3.5×10^3	30 - 1.5×10^6
E. coli[b]	45 (9)	-	5.0×10^3	70 - 4.0×10^8
EPEC[b]	4 (0.8)	-	1.0×10^4	8.8×10^2 - 4.4×10^4
ETEC[b] (LT^+ST^+)	2 (0.4)	-	1.2×10^6	2.3×10^3 - 6.3×10^7

[a] USGLN – total bacteria count 2×10^4CFU/ml, coliform count 1×10^2 CFU/ml.
[b] Presumptive enumeration.

could be the source of milk contamination and the contaminated milk could also be a cause of their illness. The Children's Hospital is a government hospital in which the middle to low socioeconomic groups of people are predominant among those who seek medical services (data not shown).

The most frequently isolated microorganism was *Klebsiella* spp (56.6%), followed by *Enterobacter* spp (41.3%) which are opportunistic pathogens of man and animals. *Klebsiella* spp were reported as a cause of the diarrheal outbreak, as some strains of *K. pneumoniae* can produce enterotoxin (Klipstein *et al*, 1977; 1978). *Klebsiella* spp and *Enterobacter* spp have also been isolated from food, air, water, soil, dust and the hospital environment (Orskov, 1981; Brenner, 1981; Tenssay and Mengistu, 1997). *Enterobacter* spp have been found from many brands of powdered milk (Muytjens *et al*, 1988). The results suggested that the source of contamination in this study were mainly from the environment, which could be water, contaminated bottle or utensils used in milk preparation. On the other hand, fecal contamination (*E. coli*) of bottle milk samples was found in only 13.4% of the samples and only 4 samples were EPEC and 2 samples were ETEC positive. However, the occurrence of ETEC and EPEC diarrhea is related to contaminated weaning food and water (Echeverria *et al*, 1982; Levine and Edelman, 1984). Furthermore, EPEC adherence factor was found in less than 3% of Thai infants under 6 months old with diarrhea (Echeverria *et al*, 1991).

Aeromonas spp were also isolated 14.4% of the total bottle milk samples with the GM of 3.5×10^3 CFU/ml. Patients with *Aeromonas* associated diarrhea have been reported among children under 6 months old (13%), and most frequently in the group of 6 months - 2 years old (57.4%) (Gracey, 1986). *Aeromonas* spp were a cause of sporadic infantile diarrhea from 0.6% in Japanese children to 6.5% in India (Yoshida *et al*, 1998; Komathi *et al*, 1998). Study in Bangladesh, *Aeromonas* and *Klebsiella* spp, following diarrheagenic *E. coli* (EAEC) were found associated with persistent diarrhea in children (Bardhan *et al*, 1998). *Aeromonas* spp which emerged as a new agent causing diarrhea in India were reported as the second commonest pathogen isolated from diarrheal stool samples (Ahmed *et al*, 1997).

Vibrio cholerae non O-1 were found in 7 samples (1.8%) but enumeration of *V. cholerae* on TCBS agar was undetectable in this study. *Vibrio* was reported to be viable but noncultured from environmental samples and frozen seafood (Colwell *et al*,1985), thus direct plate counts of those vibrios of environmental samples would be undetectable. However, *V. cholerae* non OI isolates were detected in 7 milk samples by using enrichment medium in this study.

In this study, *Salmonella, Shigella* and *Campylobacter* were not isolated. Other studies also reported low detection rates of *Salmonella, Shigella* and *Campylobacter* in food, milk and water samples (Black *et al*, 1989; Lexsomboon *et al*, 1988). These pathogens might be transmitted via direct oral fecal route as they are easily passed from person to person by direct contact or animal food origin. It was found that enteric bacteria isolated from bottle milk samples in this study were commonly found in the environment. However, the virulence factors or toxins of most enteric bacteria should be further determined. In addition, further study of factors associated with

water contamination, hygiene in milk preparation and health behavior of the caretakers could help us to understand this high contamination level of bottle milk and set up a prevention program to improve children health.

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