Original Article

Age, Viral Copy Number, and Immunosuppressive Therapy Affect the Duration of Norovirus RNA Excretion in Inpatients Diagnosed with Norovirus Infection

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SUMMARY: Norovirus is one of the leading causes of acute gastroenteritis worldwide. Although it is becoming clear that viral excretion in the stool continues even after the clinical symptoms have disappeared, the factors that determine its duration remain unknown. Between 2007 and 2009, all inpatients and medical staff at our hospital who showed symptoms of a new onset of gastroenteritis were asked to submit a sample for norovirus testing by real-time RT-PCR. One of the 273 patients included tested positive for GI norovirus, and a further 89 were positive for GII norovirus. Of these 90 norovirus-positive individuals, 76% excreted norovirus RNA in the stool for more than 7 days. The inpatient group contained more long shedders than the medical staff group (5/32 versus 1/39, P < 0.05). The median viral shedding duration was 19.3 and 15.2 days for inpatients and medical staff, respectively. Among hospitalized patients, younger individuals, those with a higher viral copy number, and individuals receiving immunosuppressive therapy tended to require a longer time to eliminate the virus. These patients should therefore be monitored and managed carefully to prevent nosocomial spread of the disease.

INTRODUCTION

Norovirus, formerly known as Norwalk-like virus, is one of the most common causes of acute gastroenteritis worldwide. Noroviruses belong to the genus Norovirus of the Caliciviridae family and contain a genome of single-stranded, positively charged RNA approximately 7.5 kb in length (1-3). The virus comprises 5 distinct genogroups (GI to GV), with the genogroups GI, GII, and GIV being associated with gastroenteritis in humans. The most prevalent circulating strain belongs to genogroup GII (4). The onset of symptoms generally occurs 15-48 h after exposure, and the illness lasts for 12-60 h (5).

Norovirus is highly infectious. According to the report by Teunis et al. (6), a dose of only 10⁷ norovirus genomes can cause infection. Norovirus infection typically result upon the ingestion of contaminated water or food and can be transmitted person-to-person via the fecal-oral route, possibly by vomit via an airborne route, or indirectly via contaminated environmental surfaces (1,7,8). Norovirus outbreaks, which are difficult to control and present a major public health challenge, have been reported in various public places and semiclosed communities, including hospitals, nursing homes, long-term care facilities, schools, cruise ships, and hotels (9-14). In particular, norovirus infections in hospitals can be detrimental to frail elderly patients, young children, and immunocompromised hosts.

A significant body of studies has revealed that norovirus continues to be shed for a long period of time after the clinical symptoms have disappeared. Thus, Murata et al. studied 71 children infected with norovirus and found that the median duration of viral shedding was 16 days (5-47 days) (15). Furthermore, 3 infants aged less than 6 months continued to excrete the virus for an extremely long period (more than 42, 44, and 47 days, respectively, after onset). Likewise, Kirkwood and Streitberg monitored 15 children for up to 36 months and detected 8 norovirus infections (16). Viral shedding for more than 25 days was detected in 4 children. In a similar study, Tu et al. reported that the average duration of viral shedding was 28.7 days in elderly patients (17), and Siebenga et al. reported that 8.4% of hospitalized patients with norovirus infections showed prolonged viral shedding (21-182 days) (18). In an experimental model of human infection, the mean norovirus excretion was 28 days post-inoculation (19). However, despite increased knowledge of the duration of norovirus shedding, the factors that determine this duration remain to be elucidated.

In this study, we used real-time reverse transcription-PCR (RT-PCR) to monitor the excretion of norovirus by infected inpatients and medical staff in order to determine the factors affecting the duration of norovirus shedding.

MATERIALS AND METHODS

Norovirus monitoring: Fecal or vomit samples were collected from patients who showed new onset of acute gastroenteritis symptoms at the Sapporo Medical Univer-
sity Hospital between March 2007 and February 2009. If the results of these tests were positive for norovirus, additional samples were collected every 7 days until the results became negative or the patient was discharged from the hospital. Infected medical staff were placed on sick leave until they tested negative for norovirus. Duration of viral shedding refers to the number of days from the day the virus was detected until the day of the first negative test.

