Changes in rotavirus genotypes before and after vaccine introduction: a multicenter, prospective observational study in three areas of Japan

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Title: Changes in rotavirus genotypes before and after vaccine introduction: a multicenter, prospective observational study in three areas of Japan

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Running title: Rotavirus genotypes in three areas of Japan
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Summary

In Japan, monovalent and pentavalent rotavirus (RV) vaccines were approved in 2011 and 2012, respectively. To monitor the changes in the RV genotypes before and after vaccine introduction, we performed a prospective observational study among children (<5 years) with gastroenteritis who tested RV-positive on antigen rapid tests. Stool samples were collected from three different sites in Japan: Tsu City, Mie Prefecture; Kurashiki City, Okayama Prefecture; and Isumi City, Chiba Prefecture. RV genotypes were determined using reverse transcription-polymerase chain reaction. In Tsu City, G3P[8] was dominant (61.0–77.1%) before vaccine introduction, but decreased there. Meanwhile, in inverse proportion of the decrease in G3P[8], G1P[8] increased until the 2013/14 season, where a sudden dominance of G2P[4] (100%) occurred. A similar trend was observed in Kurashiki City in terms of the extent of the decrease in G3P[8] and the emergence of G2P[4]. In Isumi City, G1P[8] was dominant (70.3%) before vaccine introduction, whereas G9P[8] became dominant (83.3%) in the 2013/14 season. To determine whether the genotype changes are attributable to vaccines or natural epidemiological changes, ongoing continuous monitoring of the RV genotypes is required.
1. Introduction

Group A rotavirus (RV) infection is the primary cause of severe gastroenteritis in children less than five years of age, especially in infants (1). Since 2006, live attenuated RV vaccines have been introduced as part of the national immunization programs in various countries (2), and the efficacy and safety of the vaccines have been confirmed in many countries (3-5). In 2009, the World Health Organization recommended that all nations include an RV vaccine in their national immunization programs (1). However, in Japan, the attenuated monovalent (G1P[8]) human rotavirus vaccine (RV1; Rotarix®) was not approved until November 2011, and the pentavalent (G1, G2, G3, G4, and P[8]) human-bovine rotavirus reassortant vaccine (RV5; RotaTeq®) was not approved until July 2012. Hence, these are not included as routine vaccination in the Japanese childhood immunization program even now.

In 1999, acute gastroenteritis was designated as a category V notifiable disease under the Communicable Diseases Prevention Law in Japan (6). This law requires approximately 3,000 designated pediatric sentinel sites to report the total number of patients, sex, and age category weekly (7); all of these case data are condensed to the National Epidemiological Surveillance of Infectious Diseases (NESID) system. Because
the cause of acute gastroenteritis was not required to be reported to NESID in 2007, thus we established the Rotavirus Epidemiology Study Group (RESG) in order to understand the true epidemiology and disease burden of RV gastroenteritis among children less than five years old in Japan. This group consists of one laboratory and five medical facilities in three different locations in Japan. We collected demographic information, clinical symptoms, and stool samples from RV gastroenteritis patients with a positive rapid test result. Since 2007, the RESG conducted a retrospective study (2003–2007) and a prospective active surveillance study (2008–2009) in inpatients with RV gastroenteritis in Mie Prefecture, Japan, and reported that the disease burden of hospitalization was comparable to that of other advanced countries (8,9). Our group has continued to conduct studies on the vaccine efficacy and vaccine cost-effectiveness with respect to the introduction of RV vaccines into the national immunization program.

Collected stool samples were sent to the laboratory and underwent polymerase chain reaction (PCR) and genotype testing to confirm the RV infection as well as the genotypes of the RV. In countries where the RV vaccines were introduced earlier than in Japan, studies have reported that different distributions of RV genotypes were observed before and after the introduction of the RV vaccine (10). However, whether this change is attributable to the protective effect of vaccines or to natural epidemiological changes
is controversial. Therefore, continuous monitoring of changes in the RV genotypes is necessary to evaluate the influence of the RV vaccine. Here, we summarize the results of the RV genotype changes in Japan before and after vaccine introduction.

