Changes in Anti-Group A Rotavirus Antibody Seroprevalence and Levels in the Western Gyeongnam Province of Korea Over 16 Years

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INTRODUCTION

Symptomatic rotavirus infections are common among infants and young children all over the world, both in developed and developing countries having similar overall incidences of rotavirus infection (1). In Korea, it was found that since 1980, rotavirus is the main cause of diarrhea in hospitalized children (2, 3). Since 2001, outbreaks of rotavirus in newborns at the postpartum care centers that care for postpartum mothers and their healthy newborn babies have been reported (4). In addition, there has been an increase in the incidence of symptomatic infections in Korean children older than five years of age (5, 6).

While most individuals are exposed to rotavirus at least five times during their lifetime, only the first infection causes severe acute gastroenteritis. Subsequent exposures, even to different rotavirus serotypes, only induce minor symptoms at worst. Thus, acquired immunity seems to play an important role in preventing the ill effects of rotavirus infections (7-9).

To observe how anti-group A rotavirus antibody seropositivity rates and levels have changed in the western region of Gyeongnam Province, 2,030 serum samples collected at four collection periods (1989-1990, 1994-1995, 1999-2000, and 2004-2005) were tested by Enzyme-Linked Immunosorbent Assay for IgG, and IgA antibodies reacting to recombinant VP6 protein. The seroprevalences exhibit no regular patterns over a 16-yr period. For all four collection periods, the anti-rVP6 IgG levels rose steadily during the first 5 months of life, after which they remained high. However, the 2-9 yr and 10-39 yr groups had significantly higher IgG levels in 1999-2000 and 2004-2005, respectively, than in the other collection periods. The 1-5 mo, 40- ≥ 60 yr, and 4-29 yr groups had significantly higher IgA levels in 1989-1990, 1999-2000, and 2004-2005, respectively. The 4 yr (25.0%), 5-9 yr (18.8%), 10-14 yr (41.1%), 20-29 yr (35.0%), and 30-39 yr (20.0%) groups in 2004-2005 had significant higher IgA seropositivity rate compared to the other three collection periods. These observations suggest that in the western region of Gyeongnam Province since the late 1990s, rotavirus reinfection has occurred more frequently than previously, with all ages being at risk.

Key Words: Rotavirus; VP6; ELISA; Seroepidemiology

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from patients and stores them at -20°C. Two thousands and thirty serum samples collected between 1989 to 2005 from patients without acute gastroenteritis were recruited. Of the samples collected for 16 yr, the serum samples in the Gyeongsang National University Hospital of 1989-1990, 1994-1995, 1999-2000, and 2004-2005 were examined. The sera were stratified into 16 age groups, namely, < 7 days, 1-5 months, 6-11 months, 12-17 months, 18-23 months, 2 yr, 3 yr, 4 yr, 5-9 yr, 10-14 yr, 15-19 yr, 20-29 yr, 30-39 yr, 40-49 yr, 50-59 yr, and ≥ 60 yr. The serum samples were tested for antibodies against rVP6 by ELISA (Table 1).

Expression and purification of rVP6
The rVP6 protein was expressed and the quality of the preparation was validated as described elsewhere (10). Briefly, the full-length rVP6 DNA clone (1,194 base pairs) that was donated by one of us was amplified by polymerase chain reaction (PCR), after which the PCR products were recloned into the pGEM-T vector (Promega, Madison, WI, USA). The pGEM-T/rVP6 clone was purified and digested by the del and BamHI restriction enzymes and ligated into a del- and BamHI-digested pET-15b expression vector. Escherichia coli Rosetta II strain was transformed with the recombinant plasmid and grown overnight at 37°C in LB medium containing 100 μg/mL of ampicillin. The saturated culture was then diluted 1:1,000 and, when the absorbance at 600 nm reached 0.6 to 0.8, isopropyl thiogalactopyranoside was added to a final concentration of 1 mM and the culture was maintained for 4 hr. The cell inclusion bodies containing the recombinant VP6 proteins were isolated from the sonicated cell lysate by centrifugation at 13,000 rpm for 30 min. The inclusion bodies were extracted by being stirred overnight in 8 M urea in 50 mM Tris-HCl (pH 8.0). The extracted proteins were refolded by being stirred overnight in 0.8 M L-arginine. Affinity chromatography was performed by using Probond Nickel Agarose Resin (Invitrogen, Carlsbad, CA, USA) and the purified recombinant VP6 proteins were eluted with 50 mM Tris-HCl (pH 8.0), 200 mM NaCl, and 320 mM imidazole, and then dialyzed. The quality of the rVP6 preparation was confirmed by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis, after which the preparation was preserved at -70°C.

