Reduction of Rotavirus Infection in Children Receiving Bifidobacteria-Supplemented Formula

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Abstract

This study was conducted at Pakkred Babies Home, Bangkok, Thailand; with the hypothesis that children receiving probiotic-supplemented milk-based formula may be protected from developing diarrheal diseases. Salivary rotavirus-specific IgA antibody was used as an indicator of rotavirus infection. One hundred and seventy-five children, aged 6-36 months, were enrolled in the study. They were divided into 3 groups according to the type of formula given. There were 81 episodes of diarrhea during an 8-month study period, most of which were caused by bacterial enteropathogens. Ninety-seven pairs of salivary samples were adequate for the analysis of rotavirus antibody. Among 23 children receiving milk-based follow-up formula and serving as control group, 30.4 per cent of them had $\geq$ 4-fold increase in the antibody titre, indicating subclinical rotavirus infection. The majority of children in the other 2 study groups, receiving the same formula supplemented with either *Bifidobacterium Bb12* alone or together with *Streptococcus thermophilus*, had no significant change in the antibody titres between the two time points. The results of this study support our hypothesis that children receiving bifidobacteria-supplemented milk-based formula may be protected against symptomatic rotavirus infection.

Key word : Rotavirus Infection, Probiotic-Supplemented Formula

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Probiotic is a live microbial food supplement which beneficially affects the host by improving the intestinal microbial balance. Prevention of infectious diseases, immunostimulation and improved bioavailability of nutrients are claimed to be the potential health benefits of probiotic. Lactobacilli, bifidobacteria, enterococci and streptococci used in yokurts or other fermented milk products are examples of probiotic usage[1].

Rotavirus is an important cause of acute gastroenteritis in infants and children throughout the world. In neonates, salivary rotavirus-specific IgA was found to be a better indicator of rotavirus infection than serum IgG titres. Salivary rotavirus-specific IgA was not detected in healthy neonates but was detected in 62 per cent of infected infants three weeks following infection[2]. It was also used to assess the response after rotavirus diarrhea and vaccination[3,4].

In this study, healthy infants and young children aged 6-36 months were randomized into 3 groups and received either a milk-based follow-up formula or the same formula supplemented with either *Bifidobacterium Bb12* alone or together with *Streptococcus thermophilus* during a period of 8 months. The aim of the study was the prevention of diarrheal disease in children less than 5 years of age who generally, according to the report from the Ministry of Public Health in 1994, have 1.34 episodes of diarrhea per child per year. Rotavirus-specific IgA antibody titres in the saliva samples taken at the beginning and the end of the study as well as after having watery diarrheal episodes were used as the indicators of rotavirus infection.

MATERIAL AND METHOD

One hundred and seventy-five young children aged 6-36 months from Pakkred Babies Home, Bangkok, Thailand; were enrolled in the study. Pakkred Babies Home is an orphanage. Children are distributed to live in 6 houses according to their age-groups. Those who were not suffering from malnutrition were randomized into 3 groups according to the type of formula given; 57 children aged 684± 210 days received a milk-based follow-up formula (Nan-2) and served as control group; 62 children aged 487±189 days received the same follow-up formula supplemented with *Bifidobacterium Bb12* (10^8/g) alone (Bb group) and 56 children aged 526± 193 days received the same formula supplemented with both *Bifidobacterium Bb12* and *Streptococcus thermophilus* (Bb+S.ther.group) during a period of 8 months.

Salivary samples were taken at the beginning and after an 8-month study period from all infants using sterile cotton buds. Salivary samples were also taken at varying intervals from infants who had diarrheal symptoms. Ninety-seven pairs of salivary samples were adequate for the analysis. The incidence and duration of acute diarrheal episodes were recorded. Stools from children with diarrhea were sent for culture of enteropathogens and rotavirus detection by Rotalex (rapid test based on latex agglutination.)