**Viral RNA preparation:** A 10% (wt/vol) fecal suspension was prepared with phosphate buffered saline and clarified by centrifugation. Vomit samples were used instead of fecal samples when the latter were not obtainable. Viral RNA was extracted from a 140-μl suspension using a QIAamp viral RNA kit (Qiagen, Tokyo, Japan) according to the manufacturer’s instructions. The RNA was eluted using 60 μl of AVE buffer (Qiagen) then treated with DNase. Viral RNA (24 μl) was added to a reaction mixture (6 μl) containing DNase I buffer and 3 U of DNase I (Invitrogen, Tokyo, Japan). The reaction mixture was then incubated at 37°C for 15 min to digest DNA. The enzyme was inactivated with 1 μl of 25 mM EDTA, incubated at 65°C for 10 min, then cooled immediately to 4°C.

**Real-time RT-PCR:** The real-time RT-PCR reaction was performed according to the method described by Kageyama et al. (20). Each test was run with serial 10-fold dilutions of norovirus GI and GII plasmids as controls to establish a calibration curve. In this assay, 10 copies of norovirus are equivalent to 2 × 10⁴ viral copies per gram of stool.

**Statistical analysis:** The data were analyzed statistically using Microsoft Excel®. Statistical significance (defined as P < 0.05) was evaluated using Student’s t test, and the association between age and number of days required to clear norovirus was evaluated using Pearson’s correlation coefficient.

**RESULTS**

**Patient characteristics:** Samples were obtained from 137 inpatients (72 male and 65 female) and 136 medical staff members (28 male and 108 female). A total of 50 inpatients (25 male and 25 female) and 40 medical staff members (9 male and 31 female) tested positive for norovirus. The age range of the norovirus-positive individuals was broad (1–88 years), and 89 (98.9%) of these 90 individuals tested positive for the GII genogroup. The mean duration of the symptoms was 4.2 ± 3.2 (range, 1–16) and 2.9 ± 1.3 days (range, 1–7 days) for inpatients and medical staff, respectively. As shown in Fig. 1, the viral loads of these patients at the first collection point were 2.0 × 10⁴–1.90 × 10⁹ and 2.0 × 10⁴–1.31 × 10⁸ cDNA copies per gram of stool for inpatients and medical staff, respectively.

**Duration of viral RNA shedding:** We were able to monitor the duration of viral shedding in 71 infected individuals, including 32 inpatients and 39 medical staff members. As shown in Fig. 2, the inpatient group contained more individuals who excreted norovirus RNA in stool for more than 28 days than the medical staff group (5/32 versus 1/39, P < 0.05). There was no statistical difference between the percentages for the other subgroups of inpatients and medical staff. Overall, 76.0% of individuals excreted norovirus RNA in stool for more than 7 days, and 38.0% of individuals tested positive for more than 14 days. The median period of viral shedding was 19.3 and 15.2 days for inpatients and medical staff, respectively.

**Age and viral RNA shedding:** To elucidate the factors that influence the duration of viral excretion, its association with age and viral copy number was studied. As shown in
Fig. 3A, younger infected inpatients required a longer period of time to eliminate the virus (correlation coefficient: $r = -0.504, n = 32, P < 0.01$), with 4 children under 15 years of age shedding the virus for a longer time (Fig. 3A upper panel; 22, 36, 48, and 53 days). There was no correlation between age and duration in infected medical staff (Fig. 3A lower panel).

