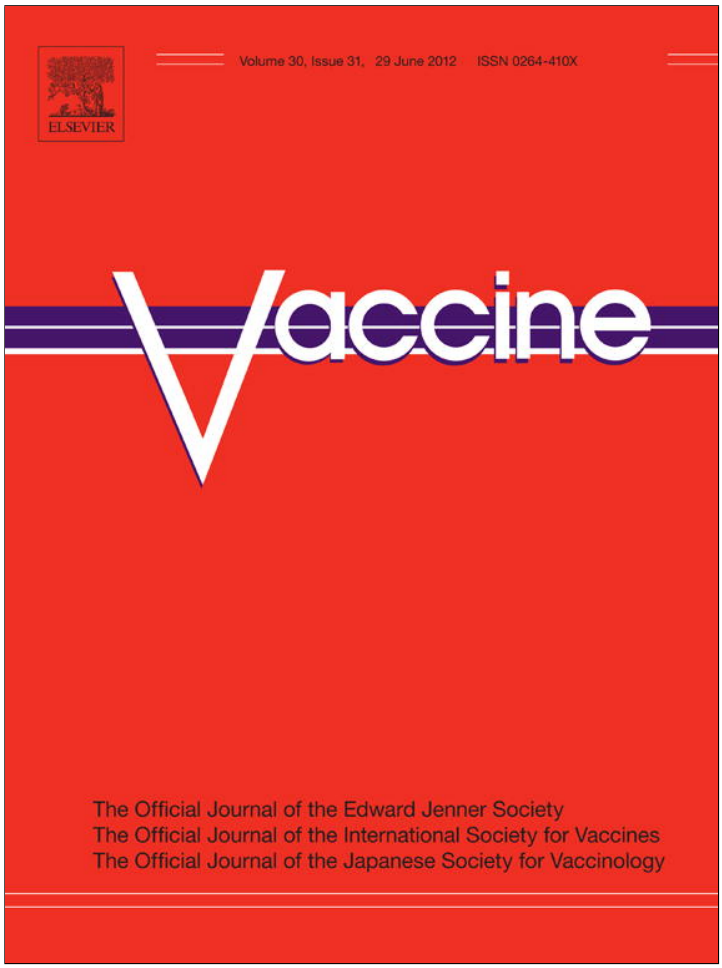

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Randomized placebo-controlled clinical trial of immunoglobulin Y as adjunct to standard supportive therapy for rotavirus-associated diarrhea among pediatric patients

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ABSTRACT

This study aims to evaluate the effect of hyperimmune immunoglobulin Y (IgY) against human rotavirus (HRV) among pediatric patients receiving standard supportive treatment for rotavirus-associated diarrhea mostly with an enteric non-cholera co-pathogen in a hospital setting. Two natural HRV reassortant clinical strains ATCC VR 2273 and ATCC VR 2274 were used as mixed immunizing antigens in poultry hens to generate anti-HRV IgY (Rotamix IgY). The Rotamix IgY was used in laboratory and clinical studies against control or placebo IgY. The control or placebo IgY was prepared using tissue culture medium from mock-infected MA104 cell line as antigen for poultry immunization. In vitro, Rotamix IgY exhibited multi-serotypic cross neutralization activities along with synergistic effects against major global serotypes G1, G2, G3, G4 and other human or animal rotavirus strains when compared with mono-specific IgY. Suckling mice (ICR strain) pre-treated orally once with Rotamix IgY and then challenged with rotavirus 3 h later showed a significant dose-dependent reduction in frequency ($p < 0.05$) and duration ($p < 0.05$) of diarrhea compared to placebo IgY-treated mice. Out of 114 children aged between 3 and 14 months and with diarrhea upon admission in a Myanmar hospital, 54 dehydrated and rotavirus-positive children were randomized into Rotamix IgY group and placebo IgY group. Of these, only 52 children had complete data with $n = 26$ children per study group. Ninety-two percent of patients in each of these groups were positive for co-infecting enteric non-cholera pathogen and all patients received standard supportive therapy for diarrhea. The patients were monitored for volume and duration of oral rehydration fluid (ORF) and intravenous fluid (IVF) intake, daily stool frequency and overall duration of diarrhea, and frequency and duration of rotavirus shedding. Compared to placebo IgY group, the Rotamix IgY group had statistically significant reduction in mean ORF intake ($p = 0.004$), mean duration of intravenous fluid administration ($p = 0.03$), mean duration of diarrhea from day of admission ($p < 0.01$) and mean duration of rotavirus clearance from stool from day of admission ($p = 0.05$). Overall, our novel approach using oral Rotamix IgY for rotavirus-infected children mostly with non-cholera enteric pathogen co-infection appears to be a promising, safe and effective adjunct to management of acute diarrhea in pediatric patients.

1. Introduction

Rotavirus is the leading single etiologic agent of severe diarrhea among infants and young children worldwide during the first five years of life. It is responsible for over 500,000 deaths in infants and young children mostly in developing countries each year which

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Table 1
Rotavirus strains used in this study.

Strain	Serotype
Human origin:	
HRV 408	Natural reassortant G3 P[?]
HRV 248	Natural reassortant G4 P[4]
Wa	G1 P[8]
KU	G1 P[8]
M37	G1 P[6]
S2	G2 P[4]
1076	G2 P[6]
YO	G3 P[8]
HK	G4 P[8]
Horse origin: HO-5	G3 P[12]
Cow origin: Shimane	G6 P[5]
Pig origin: S-80	G1 P[7]

P[?] means P genotype is unknown.

represent approximately 5% of all deaths [1]. Rotaviruses have also been implicated as causative agents of outbreaks of diarrhea occurring in nursing homes [2], among travelers [3], in day-care centers [4], and adults suffering from a variety of immunodeficiency conditions [5,6]. Among the 24 G types and 33 P types of group A rotaviruses classified so far [7], 5 G types (G1, G2, G3, G4, and G9) and 3 P types (P[4], P[6], P[8]) account for most of the G/P types of human rotaviruses detected globally. Four common G-P combinations (G1, G3, and G4 with P[8] and G2 with P[4]) are of principal epidemiologic importance being responsible for approximately 96 percent of rotavirus infections worldwide [8–11], although their relative proportions may vary by year and region. It follows therefore that any proposed immunologic intervention measures must provide good protection against these four epidemiologically significant HRV serotypes.