2. Material and Methods

Study sites and period

Five different medical facilities and one laboratory participated in this study (Figure 1). The stool samples were collected from inpatients in two hospitals in Tsu City, Mie Prefecture (National Mie Hospital, Mie Chuo Medical Center) from the 2007/08 to 2013/14 seasons. Samples were also collected from an outpatient clinic in Tsu City (Umemoto Children's Clinic); an outpatient clinic in Isumi City, Chiba Prefecture (Sotobo Children's Clinic); and from inpatients and outpatients treated in a hospital in Kurashiki City, Okayama Prefecture (Kawasaki Medical School Hospital) from the 2010/11 to 2013/14 seasons.

The annual number of births (2013) and population of children younger than 5 years (2010) in each city were 2,291 and 11,755 in Tsu City, 203 and 1,206 in Isumi City, and 4,561 and 22,058 in Kurashiki City, respectively (11).
Study design

We conducted a prospective observational study of RV genotypes using stool samples from children (<5 years old) with RV gastroenteritis who presented to a group of hospitals in Tsu City, Mie Prefecture, Japan, from the 2007/08 through the 2013/14 seasons (one season is defined as the period between November and October of the following year). Gradually, the study site was expanded to include clinics in Tsu City and hospitals and clinics located in two other areas, Okayama and Chiba, for the 2010/11 to 2013/14 seasons.

Natural defecate stool specimens were collected from children younger than 5 years old who had diarrhea (3 or more passages of watery diarrhea within the preceding 24 hours) or vomiting (once or more within 24 hours), had been given a diagnosis of acute gastroenteritis by physicians, and had positive results in the stool RV antigen rapid tests.

At the two hospitals in Tsu City, Umemoto Children’s Clinic and Sotobo Children's Clinic, stool samples were tested using a commercially available enzyme immunoassay kit (Rapid-testa; Sekisui Medical Co., Tokyo, Japan); its sensitivity and specificity is approximately 100% and 99.3%, respectively, when compared with that of PCR (data from package insert). At Kawasaki Medical School Hospital, samples were tested with a commercially available enzyme immunoassay kit (ImmunoCard ST; Fujirebio Inc.,
Tokyo, Japan); its sensitivity and specificity is approximately 93.1% and 95.8%, respectively, when compared with that of electron microscopy (data from package insert).

Stool samples were not taken if the parents did not sign the study consent form. In the outpatient setting, we asked the parents or caregivers to bring stool if their children had gastrointestinal symptoms, but we did not actively collect stool samples by using enema. Patients were excluded from the study if the stool sample volume was insufficient for G and P genotyping.

After the introduction of the RV vaccine in November 2011, we interviewed the subjects about their history of RV vaccination, including the types and dates of vaccination, to evaluate the possible influence of vaccine on the genotype distribution.

**G and P genotyping**

RV-positive stool samples were stored at -80°C on site and transported in a frozen state to the Department of Virology and Parasitology, Fujita Health University School of Medicine, for gene analysis.

The RV G and P types were determined using reverse transcription-polymerase chain reaction (RT-PCR) as described previously (12,13). First, phosphate-buffered saline
was added to the stool sample to generate a 10% stool suspension, and the supernatant was obtained by low-speed centrifugation. Thereafter, viruses were decomposed using destruction solution (sodium dodecyl sulfate, 2-mercaptoethanol, ethylenediaminetetraacetic acid), and ribonucleic acid (RNA) was extracted by treatment with chloroform and ethanol sedimentation (12). For G typing, the full-length VP7 gene was amplified using a pair of primers, 5´–GGCTTTAAAAGAGAGAAATTCTCTGG–3´ (T31) and 5´–GGTCACATCATCAATTCTAATCTAAG–3´ (T32), corresponding to the common 5´ end (strain Wa) and 3´ end (strain SA11) of the VP7 gene, respectively. In the second PCR amplification, the T32 primer was used along with G1, G2, G3, G4, G8, and G9 genotype-specific primers in order to identify the G types. For P typing, a pair of primers, 5´–TGGCTTCGTTATAGACA–3´ and 5´–CTAAATGCTTTGAATCATCCA–3´, corresponding to the common sequences of the VP4 gene, including nucleotides 11–32 and 1,072–1,094, respectively, was used for the first amplification; a mixture of primers specific to each of the variable regions: P[8], P[4], P[6], and P[9], along with a primer corresponding to nucleotides 11–32, was employed for the second amplification. The PCR products were separated by agarose gel electrophoresis, and the G and P types were confirmed based on the extent of
migration (12,13).