Enzyme-Linked Immunosorbent Assay (ELISA)
To detect rVP6-specific serum IgG and IgA antibodies, the wells of 96-well flat-bottomed EIA plates (Costar, Bloomington, MN, USA) were coated overnight at 4°C with 50 μL of a 10 μg/mL solution of rVP6 in 0.05 M carbonate, pH 8.0. The plates were washed once with 0.05% Tween 20-PBS (PBST), after which 150 μg of 3% bovine serum albumin-PBST were added to each well and incubated at 37°C for 3 hr. After three washes with PBST, 50 μL of diluted serum samples (for IgG, and IgA, the dilutions were 1:500, and 1:100, respectively) and positive and negative control serum samples were added to the wells and incubated for 1 hr at 37°C. After three washes with PBST, 50 μL of peroxide-conjugated goat anti-human IgG, or IgA (Bethyl Lab., Montgomery, TX, USA; diluted 1:10,000) were added to the wells and incubated at 37°C for 1 hr. The plates were then washed five times with PBST and 50 μL of o-phenylene diamine was added to each well. The reaction was stopped after 30 min at room temperature by adding 50 μL of 2 N H2SO4. Optical density (OD) was measured at 492 nm. The IgG, and IgA levels in each specimen were tested in duplicate and the mean ODs were obtained. The normal cutoff levels for anti-rVP6 IgG, and IgA were determined by obtaining the mean OD of all negative controls and adding three standard deviations. As a result, the normal cutoff levels for IgG, and IgA were 0.130, and 0.062, respectively. The interplate coefficients of variance for the IgG and IgA ELISAs were 10.7%, and 8.5%, respectively (10).

Statistical analysis
The data were analyzed by using SAS statistical software, version 9.1 (SAS Institute, Cary, NC, USA). How the anti-rVP6 IgG, and IgA levels and seropositivity rates in the population varied depending on age, gender, and the collection period was examined. Statistically significant differences in seroprevalence between age groups were determined by using the chi-squared test. Nonparametric tests (Kruskal-Wallis Test and Wilcoxon Scores) were used to analyze the mean anti-rVP6 IgG, and IgA antibody levels at different collection periods. P values of < 0.05 were considered to be statistically significant. Post hoc analysis using Scheffe method was performed when the difference among 4 periods was significant.

Ethics statement
This study was performed after the institutional review board reviewed and approved the research protocols of the present study (GNUHI- RB-5413). The agreement exemption was applied due to all serum samples had no genetic information and derived from the National Biobank of Korea were obtained with informed consent under institutional review board-approved protocols.

RESULTS
Gender differences and longitudinal changing pattern
Males and females did not differ in their seropositivity rates when they were divided according to age or collection period. However, compared to males, females had higher median anti-rVP6 IgG antibody levels in the 2004-2005 (P = 0.012, data not shown). By contrast, males had higher median anti-VP6 IgA levels in the 1999-2000 (P < 0.001, data not shown). No specific patterns according to age were observed during the 16-yr with regard to the anti-rVP6 IgG and IgA antibody levels and seropositivity rates.
| Age group | Median OD (range) | ELISA | Median OD (range) | Median OD (range) | Median OD (range) | Median OD (range) | Median OD (range) |
|-----------|-----------------|-------|-----------------|-----------------|-----------------|-----------------|-----------------|
| 2004-2005 | 0.334-1.866     | 0.731 | 0.097           | 0.027-1.619     | 0.113           | 0.061-1.149     | 0.109           |
| 2 yr      | 0.570-1.844     | 1.149 | 0.200           | 1.166           | 1.029           | 0.907           |
| 3 yr      | 0.338-1.805     | 0.640 | 0.138           | 0.166           | 0.146           | 0.912           |
| 4 yr      | 0.283-1.922     | 0.283 | 0.200           | 0.166           | 0.200           | 0.912           |
| 5-9 yr    | 0.191-1.342     | 0.112 | 0.166           | 0.166           | 0.166           | 0.912           |
| 10-14 yr  | 0.015-2.458     | 0.112 | 0.166           | 0.166           | 0.166           | 0.912           |
| 15-19 yr  | 0.261-1.716     | 0.112 | 0.166           | 0.166           | 0.166           | 0.912           |
| 20-29 yr  | 0.406-2.022     | 0.112 | 0.166           | 0.166           | 0.166           | 0.912           |
| 30-39 yr  | 0.210-1.229     | 0.112 | 0.166           | 0.166           | 0.166           | 0.912           |
| 40-49 yr  | 0.351-1.570     | 0.112 | 0.166           | 0.166           | 0.166           | 0.912           |
| 50-59 yr  | 0.240-1.213     | 0.112 | 0.166           | 0.166           | 0.166           | 0.912           |
| > 60 yr   | 0.353-1.703     | 0.112 | 0.166           | 0.166           | 0.166           | 0.912           |
| Subtotal  | 0.201-1.685     | 0.112 | 0.166           | 0.166           | 0.166           | 0.912           |