Conventional treatment for rotavirus diarrhea is non-specific, largely symptomatic and involves fluid and electrolyte replacement and maintenance of nutrition. The use of current vaccine regimens poses inherent limitations due to variable degrees of efficacy [12], and high cost. On the other hand, passive immunotherapy using orally administered hyperimmune chicken immunoglobulins (IgY) has been reported with various degrees of success against infectious diseases of viral, bacterial, fungal and protozoal origin in both humans and animals [13–15]. There have been reports on the experimental use of rotavirus specific IgYs in cow [16], cat [17] and mice [18,19] with promising results. So far there has been only one report of a randomized placebo-controlled clinical trial in children using IgY against rotavirus [20]. The investigators in aforesaid trial used four different strains of rotavirus to produce IgY but they did not determine the dose of IgY in terms of neutralization titer as well as the possible range of cross-reactivities of the IgY with known rotavirus serotypes. In the present study we mapped the IgY cross-reactivities against an array of clinically important human and animal strains currently circulating worldwide and defined the titer of IgY administered to rotavirus-infected patients for possible application as a general adjunct to standard supportive therapy for acute rotavirus-related diarrhea among pediatric patients.

2. Materials and methods

2.1. Viruses and cell lines

The viruses used in this study were reassortant type II subgroup human rotavirus (HRV) strains HRV 408 (ATCC 2273), HRV 248 (ATCC 2274), Wa(G1P[8]), S2(G2P[4]), YO(G3P[8]), and HK(G4P[8]) originated from human, HO-5 (G3P[12], horse), shimane (G6P[5], cow), and S-80 (G1P[7], pig) (Table 1). Rhesus monkey kidney cell line MA-104 (ATCC CRL-2378) cells were used to propagate all the above rotaviruses. MA-104 cells were maintained by using Eagle's Minimal Essential Medium (EMEM, Nissui, Japan) with Earles' salts, supplemented with 10% fetal bovine serum (FBS) and incubate at 37 °C, 5% carbon dioxide.

2.2. Preparation of Rotamix IgY and placebo IgY

Reassortant strains HRV 408 and HRV 248 were isolated from stools of 9- and 17-months old children respectively from Bangladesh [21]. These two reassortant human rotaviruses were used as antigens for the production of anti-HRV IgY according to the methods described previously [22]. Prior to their use as immunizing antigen, the above rotavirus strains were inactivated using 0.3% formalin at 37 °C for 24 h. To generate IgY, 18-week-old Hy-Line hens were immunized by intramuscular injection of an emulsified mixture of inactivated human rotavirus either as single-strain or mixed-strain emulsions. Eggs laid by the immunized hens between 3 and 10 weeks after immunization were harvested and egg yolk was isolated, pooled and processed into powder form in accordance with a method described previously [23]. The egg yolk powder from mixed vaccination with two rotavirus strains HRV 408 and HRV 248 was designated as "Rotamix IgY". Placebo IgY powder was prepared by the same method from the eggs of hens immunized using as antigen the tissue culture medium of mock-infected MA-104 cell monolayer. For in vitro and in vivo mouse studies, Rotamix IgY and placebo IgY were partially purified from egg yolk by chloroform extraction and ammonium sulfate precipitation [24]. The antigen and antibody protein concentrations were determined by Bio-Rad protein assay (Bio-Rad Laboratories, CA, USA).

2.3. Virus quantitation and fluorescent focus (FF) reduction assay on IgY

Virus titers were determined by 50% tissue culture infectious dose ($TCID_{50}$) method [16]. Briefly, tissue culture supernatants were assayed for virus infectivity on MA-104 cells based on end-point dilution of samples with cytopathic effect in a 10-fold sample dilution series. For the virus neutralization assay, fluorescent focus (FF) reduction method was employed [25]. In this assay, MA 104 cells were plated onto 96-well tissue culture plate with EMEM containing Earle's salts and non-essential amino acids and supplemented with L-glutamine, sodium pyruvate, and 5% fetal bovine serum. All rotavirus cultures were maintained with the medium containing 10 μg/ml trypsin. Cells were incubated at 37 °C with 5% CO_2 tension for 24 h or until a confluent monolayer was formed. Different dilutions of Rotamix IgY in phosphate buffered saline were prepared with final concentrations of 100, 50, 25, 12.5, 6.25, 3.13 and 1.56 mg/ml. Each dilution was mixed with an equal volume of 12 different rotavirus strains (248, 408, Wa, KU, M37, S2, 1076, YO, HK, HO-5, Shimane and S-80) at a dilution that yielded about 150–200 FF units per 0.025 ml per well and the mixtures were allowed to react at 37 °C for 1 h. About 50 μl aliquots of the IgY-virus mixtures were dispensed onto MA104 monolayers in 96-well microplate and incubated for 1 h at 37 °C. 100 μl of fresh EMEM was added, followed by 16–18 h cultivation at 37 °C, 5% CO_2. Fixation in cold (−80 °C) methanol, and reaction with first and second antibodies, were performed as described previously [26]. Neutralizing antibody titer was expressed as the reciprocal of the highest IgY dilution that reduced the FF count by >50%. The mean FF reduction titer of IgY was calculated from 3 independent assays.

2.4. Suckling mouse experiments

All procedures that involved animals were approved by the Institutional Animal Care and Use Committee of the Immunology Research Institute in Gifu, Japan. Pregnant, rotavirus-negative CD-1 SPF mice (Charles River Japan, Inc., Kanagawa, Japan) were housed

individually in an environmentally controlled animal facility until parturition. Environmental factors were closely monitored daily and maintained within the recommended range for the animals (18–23 °C, 40–75% humidity, and photoperiod of 12 h each of light and dark cycles). Mice were maintained on normal pellet diet, water was provided ad libitum and beddings and cages were changed once a week.