**Vaccine coverage rate**

Since the RV vaccine is not listed as a routine vaccine, we do not have a precise vaccine coverage rate for each study site. As a replacement, we used the vaccine shipment and recollected amount information for the study sites to estimate the vaccine coverage rate. According to this information, our study sites had estimated coverage rates of approximately 30% in the 2011/12 season, 46% in the 2012/13 season, and 55% in the 2013/14 season, except for in Isumi City, which has a policy to cover all costs of the RV vaccine to their residents since April 2013. Thus, its coverage rate is considered nearly 100% since then.

**Ethics Statement**

The study was approved in an ethical review conducted at National Mie Hospital and Kawasaki Medical School, and informed consent was obtained from the parents of all subjects before the study execution. The study, protocol, and management of personal information were in compliance with the "Ethical Guidelines for Clinical Research" of the Ministry of Health, Labour and Welfare, drafted based on the principles set forth in
3. Results

From the two hospitals in Tsu City, Mie Prefecture, a total of 258 acute gastroenteritis patients had positive results by RV antigen rapid tests between the 2007/08 and 2013/14 seasons (Figure 2A). Among these, 36 patients were excluded due to an insufficient stool sample volume. Thus, RV (G or P) genotyping was performed in 222 (86%) samples. For the other sites, 366 patients had positive test results for RV between the 2010-11 and 2013-14 seasons (Figure 2B), while 66 patients were excluded due to an insufficient stool sample volume. Thus, RV genotype testing was performed in 300 (82%) patients. The characteristics of the patients whose stool sample underwent RV genotype testing were collected. For both groups, the samples collected per year decreased after the introduction of the vaccine, whereas the median age and proportion of male patients did not change before and after the introduction of the RV vaccine (data not shown).

The distribution of genotype for each season and site are presented in Figure 3. G3P[8] was the dominant (61.0-77.1%) genotype before vaccine introduction among inpatients.
in Tsu City. However, G3P[8] drastically decreased after vaccine introduction, and G1P[8] became the dominant genotype (97.1%) in the 2012/13 season. In addition, in the 2013/14 season, G2P[4] emerged along with a decrease in the number of patients (Fig. 3A). For outpatient samples from Tsu City, G3P[8] and G1P[8] were the two major genotypes (46.7% and 41.7%, respectively) before vaccine introduction. However, G3P[8] decreased drastically in the 2012/13 season while G1P[8] increased (95.5%); in the 2013/14 season, G2P[4] emerged, which was similar to the findings in inpatients (Fig. 3B).

In Kurashiki City, G3P[8] was dominant (93.3%) among both outpatients and inpatients before vaccine introduction, but decreased drastically thereafter, whereas G1P[8] and G9P[8] increased in the first season after vaccine introduction, followed by the emergence of G2P[4] in the 2013/14 season (Fig. 3C).

Among outpatients in Isumi City, G1P[8] was dominant genotype (70.3%) before vaccine introduction. However, both G1P[8] and G3P[8] decreased after vaccine introduction, and G9P[8] became dominant (83.3%) (Fig. 3D).

Since the introduction of the RV vaccine in November 2011, eight out of 294 enrolled patients in the study had received an RV vaccine (Table). In the 2012/13 season, two
patients who received the RV1 and one patient who received the RV5 contracted RV gastroenteritis at 151, 193, and 85 days after the last dose, respectively all of which were G1P[8]. In the 2013/14 season, four patients who received the RV1 and one patient who received the RV5 contracted RV gastroenteritis at 158, 728, 756, 394, and 251 days after the last dose, respectively. The RV genotypes were G2P[4] in the patients who received the RV1 and G9P[8] in the patient who received the RV5.