**ABO blood types and viral RNA shedding:** It has been reported that norovirus utilizes carbohydrate H-1, which is expressed on the surface of epithelial cells, for attachment. The fact that antigens A and B of the ABO blood types mask H-1 should therefore reduce a host’s susceptibility to norovirus (21, 22). We evaluated the relationship between the ABO blood type and infection by determining the duration of viral RNA shedding in 7, 8, 11, and 2 inpatients with blood types A, B, O, and AB, respectively. The duration was also analyzed in 6, 3, and 5 medical staff members with blood types A, B, and O, respectively. As shown in Fig. 3B, there was no relationship between ABO blood type and duration of viral RNA shedding.

**Viral copy number and viral RNA shedding:** The viral copy number was higher in inpatients than in medical staff, and the clearance time was also longer in the former group (data not shown). However, as there was no statistical difference in this analysis, we subdivided these individuals further according to their viral copy number at initial testing. As shown in Table 1, the higher the viral copy number, the longer the time required for its clearance in inpatients. The viral copy number did not affect the duration of norovirus excretion in infected medical staff. As shown in Table 1, medical staff members with higher viral loads showed a longer viral RNA shedding duration than those with lower viral loads.

**Kinetics of viral RNA shedding:** We further analyzed the kinetics of viral excretion in inpatients and medical staff. As shown in Fig. 4B, the viral copy number was highest upon initial examination and decreased in a time-dependent manner for most medical staff. Indeed, on day 15, all medical staff showed less than 10⁵ cDNA viral copies per gram of stool (Fig. 4B). In contrast, 15.6% of inpatients showed more than 10⁵ cDNA viral copies per gram of stool on the same day (Fig. 4B, $P < 0.05$). In inpatients, 10 of the 12

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**Table 1. Norovirus copy numbers and duration of the RNA excretion**

<table>
<thead>
<tr>
<th>Inpatients</th>
<th>Viral copies</th>
<th>No. of individuals</th>
<th>Viral RNA shedding duration (range, day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>copies $&lt; 10^5$</td>
<td>7</td>
<td>10.6 ± 1.7**</td>
<td>(8-13)</td>
</tr>
<tr>
<td>$10^5 \leq$ copies $&lt; 10^6$</td>
<td>5</td>
<td>14.6 ± 3.2***</td>
<td>(12-19)</td>
</tr>
<tr>
<td>$10^6 \leq$ copies $&lt; 10^7$</td>
<td>10</td>
<td>20.9 ± 12.6**</td>
<td>(8-48)</td>
</tr>
<tr>
<td>$10^7 \leq$ copies</td>
<td>10</td>
<td>26.1 ± 14.5**</td>
<td>(8-53)</td>
</tr>
</tbody>
</table>

**Medical staff**

<table>
<thead>
<tr>
<th>Viral copies</th>
<th>No. of individuals</th>
<th>Viral RNA shedding duration (range, day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>copies $&lt; 10^5$</td>
<td>11</td>
<td>13.0 ± 10.0</td>
</tr>
<tr>
<td>$10^5 \leq$ copies $&lt; 10^6$</td>
<td>9</td>
<td>16.9 ± 5.1*</td>
</tr>
<tr>
<td>$10^6 \leq$ copies $&lt; 10^7$</td>
<td>11</td>
<td>16.3 ± 6.0*</td>
</tr>
<tr>
<td>$10^7 \leq$ copies</td>
<td>8</td>
<td>14.8 ± 4.0*</td>
</tr>
</tbody>
</table>

**Statistically not significant compared to the values of individuals with less than 10⁵ viral copies.**

**Statistically significant compared to the values of individuals with less than 10⁵ viral copies.**
individuals who excreted norovirus RNA in stool for 20 days or more were children or were receiving corticosteroids or immunosuppressive therapy.

**DISCUSSION**

In this study we compared the duration of norovirus RNA excretion in inpatients with that in medical staff and found that this duration is influenced by multiple factors, including age (Fig. 3A), viral copy number (Table 1), and use of immunosuppressive therapy (Fig. 4), especially in hospitalized patients. These factors did not affect the duration of viral excretion for medical staff. It has been reported that susceptibility/resistance to norovirus is multifactorial (21, 22). Indeed, this study revealed that both the susceptibility/resistance to norovirus infection and its duration are affected by multiple factors.