Four-day old suckling mice born after the one-week acclimatization period were randomized into several experimental groups (n = 10/each group). The challenge dose for HRV 248 and 408 strain were $10^{7.7}$ $TCID_{50}$ and $10^{7.5}$ $TCID_{50}$ respectively. They were given as 25-μl oral dose of rotavirus suspension after a 21-h fast. Rotamix IgY or placebo IgY in phosphate-buffered saline was given once as a single dose 3 h prior to challenge. For this pre-challenge IgY treatment, four different doses of Rotamix IgY were tested: 5 mg/mL, 2.5 mg/mL, 1.25 mg/mL, and 0.625 mg/mL. For control mice, 5 mg/ml of placebo IgY was used. At any time point after challenge, neither Rotamix IgY or placebo IgY was given to any mouse in the experiment. After challenge, the mice were observed daily for 3 days and were checked every 4 h daily for signs of diarrhea which is determined as loose stools on gentle palpation of the abdomen or as feces-smeared tail during the 3 days of the experiment. Stool samples were retrieved by gentle palpation of the abdomen. Stool consistency was evaluated on a five-point scale as follows: 0, normal, solid and black; 1, soft brown; 2, liquid brown; 3, soft yellow; 4, liquid yellow. Mice showing scores of 3 or higher were considered as having diarrhea. The outcome measures used were frequency of diarrhea (determined as the percentage of mice with diarrhea on a daily basis) and duration of diarrhea (determined as number of days with diarrhea).

2.5. Preparation of placebo and Rotamix IgY in sachets for oral administration to infants

Rotamix IgY or placebo IgY were mixed with maltitol and banana flavor and dispensed into sachets. One sachet contains 0.5 g anti-HRV IgY, 1.48 g maltitol and 0.02 g banana favor (total of 2 g/sachet). The protein content of the Rotamix IgY and placebo IgY preparations, were 7.5% and 7.8% respectively, with an IgY content of 20% (w/w). The fat, carbohydrate, ash and moisture contents of both IgY preparations were 12%, 5%, 0.8%, and 5% respectively.

2.6. Study design and population

The research protocol followed in this clinical trial has been approved by the Ethical Committee on Medical Research Involving Human Subjects of the Department of Medical Research (Central Myanmar). A double-blind, placebo-controlled trial of rotavirus-specific IgYs was conducted in Myanmar during the rotavirus epidemic season from January to March, 2011 in the Pediatric Infectious Disease Wards of the Defense Services Obstetrics, Gynaecology and Children's Hospital, in collaboration with the Department of Medical Research, Central Myanmar Ministry of Health. Infants and children of both sexes aged between 2 and 36 months who were brought to the above hospital with history of acute watery diarrhea and dehydration were entered in the study. Children with severe malnutrition, respiratory infections, systemic infection and a history of bloody or mucoid diarrhea were excluded. Selected children were taken to the study ward and observed for 4 h during which rehydration with oral rehydration fluids (ORF) or intravenous fluids (IVF) was performed while their stools were screened for rotavirus antigen by the commercial Dipstick 'Eiken' Rota kit (SA Scientific, USA). Those with positive results were finally enrolled in the study after obtaining informed consent from their parents/guardians.

Table 2
Medications given to Rotamix IgY and placebo IgY groups as routinely prescribed by attending physician.

Treatments	No. of patient	
	Rotamix IgY group	Placebo group
Antibiotics		
Ampicillin	1	1
Metronidazole	25	23
Cotrimoxazole	10	9
Amikacin	2	3
Gentamycin	11	9
Cefotaxine	0	1
Ceftriazone	0	1
Chloramphenicol	0	1
Vitamins/minerals		
Astymin C	2	4
Folic acid	12	12
Zinc	7	10
Becozinc	1	1
Burplex	3	1
Probiotics		
Biovita:	4	3
Lactobacillus helveticus R0052		
Bifidobacterium longum R0175		
Bioflor: *Saccharomyces boulardii*	0	1
Medilac-S: *Streptococcus faecium*	4	1
Bacillus subtilis		

2.7. Treatment protocol

Out of 114 children screened, 54 children qualified for the study based on above-mentioned criteria with 2 children being dropped from the study later due to incomplete data. The selected children were divided into two groups by randomization through alternate assignment of the placebo IgY and Rotamix IgY sachets labeled A and B respectively to patients according to the order in which they were admitted at the hospital for confinement. The appearance of the sachets was identical except for the A or B label but the enroller (field scientist) did not know which of the sachets was placebo or Rotamix IgY. One sachet of the Rotamix or placebo IgYs was administered four times daily for 8 consecutive days in addition to rehydration therapy. The attending clinicians monitored other possible untoward reactions to Rotamix IgY. Under the supervision of pediatricians who did not know the group status of any of the study children or infants, the ongoing water loss was replaced by equivalent amount of ORF but intravenous fluid solution was used if ORF therapy failed to compensate for ongoing water loss. The IVF was discontinued as soon as dehydration was corrected and oral rehydration was resumed. Routine medical treatments given to participants in the form of antimicrobials or supplementary vitamins/probiotics were noted and recorded for all patients in both groups (Table 2). Exclusively breast-fed infants continued to receive mothers' milk while weaned children received a milk-based formula in addition to breast milk. Non-breastfed children were given milk formula or semisolid and solid foods appropriate for their age and food habits. The solid or semi-solid food is usually rice-based and may contain non-fermented milk or vegetable materials. There were no prebiotics or probiotics in any of the home-made diet for infants.

2.8. Clinical assessments and laboratory investigations

Baseline physical, clinical and microbiological assessments were made on the first visit at day 0. At this time, all selected patients passed a thorough physical examination and assessment of hydration by physicians who managed the subsequent ORF and IVF administrations while recording stool frequency and duration of

Table 3
In vitro cross neutralization activity of anti-HRV IgY preparations with human and animal rotavirus strains as determined by FF reduction assay.