*Statistically significant differences between the four time periods in terms of optical density. OD, optical density.
Anti-rVP6 IgG antibody levels and seropositivity rates

As shown in Table 1 and Fig. 1, the < 2 yr age groups had similar anti-rVP6 IgG levels to the ≥ 40 yr age groups. For all collection periods, the anti-rVP6 IgG levels started rising in the 0-5 months of age, after which they remained high in all age groups. However, the 1999-2000 collection period was associated with differences between the 2-39 yr age groups with regard to anti-rVP6 IgG levels. In particular, the 2-9 yr age groups had significantly higher median ODs than the 10-39 yr groups (P < 0.001).

In addition, in the 2004-2005 collection period, the median ODs of the 10-39 yr groups were significantly higher than the median ODs of the same groups in the other three collection periods (P = 0.05) (Fig. 1). Moreover, the 1994-1995 period was associated with lower neonatal anti-rVP6 IgG levels than the other three periods, although this trend did not show statistical significance. The anti-rVP6 IgG seropositivity rates for all age groups in all collection periods were almost 100% (Fig. 2).

Anti-rVP6 IgA antibody levels and seropositivity rates

For all collection periods, all age groups had lower median anti-rVP4 IgA ODs than anti-rVP6 IgG ODs (Table 1 and Fig. 3). The median IgA OD of the 1-5 months age group was significantly higher in the 1989-1990 collection period than in the other three periods (P < 0.001), while the 4-29 yr age groups had significantly higher ODs in 2004-2005 than in other periods (P < 0.001). Moreover, the 40- ≥ 60 yr age groups had significantly higher ODs in 1999-2000 than in the other periods (P < 0.001).

In terms of anti-rVP6 IgA seropositivity rates, the 1989-1990,
1994-1995, and 1999-2000 periods exhibited bimodal patterns, where seroprevalence first peaks in early childhood (between 1 month and 2 yr); this is followed by a reduction in seroprevalence until a trough appears at 2-4 yr. Thereafter, seroprevalence climbs before reaching a high value at 10-19 yr that is similar to the values of older age groups. The remaining period, 2004-2005, showed consistently high seropositivity rates after the age of 1 yr (Fig. 4). The 1-5 months group had a higher seropositivity rate in the 1989-1990 period than in the other periods ($P = 0.067$). Moreover, the 2-14 yr age groups had significantly higher seropositivity rates in 2004-2005 than in the other periods ($P < 0.001$). The high seropositivity rates of the 1-yr-olds in 2004-2005 were thus also observed in older age groups of that collection period.

**DISCUSSION**

In terms of group A rotavirus seroprevalence, no regular changing patterns were seen over the 16-yr study period (1989-2005). This suggests that rotavirus infections are random events that depend on environmental or social factors at each specific time period. In addition, no specific changes in group A rotavirus seroprevalence were observed throughout the study period, despite the fact that symptomatic neonatal rotavirus infections have been increasing steadily in Korea since 2001 (4-6).

All age groups in the all periods had anti-VP6 IgG seropositivity rates of almost 100%, which indicates that all age groups had been exposed repeatedly to rotavirus during the 16-yr study period. Since the serum samples were from patients without...
acute gastroenteritis, this repeated rotavirus exposure appears to boost anti-rotavirus immunity in children and adults rather than causing disease. Notably, a study analyzing cord blood from Indian infants revealed that their anti-rotavirus IgG levels declined at 6 months of age (11). A serological analysis in Brazil also showed clearly that the anti-rotavirus IgG levels decrease during the first 6-9 months of age (12). By contrast, our study showed no significant decline in the anti-rVP6 IgG levels during the first year of life; indeed, the high IgG levels that were observed in the 6-11 months age group were sustained in all subsequent age groups. This discrepancy may relate to the fact that the VP6-specific IgG levels produced by the Koreans participating in this study are higher than those generated by the Indians and Brazilians (11, 12). The surveillance of acute gastroenteritis in Seoul, Korea showed rotavirus is the most common pathogen in infants with viral gastroenteritis (42.7%, 496/1,161) (13).