We reviewed the underlying disorders of 8 inpatients, other than children, who showed positive results for 20 days or more (Fig. 4). These disorders included tumors of the central nervous system, non-Hodgkin’s lymphoma, nephrotic syndrome, and polymyositis. Six of these patients were receiving corticosteroids or immunosuppressive therapy before or during the infection. Moreover, 3 of the 4 children in this study were receiving immunosuppressive treatment for a preexisting hematological disorder. These observations show that immunosuppressive therapy may delay recovery from norovirus infections. Acquired immunity may also play an important role in viral clearance. Thus, Siebenga et al. reported that 5 out of 8 norovirus-infected individuals who excreted norovirus for more than 3 weeks had impaired immunity (18). Likewise, Roddie et al. showed that norovirus RNA can be detected in stool for an extremely long time (0–27 months) in patients who have undergone allogeneic hematopoietic stem cell transplantation (23). It has been reported that sero-positivity against norovirus is lowest in the first year of life and increases thereafter (24–26). In this study, when monitoring the duration of viral shedding, we found that younger patients tended to require a longer period for complete clearance (Fig. 3A). As with influenza, the development of vaccinations as a result of epidemiologic surveillance and reformulations might be helpful to protect the community against norovirus infections (27,28).

A young age was previously reported to be associated with a longer duration of norovirus shedding (15,29). The results obtained in this study are consistent with previous reports that hospitalized children have a higher risk of excreting norovirus RNA in stool for a longer time than other infected individuals (Fig. 3A).

To the best of our knowledge, this is the first study to examine the relationship between ABO blood type and the duration of norovirus RNA excretion. We were able to obtain information regarding viral shedding duration and ABO blood type for 28 inpatients and 14 medical staff members, but found no correlation between them (Fig. 3B). The part of the norovirus capsid protein that binds to the ABH histo-blood group antigens on host cells shows high immune selection and evolves over time by antigenic drift, thereby enabling the virus to reinfect human populations (28). One limitation of RT-PCR method used in this study is that it cannot determine the genotypes of the norovirus; genogroups I and II contain more than 14 and 17 genotypes, respectively, and intragenotype variants have also been identified (30). It is therefore plausible that both host factors and virus-related variations affect the duration of viral excretion.

In the medical staff group, one individual shed norovirus RNA for 36 days (Fig. 4B). We found no particular preexisting disorder or immunosuppressive drug use in this individual. Full nucleotide sequencing of the viruses may therefore be required to determine why this individual showed longer viral RNA excretion than the others.

More than 70% of norovirus-infected subjects, including medical staff, excreted the virus for more than 7 days (Fig.
transmission of the disease.

beneficial for the management of health care-associated
indicating that weekly monitoring of viral excretion may be
these 2 periods. The absolute number of norovirus-infected
environmental cleaning and hygiene practices were used in
their stool samples tested negative for norovirus. The same
we were unable to control the epidemic. In contrast, in the
norovirus infection was either isolated or placed on sick
break at a long-term-care facility: the role of environmental surface
contamination. Infect. Control Hosp. Epidemiol., 26, 802

In the winter of 2007–2008, any individual diagnosed with
norovirus infection was either isolated or placed on sick
leave for a week. The results of this sub-study showed that
were unable to control the epidemic. In contrast, in the
winter of 2008–2009 we isolated infected individuals until
their stool samples tested negative for norovirus. The same
environmental cleaning and hygiene practices were used in
these 2 periods. The absolute number of norovirus-infected
individuals decreased from 67 to 23 in these 2 periods, thus
indicating that weekly monitoring of viral excretion may be
beneficial for the management of health care-associated
transmission of the disease.

Acknowledgments We thank Dr. Osamu Nishio (National Institute of Infectious Diseases) for providing us with positive controls for norovirus RT-PCR.

Conflict of interest None to declare.

REFERENCES