IgY samples	Neutralization titer/0.1 ml IgY[a] against different human rotavirus strains											
	408	248	Wa	KU	M37	S2	1076	YO	HK	HO-5	Shimane	S-80
Anti-408	5120	2560	10240	5120	10240	2560	>40960	40960	10240	5120	<20	1280
Anti-248	2560	>40960	5120	5120	5120	5120	>40960	20480	40960	1280	<20	640
Rotamix	10240	>40960	20480	20480	20480	10240	>40960	>40960	>40960	5120	<20	2560
Control	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20

[a] IgY titer is expressed as dilution factor of 1 g hyperimmunized IgY powder that reduced the fluorescent focus (FF) count by >50% in the FF reduction assay. Results are presented as the mean of three independent experiments.

diarrhea daily until day 8. Fecal samples were collected for stool culture to detect viral and bacterial infection using standard direct and enrichment enteric media as described elsewhere [27]. Detailed identification of bacterial serotypes using serology was not done. Stool specimens were obtained daily for 8 days to assess the duration of viral shedding. Detection of rotavirus in stool during the study was made by ELISA method [28] as described below. Technicians of the diagnostic laboratory were not aware of the group each patient belonged to. The code for the sachets was broken only when all the laboratory and clinical datas were compiled for data analysis. The randomized group assignment of patients resulted in Rotamix IgY and placebo IgY groups with demographic and co-pathogen infection profiles as shown in Tables 3 and 4 respectively. The patient exclusion criteria followed in this study (severe malnutrition, respiratory infections, systemic infection and a history of bloody or mucoid diarrhea) may have excluded *Vibrio cholera*-infected children in these groups.

2.9. Detection of rotavirus antigen in stool

A total of 416 stool specimens were collected from 52 patients at different time points (days 1–8) post-treatment and stored at −20 °C. Ten percent fecal suspensions were prepared in phosphate buffered saline (PBS), clarified by centrifugation (1000 × *g* for 5 min) and analyzed by ELISA as described previously [28] with some modifications. Briefly, polyvinyl 96-well microtiter plates (Nalgene Nunc International, Rochester, NY) were coated with 1:10,000 dilution of a monoclonal antibody (Mab) YO-156 and incubated overnight at 4 °C. The MAb YO-156 (IgG2a) is highly reactive with VP6, a common epitope of all group A rotaviruses that have been examined to date [29]. After a routine 3 times wash with PBS-Tween 20 (PBST), the wells were incubated with 1% bovine serum albumin overnight at 4 °C and washed as before. A mixture of 10% stool suspension (375 μl) and 10% skim milk (125 μl) was then incubated in the wells overnight at 4 °C. After a routine PBST wash, 50 μL of anti-human rotavirus hyperimmune rabbit serum diluted 1:5000 with PBST that contained 2.5% skim milk were added to each well. The plate was incubated at 37 °C for 1.5 h. After a routine PBST wash, wells were incubated with 1:5000 dilution of peroxidase-conjugated donkey anti-rabbit immunoglobulin G (Jackson Immuno Research Laboratory, Inc, West Grove, PA) at 37 °C for 1.5 h. After a 4× PBST wash, substrate was then added and MAb bound to rotavirus VP6 antigen in test samples were detected by a micro-ELISA reader (EAR400; SLT-Lab instruments, Salzburg, Austria) at 492-nm. To establish an appropriate cutoff value, we tested 22 fecal samples collected from control or placebo subjects prior to treatment. The optical density (OD) of 0.13 was used as the cut-off value for a positive result.

Table 4
Baseline characteristics of the study children upon admission prior to IgY treatment.[*]

Characteristics	Test ($n=26$)	Placebo ($n=26$)
Male:female	13:13	17:09
Resident (urban:rural)	16:10	12:14
Age (months)	13.8 ± 10.6[†]	13.5 ± 6.3
Weight (lb)	17.5 ± 4.5	18.8 ± 3.1
Breast-feeding frequency (number/day)	7.1 ± 7.0	7.8 ± 6.7
Temperature (°F)	100.5 ± 1.5	100.4 ± 1.8
Fever rate (%)	22/26 (85%)	21/26 (81%)
Fluid intake (ml/day):		
ORS and others supplement	767.3 ± 538.6	1005.4 ± 628.0
Intravenous fluid (IVF)	345.0 ± 347	592.3 ± 491.3
Diarrhea duration (h)	69.6 ± 33.6	74.4 ± 38.4
Stool frequency (number/day)	9.2 ± 5.6	8.5 ± 7.3

[*] Values are not statistically significant between groups.
[†] Mean ± SD.

2.10. Data analysis

All data are presented as the means ± standard deviations (SD). The proportion of mice with diarrhea between groups and rotavirus shedding among patients in test and placebo groups, were analyzed by Chi-square test. The comparison of ORF intake and stool frequency among the study children between test and placebo groups were compared by the Student's *t*-test. Probability (p) of ≤0.05 was defined as statistically significant.

3. Results

3.1. FF reduction titer of Rotamix IgY and placebo IgY

The reactivity of the anti-HRV IgY is shown in Table 3. The neutralization titers of Rotamix IgY, as measured by FF reduction assay against the HRV strain 408 and 248 serotypes were 10,240 and 40,960 respectively. All 3 test IgY samples cross-reacted with all rotavirus strains tested except for the cow strain Shimane but the Rotamix IgY showed higher cross-reactivity against strains 408, Wa, KU, M37, S2, and S-80 compared to the 2 monovalent IgY samples (Table 3). Placebo IgY showed no reaction with all serological strains (neutralization titer was <20).

3.2. Suckling mouse experiments

There was no mortality in both placebo and test mouse groups. Feeding mice with Rotamix IgY prevented diarrhea in a dose-dependent manner. Among mice challenged with HRV 248, feeding higher doses of Rotamix IgY (5 mg/ml or 2.5 mg/ml) significantly reduced the frequency of diarrhea among test mice on day 2 (1/10 vs. 5/10 among placebo mice) and day 3 (1/10 vs. 7/10 among placebo mice) ($p=0.006$). Feeding with the lowest dose had the same effect on the frequency of diarrhea as the placebo group on day 3 (70% for 0.625 mg/ml compared to 70% for controls, $p>0.05$) (Fig. 1). Among mice challenged with HRV 408, feeding of 5 or 2.5 mg/ml Rotamix IgY had a significantly reduced frequency of diarrhea in the test group on days 2 and 3 ($p\leq0.02$). Feeding with the lowest dose had the same effect on the frequency of diarrhea as the placebo group on day 3 (90% for 0.625 mg/ml compared to 80% for placebo, $p>0.05$) (Fig. 1). No clinical symptoms other than diarrhea were observed in any of the mouse groups.

Table 5
Infection status among study children as revealed by stool microbiological tests upon admission to hospital.

