Journal of Microbiology, Immunology and Infection 55 (2022) 1188—1194

Available online at www.sciencedirect.com

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Original Article

Clinical and virological characteristics of viral shedding in children with norovirus gastroenteritis

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Received 19 January 2021; received in revised form 5 October 2021; accepted 20 October 2021
Available online 30 October 2021

KEYWORDS
Norovirus gastroenteritis;
Viral shedding;
Children

Abstract *Background:* The correlation between the clinical manifestations and fecal viral load of norovirus (NoV) infection remains unknown.
Methods: We established a SYBR® Green-based real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) method to quantify NoV and then sequenced its genomes from the feces of patients admitted at the Chang Gung Memorial Hospital from 2017 to 2018.
Results: NoV GII.4 Sydney ($n = 21$, 36.2%) and GII.P16-GII.2 ($n = 19$, 32.8%), the two predominant genotypes found among 58 isolates, were closely related to the Taiwan variant 2012a cluster in the VP1 region and genotypes of China strain. An increase in viral load could be observed on Day 3 following the onset of NoV infection. The viral load then declined rapidly from days 10—15 but remained high for >1 month in a severe combined immunodeficiency patient. Significantly longer shedding was found in patients with fever ($p = 0.03$) or infected by the GII.4 Sydney strain ($p < 0.01$).

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https://doi.org/10.1016/j.jmii.2021.10.006

Conclusion: The qRT-PCR-mediated method proposed in this work could quantify the viral load in patients with NoV infection. Significant viral shedding over a period of 2 weeks in children with acute gastroenteritis and >1 month in an immunodeficient patient was observed. Significantly longer shedding could be correlated with infection by the GII.4 Sydney strain and febrile patients.

Introduction

Following the introduction of rotavirus vaccines, norovirus (NoV) has become the leading pathogen of acute gastroenteritis (AGE) in children in the United States; NoV infection is correlated with costs of $273 million for treatment and $1 million for health care each year.[1] NoVs belong to the Caliciviridae family, which feature a genome comprising single-stranded RNA measuring approximately 7.5 kb in length and coding three DNA open reading frames, namely, ORF1, ORF2, and ORF3.[2,3] ORF1 encodes a polyprotein required for replication, such as RNA-dependent RNA polymerase (RdRp); ORF2 encodes the capsid protein (VP1); and ORF3 encodes the minor capsid protein (VP2).[4] NoV is highly infectious; in fact, exposure to 1000 NoV particles is sufficient to cause infection.[5] Rapid transmission of NoV via person-to-person contact renders a massive problem for infection control. The duration of NoV illness is typically 12—72 h, but the illness may be prolonged and severe among the very young and immunocompromised patients.[6] Patients infected by NoV should be managed carefully to avoid further spreading the disease. Previous studies showed that NoV shedding is influenced by multiple factors, including age and viral copy numbers.[7] Although real-time reverse transcriptase polymerase chain reaction (RT-PCR) is an effective tool to identify and monitor NoV transmission, previous RT-PCR techniques for NoV RNA could only yield qualitative results. Few studies have evaluated the correlation between the fecal viral load of NoV, NoV infection, and viral shedding. As a proxy measure for fecal viral load, RT-PCR and the threshold cycle (*CT*) may distinguish patients with asymptomatic viral shedding from those with significant clinical diseases[8] but cannot precisely determine the viral load and viral shedding in the clinical setting. A quantitative method is of great importance in the management of NoV infection in immunocompromised hosts, including transplant and cancer patients. Earlier studies indicated that infection by the NoV GII strain is associated with higher fecal viral loads in cancer patients following NoV gastroenteritis.[9] The recombinant NoV GII.P16-GII.2 strain was first detected in 2016, and the continuous increase in its occurrence since early 2017 warrants further investigation to assess NoV evolution and viral shedding in immunocompromised hosts.[10] In this study, we established a standardized method to quantify NoV in the feces of NoV-infected children and evaluated the association between viral shedding and the clinical presentation of these patients.