4. Discussion

We reported the distribution of the RV genotypes before and after the introduction of the RV vaccine in Japan. Samples were collected from three different sites. The genotype distribution was similar among Tsu City and Kurashiki City, where G3P[8] was the dominant genotype before vaccine introduction, but was taken over by G1P[8] for two seasons, and then by G2P[4]. However, in Kurashiki City only, G9P[8] was observed after the introduction of vaccine. In several studies, the rapid spread and predominance of unusual DS-1-like G1P[8] rotavirus were reported during 2011-2013 in various locations across Japan. The increase in G1P[8] strains after vaccination in Tsu City and Kurashiki City may be related to increase in this unusual strain (14-16). On the other hand, the genotype distribution was quite different in Isumi City. There, G1P[8] was the
dominant genotype prior to the introduction of the vaccine, and G9P[8] became the major genotype in the 2013/14 season. Isumi City is the only area where the RV vaccine is recommended and funded by the city government. Thus, the coverage rate of the vaccine was close to 100% during this period, which was much higher than in the other sites. In addition, the RV5 was used at a much higher rate. Differences in the coverage rate and the kind of vaccine used may play major roles in the genotype distribution. One caveat, however, is that the major genotype in Isumi City differed from that in other sites before the introduction of the vaccine. Thus, we could not conclude that the change in the genotype distribution was caused by vaccine alone. For Tsu City, we were able to compare the genotypes between inpatients and outpatients. The trend was very similar among both groups, implying that the genotype is not related to the severity of the disease.

Marked changes in genotype prevalence following vaccine introduction have been observed in other countries. In Brazil, predominance of G2P[4] after RV1 introduction has been reported (17). In the USA, the prevalence of G3P[8] increased after RV5 introduction (18). In Australia, G2P[4] and G3P[8] increased distinctly in sites using RV1 and RV5 (19), respectively. However, these phenomena were temporary, and no
increase in severity has been reported. It is difficult to determine the cause of these changes, but monitoring of such changes is necessary to ensure the long-term safety of the RV vaccine.

In the present study, we found eight infants with a history of RV vaccination before they contracted RV gastroenteritis. The genotypes of RV of these patients were all G1P[8] in the 2012/13 season and G2P[4] for the majority of the patients in the 2013/14 season. Especially, for patients with the G2P[4] genotype, all patient received the RV1 prior to the onset of gastroenteritis. Some studies have implied that the selection pressure of the vaccine may have caused this phenomenon (18); however, in our results, the genotype of the vaccinated patients matched the dominant circulating genotype for each season. However, Korea where the RV5 and RV1 were approved in 2007 and 2008, respectively, experienced an outbreak of G2P[4] in 2013, six to seven years after the introduction of the RV vaccines. Forty-four patients infected with the G2P[4] strain, including nine and five patients with a history of RV1 vaccination and RV5 vaccination, respectively, were reported (20). Thus, continuous monitoring of genotypes from patients with and without vaccination is important.
In our study, all inpatients and outpatients with acute gastroenteritis were asked to bring a stool sample and be tested for RV infection. However, some patients with RV gastroenteritis do not visit hospitals because infection is not apparent or the symptoms are mild. Even if they visited clinics, it is difficult to collect stool samples at the time of an outpatient visit. Thus, especially in outpatients, the changes in the number of RV-positive cases are not likely to reflect the true picture of the epidemic. Furthermore, because our study sites were very limited, it is possible that other parts of Japan may have different distributions of the RV genotypes. Therefore, we may see different distributions of the RV genotype in Japan if we add more sites around the country.

Usually, when evaluating vaccine efficacy and changes in genotypes, the types of vaccine used and the vaccination rates in the population are considered essential information. Since the RV vaccine is still not recommended as a routine vaccine in Japan, the precise vaccination coverage rate and the type of vaccine used is unclear. We estimated the vaccine coverage rate of the studied areas based on the vaccine shipment and recollected vaccine information. This is our best estimate at the moment, and the vaccine coverage rate was estimated around 30-46%, except for in Isumi City (nearly 100%).
Nevertheless, despite the above-mentioned limitation, it is still safe to say that after the introduction of the vaccine, the circulating genotype of RV has changed. We may observe more definite changes once the vaccination coverage rises and we may see more cases with a vaccine history. Continuous monitoring of the RV genotype is hence of great importance.

In conclusion, this study presented the changes in RV genotypes distribution after RV vaccine introduction in Japan. However, it is difficult to evaluate whether these changes were due to the effect of vaccination or represented natural epidemiological changes. Long-term monitoring of the RV genotypes is necessary.

5. Acknowledgements

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novel influenza."