Organisms co-infecting with human rotavirus	Rotamix IgY, $n = 26$ (%)	Placebo IgY, $n = 26$ (%)	Total, $n = 52$ (%)
Escherichia coli	10 (38.5)	8 (31)	18 (35)
Shigella dysenteriae	1 (4)	6 (23)	7 (14)
Shigella sonnei	2 (8)	2 (8)	4 (8)
Shigella flexneri	1 (4)	1 (4)	2 (4)
Proteus vulgaris	4 (16)	1 (4)	5 (10)
Staphylococcus aureus	3 (12)	0	3 (6)
Salmonella paratyphi A	1 (4)	1 (4)	2 (4)
Klebsiella aerogenes	1 (4)	1 (4)	2 (4)
Yersinia enterocolytica	0	2 (8)	2 (4)
Aeromonas hydrophila	1 (4)	0	1 (2)
Fungal origin	0	2 (8)	2 (4)
No concurrent infection	2 (8)	2 (8)	4 (8)

3.3. Baseline characteristics of study population

Patients admitted to the hospital were screened and randomly assigned to Rotamix IgY or placebo group according to flow diagram in Fig. 2. Table 4 presents the baseline characteristics of patients upon admission. The demographic and clinical background of subjects in the test and placebo groups were generally comparable. The mean ± SD of duration of diarrhea before enrollment in the study was 69.6 ± 33.6 and 74.4 ± 38.4 h for children in the test and placebo groups, respectively. The mean ± SD of stool frequency (number/day) before enrollment in the study was 9.2 ± 5.6 versus 8.5 ± 7.3 for children in the test and placebo groups, respectively. The mean of oral fluid intake (ml/day) was 767.3 and 1005.4 ml for children in the test and placebo groups, respectively.

Upon admission, 48 children (24 in test and control groups) showed mixed infection with one or more co-pathogens in their stool culture (18 *Escherichia coli*, 13 *Shigella* spp, 5 *Proteus* spp., 2 *Salmonella* spp., 8 multiple pathogens, and 2 others with fungal infection (Table 5).

3.4. Effect of anti-HRV IgY on study outcome

Fig. 3 shows the comparative data of the oral rehydration fluids (ORF) intake between Rotamix IgY and placebo IgY groups on a daily basis for 8 days. There was statistically lower volume of fluid intake among Rotamix IgY children on days 2 ($p = 0.001$), 6 ($p = 0.04$) and 8 ($p = 0.02$). Average ORF intake during the 8 days of observation was significantly less in the Rotamix IgY treated group (Rotamix IgY vs. placebo: 699.3 ± 111.1 vs. 919.1 ± 171.31, $p = 0.004$).

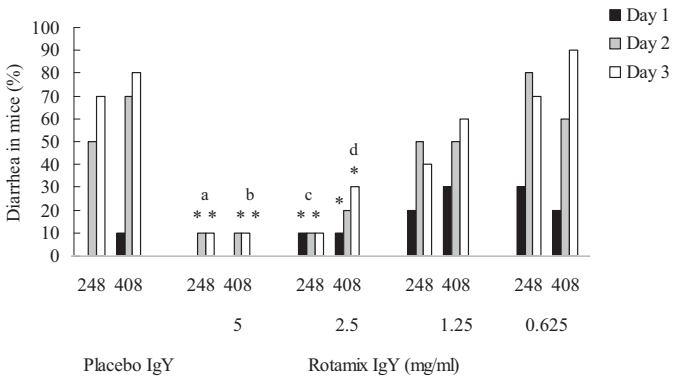

Fig. 1. Preventive effect of orally administrated different doses of Rotamix IgY against HRV 248 and HRV 408-induced diarrhea in suckling mice. *: Significant difference between placebo and Rotamix IgY groups ($p \leq 0.05$, Chi-square test). a: Rotamix IgY 5 mg/ml vs. control: Day 2: $\chi^2 = 3.8$; $p = 0.05$; Day 3: $\chi^2 = 7.5$; $p = 0.006$. b: Rotamix IgY 5 mg/ml vs. control: Day 2: $\chi^2 = 7.5$; $p = 0.006$; Day 3: $\chi^2 = 9.9$; $p = 0.002$. c: Rotamix IgY 2.5 mg/ml vs. control: Day 2: $\chi^2 = 3.8$; $p = 0.05$; Day 3: $\chi^2 = 7.5$; $p = 0.006$. d: Rotamix IgY 2.5 mg/ml vs. control: Day 2: $\chi^2 = 5.1$; $p = 0.02$; Day 3: $\chi^2 = 5.1$; $p = 0.02$.

The mean duration of IVF administration was 5 days for the Rotamix IgY group and 8 days for the placebo IgY group. Comparing day-to-day results, the Rotamix IgY group showed significantly less IVF administration only on day 1 ($p = 0.03$, data not shown) of intervention. From days 2 to 8, the difference in daily mean volume (ml/day) between the two groups is not statistically significant (77.4 ± 121.4 vs. 93.3 ± 196.7 ml/day for test and placebo groups respectively, $p = 0.42$).

There was a decreasing trend in daily frequency of diarrhea among children in the Rotamix group (Fig. 4) but the difference from placebo IgY group was statistically significant only on day 2 ($p = 0.03$) and day 3 ($p = 0.05$). The overall difference in mean duration of diarrhea (test vs. placebo: 135.3 ± 42.0 vs. 185.5 ± 41.7 h respectively) was also statistically significant ($p = 0.01$) (data not shown in figure).

All pediatric patients received the usual medical treatment according to the nature of mixed infection as judged by attending physicians. These consisted of several antimicrobials, vitamin/minerals and probiotics (Table 2). Overall, both test group and placebo group were similar in terms of the kind of antimicrobials and supportive vitamins and probiotics given during the term of the study.

At the time of discharge, no stools from patients in the test group showed rotavirus shedding, while 4 patients had rotavirus positive stool in the placebo group (statistically significant ($\chi^2 = 4.3$; $p = 0.04$). The frequency of rotavirus shedding was significantly higher in children treated with placebo IgY than in those treated with Rotamix IgY on day 3 ($\chi^2 = 7.7$, $p = 0.005$) and on days 6–8 ($\chi^2 = 5.9$, $p = 0.02$) (Fig. 5). The difference in mean duration of rotavirus excretion between the two groups is statistically significant (3.0 ± 1.6 vs. 4.2 ± 2.9 days for test vs. placebo group respectively, $p = 0.05$).