Total protein determinations

The bicinchoninic acid (BCA) micro method was used (Pierce, Rockford, USA). Triplicate determinations were set up in Microtiter plates (Microtec, Embrach-Embraport, CH). To 10 µl of saliva or standard (2-fold dilutions from 2.0-0.0625 mg/ml) was added 200 µl working reagent. Plates were mixed well and incubated at 37°C for 30 min before reading on a Microtech MR 5000 plate reader (Microtec, Embrach-Embraport, CH) at 550 nm with the programmable EIA-Calc system using a linear regression curve fit.

Total S-IgA determinations by ELISA

A capture ELISA technique was used for S-IgA determination. Microtiter plate wells (Dynatech) were coated overnight at 4°C with 100 µl per well of anti-human IgA α-chain specific (Sigma), 20 µg/ml in coating buffer. For each sample to be tested a control well was left uncoated. All washing steps in the assay were performed 3x with PBS containing 0.05 per cent Tween-20. After being washed, the wells were blocked with 100 µl of PBS-Tween containing 0.5 per cent caseinate K, and incubated 1 hour at 37°C. Plates were again washed and 100 µl of standards (2-fold dilutions from 1.0-0.0625 µg/ml) or saliva samples (at dilution 1:200 to 1:500) diluted in PBS-Tween 0.05 per cent were added to wells in triplicate and to control wells, and incubated 1 hour at 37°C. Purified S-IgA (Nordic Immunological Laboratories) was used as standard. Unbound antibody was removed by washing and 100 µl of anti-human IgA α-chain specific conjugated with alkaline phosphatase (Sigma) was added at dilution 1:5,000 in PBS-Tween and incubated 1 hour at 37°C. Plates were again washed and 200 µl of paranitrophenyl phosphate 1 mg/ml

(Sigma 104) in substrate buffer was added and incubated 30 min at 37°C, and the optical density measured at 405 nm. Concentrations were determined using the programmable EIA-Calc system using a linear regression curve fit.

Total S-IgA determinations by RID

Secretory IgA (S-IgA) was determined in saliva by radial immunodiffusion (RID) using the ML plates (range 8.5 to 85 mg/L) for the determination of monomeric IgA (The Binding Site, Birmingham, UK). For each series of determinations, a standard curve using purified human secretory IgA at 450, 270, 135, and 45 µg/ml was included, as well as monomeric IgA controls. All samples and standards were applied in duplicate and distributed randomly on different plates. After a diffusion period of 72 h, the horizontal and vertical diameters were measured using an electronic RID reader (The Binding Site, Birmingham, UK) and concentrations calculated from the standard curve.

Specific IgA anti-rotavirus antibody in saliva

A direct ELISA was used to determine specific IgA anti-rotavirus antibody in saliva. Microtiter plate wells (Dynatech M 29 AR = Greiner 655171) were coated overnight at 4°C with 100 µl per well of rotavirus antigen at a concentration of 20 µl/ 10 ml in coating buffer. The antigen prepared by H. Brussow was a bovine rotavirus V1005 specific for the rotavirus group antigen VP6 containing single shelled particles and was purified by sucrose gradient centrifugation. For each sample to be tested, a control well was left uncoated to test for non-specific binding and samples and standards were applied in triplicate. All washing steps in the assay were performed 3x with PBS containing 0.05 per cent Tween-20. The standard was pooled human serum and was applied in triplicate using two-fold dilutions from 1:50-3,200. To avoid non-specific adsorptions to the plastic, both saliva (usually diluted 1:10) and standards were diluted in PBS 0.5 per cent Tween-20. To each well was added 100 µl of sample and standards and plates were held at 4°C overnight. The plates were washed 3x and 100 µl of biotin-conjugated anti-human IgA α-chain specific (Sigma) added at dilution 1:5,000 in PBS-Tween 0.05 per cent and incubated 2 h at room temperature with gentle shaking. Plates were washed 3x and 100 µl streptavidin peroxidase conjugate dilution 1:1000 in PBS-Tween 0.05 per cent were added. Plates were incubated at room temperature for 30 min with gentle shaking. Plates were again washed 3x and 100 µl of TMB peroxidase substrate system was added and plates incubated 30 min at room temperature with gentle shaking. The reaction was stopped by the addition of 100 µl 1 M phosphoric acid. The optical densities were read on a Dynatech plate reader (MR 5000 with EIA-Calc soft wear) at A450/550 nm, and titres were calculated from the linear regression data of the standard curve using an absorbance cut-off value of 0.1. If sample OD values did not fall within the standard curve they were repeated at a suitable dilution (between 1:2 and 1:50).