In a study of rotavirus surveillance testing on all the newborns who were admitted to the nursery in Korea, 47 of 61 neonates had no symptoms of gastroenteritis such as fever, vomiting and diarrhea (14). Both results supported that the higher level of IgG in 6-11 months of infants in the present study. It may also be due to the fact that Koreans are earlier exposed and more frequently re-exposed to rotavirus. Infants who did not have rotavirus infections had significantly higher IgG level in cord blood and serum samples at 6 months than infants who had symptomatic/asymptomatic rotavirus infections. This result suggested that fewer rotavirus infections occur when cord blood retains higher level of anti-rotavirus IgG antibodies (11). In the present study, the newborns (< 7 day) in all four collection periods had lower anti-rVP6 IgG levels than most of the other age groups, yet concomitant decreases in anti-rVP6 IgG levels were not observed in the women of reproductive age (20-39 yr of age). Thus, our data do not support the notion that the recent increases in symptomatic neonatal infection are due to low maternal serum IgG levels. Indeed, we observed that compared to men, women had significantly higher anti-rVP6 IgG levels in 2004-2005. This may reflect greater exposure of mothers to their children than fathers and thus their greater chance of re-exposure to rotavirus, which boosts their anti-rotavirus IgG levels. However, this theory does not explain why women did not differ from men in their anti-rVP6 IgA levels; indeed, in several collection periods, men had higher anti-rVP6 IgA levels than women (data not shown). Further studies are needed to resolve this issue.

The prevalence of rotavirus infection is generally underestimated because asymptomatic infections occur frequently and adults who present with diarrhea are not routinely tested for rotavirus infection. The anti-rVP6 IgA antibody pattern in the present study suggest that, compared to other collection periods, 1) rotavirus re-exposure of the adult population occurred less frequently in the late 1980s, and 2) the entire population was less frequently re-exposed until the late 1990s. In the 2004-2005 period, the adults had very high IgA levels and seropositivity rates, which is suggestive of frequent re-exposure to rotavirus infection after a long period of limited exposure. Notably, long-term hospitalization is a risk factor for rotavirus-induced illnesses because such patients tend to live in closed communities which may impair immune response (15). Gyeongsang National University Hospital is located in Jinju, which is a city of 350,000 people in the western part of Gyeongnam Province. Jinju is a relatively small city by Korean standards and is surrounded by rural communities. The annual growth of the population is 0.4% since 1995. Jinju is known as the city of education and there are 6 universities, 26 high schools, 20 middle schools and 45 elementary schools. In Korea, most of the middle and high school students stay in the same class from early in the morning until late in the evening; this can also be true for college students. Thus, many students live in closed-type communities, which may make them more vulnerable to rotavirus, which in turn could result in epidemics of rotavirus infections.

Mucosal and serum IgA antibodies protect against rotavirus infection (16, 17). A cohort study of 200 infants also found that serum anti-rotavirus IgA antibodies are a stronger marker of protection than serum anti-rotavirus IgG antibodies (17). Thus, the IgA seropositivity rates may be reflective of recent rotavirus infections and epidemics. In the present study, the first three collection periods were associated with bimodal patterns of anti-rVP6 IgA seropositivity. This suggests that during these periods, the first infection occurred in very young children and re-infection occurred in over the age of 10-19 yr. By contrast, in the 2004-2005, most of the age groups showed high anti-rVP6 IgA seropositivity rates. This suggests that, by this time, most people had been exposed repeatedly to rotavirus.

The present study suffers from two limitations. First, the rotavirus infection history of the patients was not known. The second is that laboratory-based surveillance of stool rotavirus was not performed when the sera were obtained. As a consequence, it is not clear whether other rotavirus groups were prevalent, which could potentially affect the immune responses to the rVP6 antigen of group A rotaviruses. Alternatively, the antibodies raised by the other rotavirus groups may be crossreactive and could have led to false positives in the ELISA used in the present study. However, it has also been shown that the antibodies elicited by different rotavirus strains are not crossreactive in VP6-based ELISAs (18).

In late 2008, the rotavirus vaccine started to be used in Korean infants on a selective basis. A recent review of the efficacy of this vaccine has shown that it was more efficacious in developed and middle-income countries than in lower income countries (19). It was suggested that the impact of national rotavirus vaccination should be assessed by postmarketing surveillance. Moreover, the circulating rotavirus strains should be monitored con-
continually.

In summary, the anti-rVP6 IgG and IgA antibody responses examined in this study show that people in the southern central part of Korea have been frequently and repeatedly exposed to rotaviruses since the late 1990s despite socioeconomic, housing, and environmental-sanitation conditions improve.

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