Table 6 shows a summary of key observations and the total outcomes from day of admission of the patient to the hospital for confinement until day 8. Moreover, Rotamix IgY was not associated with any adverse clinical event after oral dosing for 8 consecutive days among infants and children with pre-existing diarrhea and dehydration.

4. Discussion

The serotypic diversity among rotaviruses is due to genetic reassortments arising from interspecies transmission and/or mixed infection. Reassortant serotypes are clinically important in as much as they cause severe forms of diarrhea among children worldwide. In this study, two natural unique human rotavirus reassortant clinical isolates ATCC VR 2273 (HRV 408) and ATCC VR 2274 (HRV 248) were selected and used to prepare the Rotamix IgY powder produced by mixing the above serotypes as antigens. These two viruses

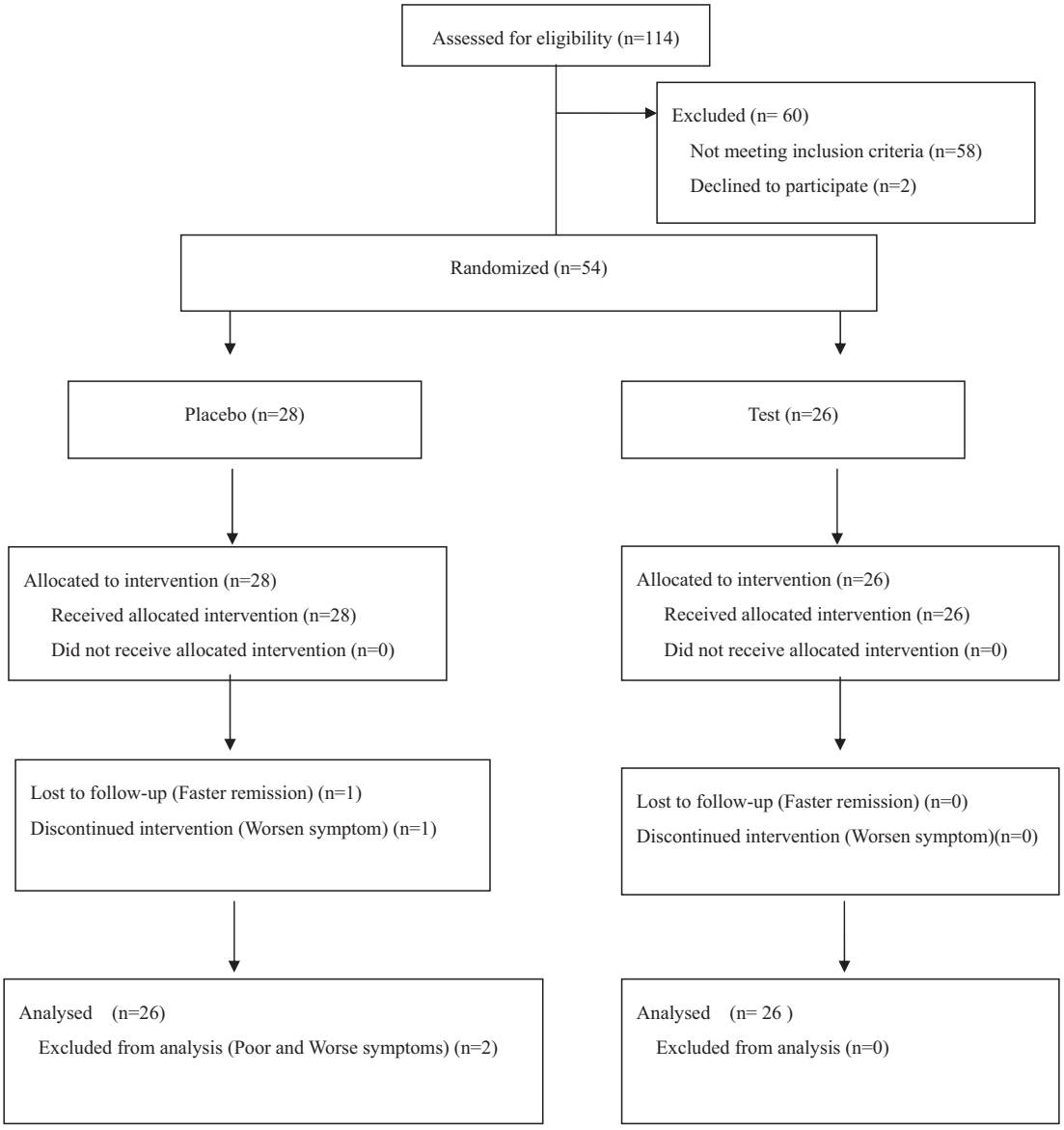

Fig. 2. Diagram showing the decision flow on study subjects throughout the study.

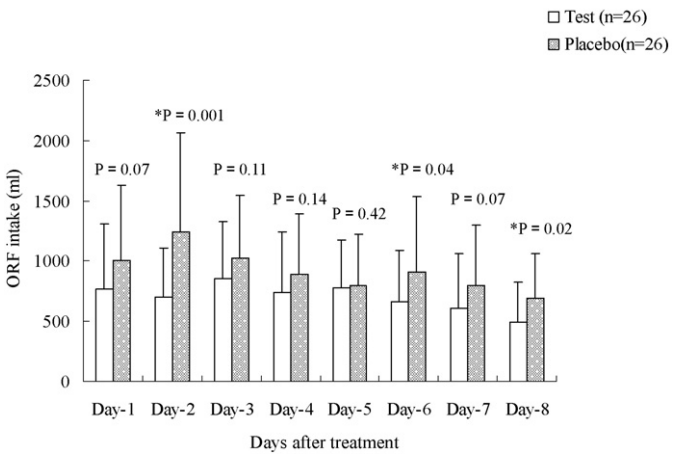

Fig. 3. Comparative analysis of mean daily oral fluid intake in study children between Rotamix IgY and placebo IgY groups. *: Significant differences between the Rotamix IgY and placebo IgY groups (*$p \leq 0.05$, Student's *t*-test).