All of the laboratory tests were performed at Research & Development, Nestle' Research Center, Lausanne, Switzerland.

Statistical analysis

The statistical analyses were performed by J.M. Aeschlimann and the p-values indicated were for the student *t*-test comparing the 2 time points within each group.

RESULTS

The initial average salivary values of total protein, total secretory-IgA (S-IgA) and specific IgA anti-rotavirus antibody in all groups of children are shown in Table 1. The average values for total S-IgA and total protein for the two time points are shown in Table 2. Total protein in Bb group was higher than that in the other two groups at the beginning of the study, and there was a significant decrease in total protein in this group. At the end of the study, all total protein values were similar.

In Bb+S.ther. group, total S-IgA was higher at the beginning of the study than that in the other two groups and there was a significant decrease in total S-IgA between the two time points. In Bb and control groups, total S-IgA levels were

Table 1. Initial average salivary values in all groups.

Parameter		
Total protein	1.13	mg/ml
Total S-IgA	94.8	µg/ml
IgA anti-rotavirus	35	GMT
IgA anti-rotavirus	465	GMT/mg protein
IgA	8.73	% protein

Table 2. Average values for salivary total protein and total IgA in various groups.

Group (n)	Control (23)	Bb (40)	Bb + S. ther. (34)
Total protein (mg/ml)			
start	0.91	1.33	1.16
end	0.99	0.98	1.12
* p - value	0.59	0.02	0.76
Total IgA (μg/ml)			
start	68.7	83.9	131.9
end	69.9	76.0	92.5
* p – value	0.92	0.57	0.03
IgA as % protein			
start	7.96	7.27	10.96
end	7.52	7.73	8.85
* p – value	0.79	0.92	0.14

* p - values for change comparing the two sample times

Table 3. Salivary IgA anti-rotavirus titres.

Group (n)	Control (23)	Bb (40)	Bb + S. ther. (34)
IgA anti-rotavirus titres/ml			
GMT start	21.7	28.6	53.1
GMT end	47.8	39.4	49.4
Fold rise	2.20	1.38	0.93
* p - value	0.01	0.20	0.83
IgA anti-rotavirus titres/mg total IgA			
GMT start	424	480	492
GMT end	843	718	642
Fold rise	1.99	1.50	1.30
* p - value	0.01	0.60	0.33

* p - value for change in specific antibody titres comparing the two sample times

very low and thus IgA expressed as a percentage of total protein was also low. There was a correlation coefficient (0.496 for n = 184) between total protein and total S-IgA.

The geometric mean titre (GMT) for rotavirus-specific IgA antibody titres expressed per ml and per mg total S-IgA are shown in Table 3. A significant increase in salivary anti-rotavirus titres with a 2-fold increase was observed in control group. As the number of infants in each group were not comparable, the changes in salivary anti-rotavirus titres per mg total S-IgA were expressed in

Table 4. Change in salivary anti-rotavirus titres.

Group (n)	% of infants with rising titer (fold)		
	< 0.3	0.3 - 3.9	> 4
Control (23)	13.0	56.5	30.4
Bb (40)	12.5	65.5	22.5
Bb + S. ther. (34)	14.7	64.7	20.6

Table 5. The incidence of diarrhea in children at Pakkred Babies Home, Bangkok, Thailand. (August 1996 - March 1997).