6. Conflict of interest

7. References


7. IDSC/NIID. The National Epidemiological Surveillance of Infectious Diseases in compliance with the enforcement of the new Infectious Diseases Control Law. IASR.


8. Figure legends

Fig. 1. Samples were collected from pediatric patients with rotavirus gastroenteritis who visited 6 institutions located in 3 areas of Japan.

Fig. 2. Flow diagram for the selection of patients for rotavirus (RV) genotyping.

Fig. 3. Distribution of the genotype for each season and site.

NOTE: n=the number of rotavirus (RV)-positive cases detected with the antigen rapid test / the number of RV-positive cases detected with polymerase chain reaction.
Table. RV gastroenteritis in patients with a history of RV vaccination

<table>
<thead>
<tr>
<th>No.</th>
<th>Season</th>
<th>Age (Months)</th>
<th>Sex</th>
<th>Address (City)</th>
<th>Hospitalization</th>
<th>Dehydration*</th>
<th>Type of vaccine</th>
<th>Time from the final vaccination to onset (Days)</th>
<th>RV genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2012/13</td>
<td>9</td>
<td>M</td>
<td>Tsu</td>
<td>Yes</td>
<td>No</td>
<td>RV1</td>
<td>151</td>
<td>G1P[8]</td>
</tr>
<tr>
<td>2</td>
<td>2012/13</td>
<td>10</td>
<td>F</td>
<td>Tsu</td>
<td>No</td>
<td>No</td>
<td>RV1</td>
<td>193</td>
<td>G1P[8]</td>
</tr>
<tr>
<td>3</td>
<td>2012/13</td>
<td>7</td>
<td>M</td>
<td>Isumi</td>
<td>No</td>
<td>NA</td>
<td>RV5</td>
<td>85</td>
<td>G1P[8]</td>
</tr>
<tr>
<td>4</td>
<td>2013/14</td>
<td>9</td>
<td>M</td>
<td>Tsu</td>
<td>No</td>
<td>No</td>
<td>RV1</td>
<td>158</td>
<td>G2P[4]</td>
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<tr>
<td>5</td>
<td>2013/14</td>
<td>28</td>
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<td>Tsu</td>
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<td>No</td>
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<td>756</td>
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<td>6</td>
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<td>7</td>
<td>2013/14</td>
<td>16</td>
<td>M</td>
<td>Kurashiki</td>
<td>Yes</td>
<td>No</td>
<td>RV1</td>
<td>394</td>
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</tr>
<tr>
<td>8</td>
<td>2013/14</td>
<td>12</td>
<td>F</td>
<td>Kurashiki</td>
<td>Yes</td>
<td>No</td>
<td>RV5</td>
<td>251</td>
<td>G9P[8]</td>
</tr>
</tbody>
</table>

NA: not available  RV1: monovalent rotavirus vaccine  RV5: pentavalent rotavirus vaccine

*Dehydration suggests loss of more than 5% of body weight
Gene analysis
Department of Virology and Parasitology,
Fujita Health University School of Medicine

Kurashiki City, OKAYAMA
Kawasaki Medical School Hospital
(2010/11-2013/14, inpatients and outpatients)

Isumi City, CHIBA
Sotobo Children's Clinic
(2010/11-2013/14, outpatients)

Tsu City, MIE
National Mie Hospital,
Mie Chuo Medical Center
(2007/08–2013/14, inpatients)
Umemoto Children's Clinic
(2010/11-2013/14, outpatients)
258 patients tested positive in stool RV antigen rapid tests (2007/08-2013/14)

- 2 hospitals in Tsu City (inpatients)

- 36 patients excluded from the study
  - Insufficient volume of stool sample

- 222 patients underwent RV genotyping

366 patients tested positive in stool RV antigen rapid tests (2010/11-2013/14)

- 1 clinic in Tsu City (inpatients)
- 1 hospital in Kurashiki City (outpatients, inpatients)
- 1 clinic in Isumi City (outpatients)

- 66 patients excluded from the study
  - Insufficient volume of stool sample

- 300 patients underwent RV genotyping
3A. Inpatients in Tsu City

3B. Outpatients in Tsu City

3C. Inpatients and outpatients in Kurashiki City

3D. Outpatients in Isumi City