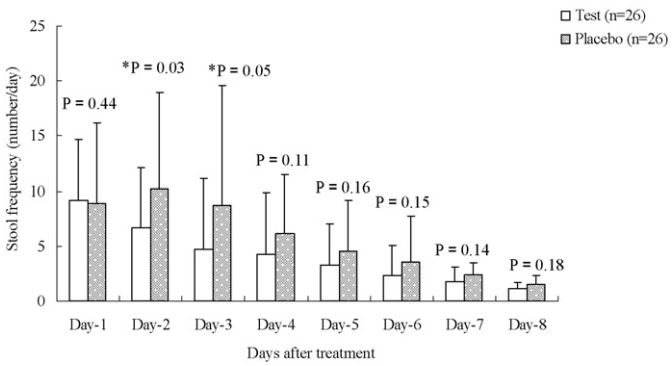

Fig. 4. Comparison of stool frequency (number/day) between children treated with Rotamix IgY or placebo IgY. *: Significant differences compared with placebo and Rotamix IgY groups (*$p \leq 0.05$, Student's *t*-test).

Table 6
Comparative analysis of Rotamix IgY and placebo effects on study groups according to outcome measures.

Parameters	Placebo IgY group	Rotamix IgY group	Statistics
1. Daily oral rehydration fluid volume	Day 2 = 1244.1 ± 818.4 ml Day 6 = 912.5 ± 623.0 ml Day 8 = 688.5 ± 372.7 ml	Day 2 = 704.4 ± 403.8 ml Day 6 = 660.6 ± 429.8 ml Day 8 = 493.9 ± 329.8 ml	Day 2; $p = 0.001^*$ Day 6; $p = 0.04^*$ Day 8; $p = 0.02^*$ (See Fig. 3)
2. Total oral rehydration fluid volume from day of admission	919.1 ± 171.31 ml	699.3 ± 111.1 ml	$p = 0.004^*$
3. Mean duration of intravenous fluid administration	8 days	5 days	$p = 0.03^*$
4. Mean volume of intravenous fluid administered daily	93.3 ± 196.7 ml	77.4 ± 121.4 ml	Not significant ($p = 0.42$)
5. No. of stools/day	Day 2 = 10.2 ± 8.8 Day 3 = 8.7 ± 11.2	Day 2 = 6.7 ± 4.3 Day 3 = 4.7 ± 4.1	Day 2; $p = 0.03^*$ Day 3; $p = 0.05^*$ (See Fig. 4)
6. Total duration of diarrhea from day of admission	185.5 ± 41.7 h	135.3 ± 42.0 h	$p = 0.01^*$
7. Daily frequency of viral shedding	Day 3 = 88 Day 6 = 25 Day 7 = 20 Day 8 = 25	Day 3 = 42 Day 6 = 0 Day 7 = 0 Day 8 = 0	Day 3; $p = 0.005^*$ Day 6; $p = 0.02^*$ Day 7; $p = 0.04^*$ Day 8; $p = 0.02^*$ (See Fig. 5)
8. Total duration of viral shedding from day of admission	4.2 ± 2.9 days	3.0 ± 1.6 days	$p = 0.05^*$

* Statistically significant.

derived their genomic segments from parental strains of different genogroups and/or serotypes. The HRV 408 was shown to have subgroup II specificity (I1 genotype according to recent nomenclature) [30], and its RNA profile is long which is characteristic of subgroup II rotaviruses [21]. HRV 408 has G3 specificity, but could also be neutralized by polyclonal antisera to G1, G2, and G3, and may induce neutralizing antibodies to at least G1 and G2 [30]. The HRV 248 was shown to have G4P[4] specificity, subgroup II specificity (I1 genotype of VP6), and a long RNA pattern. By RNA–RNA hybridization assay, it was found that 7 of 11 RNA segments of the HRV 248 are derived from Wa genogroup and the remaining 4 are from DS-1 genogroup. Thus, HRV 248 is shown to be an intergenogroup reassortant [31] strain.

In order to determine cross reactivity profile, several rotavirus serotypes originally derived from human, horse, cattle and swine were reacted in vitro against Rotamix IgY. Results revealed that Rotamix IgY strongly cross-neutralized all the major HRV serotypes (G1, G2, G3, and G4) along with other human and animal strains (Table 3). In addition to a broad spectrum of cross-reactivity, synergism was observed in vitro with Rotamix IgY relative to the lower titers of IgY developed individually from HRV 408 and HRV 248 strains. This in vitro cross-reactivity profile suggests that Rotamix IgY might provide a general multi-serotypic passive protection in vivo particularly among rotavirus infected infants and young children considering the broad serotypic diversity of human rotaviruses in different regions of the world.

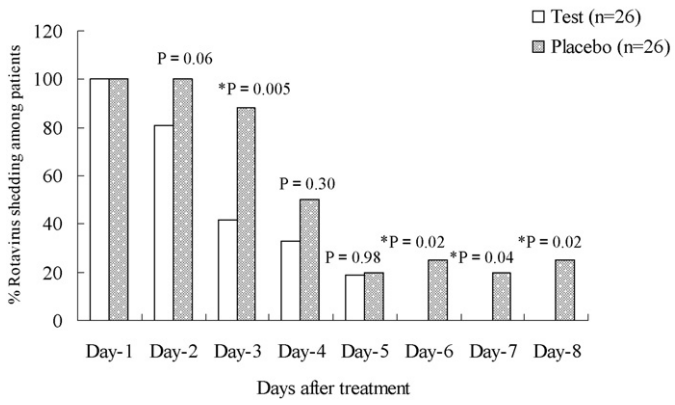

Fig. 5. Effect of Rotamix IgY on daily frequency of rotavirus shedding in stools of children. *: Significant differences between Rotamix IgY and placebo IgY groups (*$p \leq 0.05$, Chi-square test).

As a first step towards assessment of protection against HRV-induced diarrhea in pediatric patients using Rotamix IgY, we used the latter IgY in a placebo-controlled mouse challenge experiment. There was significant reduction in the frequency of diarrheal episodes among challenged mice using the dual-specificity Rotamix IgY. The rate of protection was specific based on the dose-dependent protection rate afforded to mice with protection rate reaching up to 90% at the highest IgY concentration used (Fig. 1). These findings are consistent with previous applications of IgY as a therapeutic agent in cat [17] and calves [16,32] and as prophylactic agent in mice [33]. However, these earlier studies were based on the use of IgY with single rotavirus strain specificity and are therefore of limited application in clinical settings with multi-serotypic etiology. The results on dose-dependent protection effect together with the safety of Rotamix IgY in infected suckling mice encouraged us to evaluate the therapeutic activity of Rotamix IgY among children with diarrhea concurrently infected with HRV and other enteric pathogens during their hospital confinement.