Group	Children (n)	Diarrhea	
		episodes	episodes/ child/ year
Control	57	14	0.56
Bb	62	40	1.22
Bb + S.ther.	56	29	1.01
Total	175	83	0.93

percentages (Table 4). The control group had 30.4 per cent with a >4-fold increase in titre as compared with 22.5 per cent and 20.6 per cent for Bb group and Bb + S. ther. group, respectively. For the majority of infants (57- 65%), there were no changes in titres between the two time points and for a small percentage of infants, there was a decrease in the titre.

The incidence of diarrhea in children at Pakkred Babies Home during the study period of 8 months are shown in Table 5. The average episodes/child/year was 0.93. Among 81 diarrheal episodes, shigella was responsible for one fifth of these episodes (Table 6). Rotavirus was proved to be the cause of diarrhea in only 3 episodes.

DISCUSSION

The incidence of diarrheal diseases in this study is lower than the national figure. This is probably due to the improved hygenic condition in the institution during the study period. One of the weaknesses of this study at the actual recruitment, is the difference in the ages that clearly advantages the control group which are more aged and may be less sensitive to develop diarrhea. Regarding the

Table 6. Enteropathogens isolated from stool cultures in 81 episodes of diarrhea. (August 1996 - March 1997).

Enteropathogen	Group n (%)			Total	Per cent
	Control	Bb	Bb + S. ther.		
E. coli	8 (17)	5 (53)	14 (30)	47	58
Shigella sp.	4 (23)	5 (30)	8 (47)	17	21
Salmonella sp.	-	-	1 (100)	1	1.2
Rotavirus	2 (67)	1 (33)	-	3	3.7
Nonspecific	-	-	-	13	16.1
Total	14 (20)	31 (46)	23 (34)	81	100

etiologic agents, bacteria such as shigella, salmonella and *E. coli* seem to be the leading causative enteropathogens. Rotavirus accounts for only 3.7 per cent of the 81 diarrheal episodes, 2 in the control group, 1 in Bb group and none in the Bb+S.ther. group. This is in contrast with previous studies, as most of them show that rotavirus is the most frequent viral cause of severe diarrhea in infants, in both developing and developed areas of the world(5-7).

At the first sample time, the Bb+S. ther. group had higher total IgA levels per ml saliva and also higher anti-rotavirus titres per ml than the other two groups, but the initial anti-rotavirus titres in the 3 groups were similar when calculated per mg total IgA. The control group had the highest percentage of infants (30.4%) with ≥ 4-fold increase in anti-rotavirus titres compared with 22.5 per cent and 20.6 per cent for Bb and Bb+S.ther. groups, respectively; and a 2-fold increase in the geometric mean titre, indicating perhaps more subclinical rotavirus infections. A lower incidence of symptomatic or silent rotavirus infection in the majority of children receiving bifidobacteria-supplemented formula was shown by a lower antibody response in their saliva. For those who had rotavirus infection, there was an increase in salivary anti-rotavirus titres between 2-3 weeks after infection (Fig. 1).

This clinical study together with salivary rotavirus-specific IgA antibody determination support the hypothesis that a milk-based follow-up formula supplemented with bifidobacteria may protect against rotavirus infection.

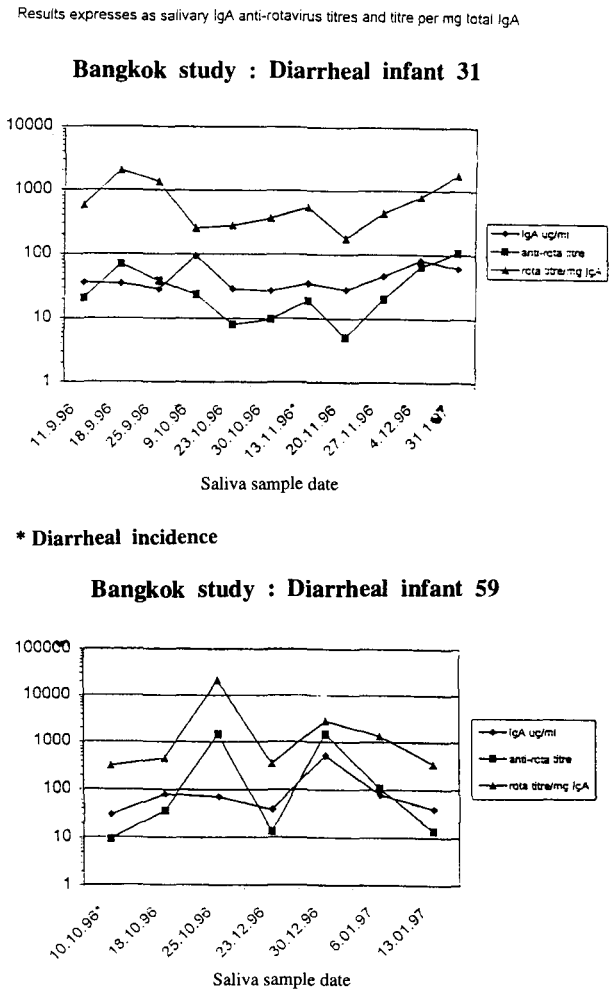

Fig. 1. Salivary specific IgA anti-rotavirus titres of 2 infants with rotavirus infections.

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การลดลงของการติดเชื้อไวรัสโรต้าในเด็กที่ได้รับนมผงที่เติมเชื้อไบฟิโดแบคทีเรีย

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การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อพิสูจน์สมมุติฐานที่ว่า เด็กที่ได้รับนมผงที่เติมโปรไบโอติกจะมีอุบัติการของโรคอุจจาระร่วงน้อยลง ได้ทำการศึกษาที่สถานเลี้ยงเด็กอ่อน ปากเกร็ด เด็กอายุระหว่าง 6-36 เดือน จำนวน 175 คน ได้เข้าร่วมในการศึกษา โดยแบ่งเด็กเป็น 3 กลุ่มตามชนิดของนมผงที่ได้รับ ได้ใช้แอนติบอดี้ต่อเชื้อไวรัสโรต้าในน้ำลายเป็นตัวบ่งชี้ถึงการติดเชื้อไวรัสโรต้า ในช่วงที่ทำการศึกษาเป็นเวลา 8 เดือน มีอุบัติการของโรคอุจจาระร่วงเกิดขึ้น 81 ครั้ง ส่วนใหญ่เกิดจากการติดเชื้อแบคทีเรียในลำไส้ มีจำนวนน้ำลาย 97 คู่ที่มีปริมาณเพียงพอสำหรับการตรวจหาแอนติบอดี้ต่อเชื้อไวรัสโรต้า ในกลุ่มควบคุมที่ได้รับนมผงสูตรต่อเนื่อง จำนวน 23 คน ร้อยละ 30.4 มีภูมิต้านทานต่อเชื้อไวรัสโรต้าในน้ำลายสูงขึ้นมากกว่าหรือเท่ากับ 4 เท่า แสดงถึงการติดเชื้อไวรัสโรต้าโดยไม่มีอาการ ในกลุ่มศึกษา 2 กลุ่ม เด็กซึ่งได้รับนมผงสูตรต่อเนื่อง ที่เติมเชื้อ *Bifidobacterium Bb12* อย่างเดียว หรือร่วมกับ *Streptococcus thermophilus* ส่วนใหญ่ไม่มีการเปลี่ยนแปลงในระดับภูมิต้านทานต่อเชื้อไวรัสโรต้าในน้ำลาย ผลของการศึกษาครั้งนี้สนับสนุนสมมติฐานที่ว่าเด็กที่รับนมผงที่เติมเชื้อ bifidobacteria อาจจะสามารถป้องกันการติดเชื้อไวรัสโรต้าที่แสดงอาการได้

คำสำคัญ : การติดเชื้อไวรัสโรต้า, นมผงที่เติมโปรไบโอติก

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