Out of 114 young children and infants admitted with diarrhea and other complaints in a Myanmar hospital, 54 of them (47.4%) were initially entered in this study using our inclusion criteria but 2 of them were excluded later due to incomplete data (Fig. 1). This frequency of rotavirus infection is almost similar to earlier studies in Myanmar (57% and 55% in 2004 and 2005 respectively) [11]. Of the remaining 52 subjects, 92% had a second enteric non-cholera pathogen (Table 5). Mixed infection with different species of enteric pathogens as observed in this study may be representative of clinical conditions in developing countries as earlier observed [27,34].

Rotamix IgY, with a neutralization titer of 10,240–40,960 against a broad range of human rotavirus strains known to be currently circulating worldwide, was administered orally at the rate of 500 mg × 4 doses daily (containing about 30 mg of IgY/day) together with standard medical treatment (Table 2) to control bacterial infections, replace fluid loss and/or provide nutritional supplements to mitigate the effects of diarrhea. As such, Rotamix IgY was used as an adjunct to standard supportive treatment of rotavirus diarrhea with an intention to directly neutralize or suppress rotavirus infectivity in the gut and thereby prevent further cell-to-cell spread of infection and resulting fluid loss.

Since the first 1–3 days in the hospital is usually the most severe period of the illness, it follows that any beneficial effect arising from Rotamix IgY administration within this period (as seen in Fig. 4) signifies clinical relevance [20]. From the public health standpoint, neutralizing rotavirus infectivity in acute enteritis and reducing the duration of viral shedding by specific IgY may potentially prevent further spread of infection in a community or hospital setting. It is worth noting that Rotamix IgY influenced the above clinical parameters despite the presence of co-infecting enteric pathogens in 92% of the subjects indicating an important role played by Rotamix IgY's as an adjunct to supportive therapy for infant rotavirus diarrhea. This finding also highlights the contribution of rotavirus as a pivotal virulence factor in determining the course of infection during mixed infection with other non-cholera enteric pathogens.

While certain observations were statistically significant only on certain days as shown in Figs. 3–5, the totality of data over the whole 8-day observation period will bear out statistically significant differences in observations between the two groups particularly in terms of total oral rehydration fluid volume from day of admission, mean duration of intravenous fluid administration, total duration of diarrhea starting from the day of admission, and total duration of viral shedding from day of admission (Table 6). These significant observations may translate into real benefits in terms of earlier termination of IV fluid by 3 days, earlier recovery from diarrhea by 2 days, and earlier cessation of rotavirus shedding via stool by 1 day.

Data in Tables 2, 4 and 5 indicate that patients in both test and placebo groups had similar demographics, had comparable frequency and type of co-infecting pathogen burden as well as comparable treatments received during the clinical trial. Among test group patients, oral administration of Rotamix IgY containing antibodies known to neutralize or disable a broad spectrum of rotavirus serotypes in vitro (Table 3), some of which were circulating in the study area [11], significantly improved the clinical picture as outlined in Table 6. This means the favorable outcomes among Rotamix IgY treated patients were mainly due to the specific action of the IgY received as can be gleaned from the lack of similar outcomes among placebo patients.

A 2009 report showed that among children less than 5 years of age who were admitted to Yangon Children's Hospital in Myanmar, the most common serotypes were G3, P[4] and P[8] while the most common G and P combinations were G3P[8] and G1P[8] [11]. Representatives of these strains tested in this study (Table 1) could be cross-neutralized by Rotamix IgY (Table 3) which may further account for the adjunctive value of oral Rotamix IgY administration in our clinical setting.

None of the children in either test or placebo group in this study recovered before day 5 from the day of admission to hospital. This is inconsistent with an earlier observation wherein rotavirus diarrhea in children normally lasts for 1–6 days, is self-limiting and children usually recover before day 5 [35]. The generally longer episode of diarrhea observed in this study may be explained by: 1) the presence of a co-infecting pathogens in almost all subjects examined, dual infection being known to produce a more severe illness [36], and 2) malnutrition. In developing countries, diarrhea has been observed to last longer in children with less than ideal nutrition [37].

Passive immunotherapy using IgY has advantages over vaccination due to its: 1) rapid and local onset of action, 2) highly specific activity, 3) applicability to a broader age range of patients from infants to adults including immunodeficient patients, and 4) is nontoxic being a normal part of the human diet [38,39]. Notably, none of the study patients treated with Rotamix IgY or placebo IgY presented with complaints other than the ongoing diarrhea and its resulting dehydration. The absence of any adverse clinical event attributable to or associated with oral IgY administration for 8 days is consistent with our clinical findings in the mouse protection experiment (Fig. 1), as well as in earlier published passive immunization trials with hyperimmune IgY against *Streptococcus mutans* [40] and *Helicobacter pylori* [15] among human subjects. With the recent findings by our group on the application of IgY against *Vibrio cholerae* [41] and Shiga-toxin [42], the present study paves the way toward combined use of IgYs against Shiga toxin, cholera and rotavirus for prevention and treatment of pediatric diarrhea in the future.

Considering that Rotamix IgY is strongly reactive with a broad range of serotypes responsible for the vast majority of rotavirus-associated diarrhea in infants and young children worldwide, and may ameliorate patient condition when administered orally even to those with mixed (rotavirus plus non-rotavirus) enteric infections, it comprises a potentially useful adjunct to general supportive therapy of acute rotavirus infection in pediatric patients.

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Contributors: SR participated in the design, organization and coordination of the study, participated in the statistical analysis and drafted the manuscript. KHM supported the in vitro analysis, KWH carried out the clinical work, participated in the clinical data analysis. KT provided his lab facilities to carry out in vitro analysis, advised on overall data analysis; FCI advised on data interpretation and did critical revision and finalizing of manuscript; TT coordinated the working team. YK NVS, KU, NNDF KO participated in drafting of the manuscript and coordinated with the working team. HNO, YYM, TH, SSM, KT participated in the clinical works and coordinated with the working team. All authors have read and approved the final manuscript. *Conflict of interest statement*: The authors declare that they have no competing interests.

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