Methods

Study design and data collection

This study was approved by the Institutional Review Board of Chang Gung Memorial Hospital (CGMH103-5084A3 and CGMH104-9820A3). Fecal specimens were collected from all hospitalized pediatric patients with NoV gastroenteritis confirmed by RT-PCR in Chang Gung Memorial Hospital from January 2017 to December 2018. We also analyzed the clinical characteristics of the patients according to the VP1 gene genotypes described previously.[11,12] Fecal samples were collected from patients on days 2—3, 7—10, 10—17, and 17—24 after admission. Longer follow-up at 1 month intervals was performed if no shedding was noted. This study included an immunocompromised patient who had chronic diarrhea and later underwent bone marrow transplantation (BMT); the clinical symptoms of this patient were traced for 5 months.

Nucleic acid isolation and sequencing

Viral nucleic acid extraction from fecal samples was performed using a QIAamp Viral RNA Mini Kit (Qiagen) according to the manufacturer's recommendations. The concentration of viral nucleic acids was determined using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). First-strand cDNA synthesis and PCR were performed according to the manufacturer's recommendations (SuperScript III First-Strand Synthesis System, Invitrogen). The cDNA products were cloned into a plasmid (pCR-XL-TOPO vector, Invitrogen), and the recombinant plasmid was transferred into competent *Escherichia coli*. The plasmids were purified and assessed for the DNA insertion sequence by using an ABI 3730 DNA analyzer (Applied Biosystems). The reference sequences of all NoVs used for comparison were obtained from the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov).

Phylogenetic analysis

The nucleotide sequences of the virus were genotyped using the RIVM online NoV genotyping tool (RIVM, https://www.rivm.nl/mpf/typingtool/norovirus/) and aligned using the SnapGene software package (GSL Biotech). The obtained alignment was entered into MEGA X software to reconstruct the corresponding phylogenetic tree.[13] The

neighbor-joining algorithm was employed, and the Tamura three-parameter substitution model was adopted. The substitution rates among different sites were assumed to be uniform, and gaps within the alignment were completely deleted when calculating pairwise distances. The reliability of the interior branches of the phylogenetic tree was assessed using the bootstrap method with 1000 replications. Sequences of the NoV GII.P16-GII.2 and GII.4 Sydney strains obtained in this study were deposited at the GenBank sequence database under the accession numbers MH979229 and MH979230, respectively. The nucleotide sequences determined in this study were uploaded to GenBank under accession numbers KY421122, MG746025, MG745988, MG746043, MG746044, KY421121, MG745996, MG745987, MG746048, KY865307, KY771081, LC209434, LC209481, LC209459, LC209478, LC209479, LC228948, LC209434, LC209458, LC209476, LC209477, LC145808, KF712507, FJ514242, JQ622197, AB543808, JX989073, JX448566, LN854565, MG786781, KF306214, KC517378, KC517377, JQ798158, KY424340, KC013592, HM748973, KC960615, KF509946, JQ613552, HF952135, and LC145800.

Norovirus fecal viral load

Viral loads were determined according to the following copy number quantification methods devised in this study. The primer sequences were designed on the basis of the RdRp gene sequence, and the amplicon was cloned into the PCR-XL-TOPO plasmid.[14] The pRdRp plasmid was linearized using *Kpn*I prior to *in vitro* transcription (MEGAscript® T7 Kit). The transcript of the RdRp gene was reverse-transcribed into cDNA and dissolved in DNase-free distilled water. EndMemo number calculation (EndMemo, 2019) indicated that the cDNA quantity was equivalent to approximately 3.1 × 1012 copy numbers.[15] A standard measurement method was established using a standard curve to analyze the cDNA quantity. The standard curve was obtained using 10-fold serial dilutions of the cDNA (10−1−10−10 copies/ml; Fig. 1). The copy number of the RdRp gene and the *CT* value were determined by SYBR® Green-based real-time PCR. The coefficient of determination (*R2*) obtained from linear regression analysis was 0.99; thus, the copy number and *CT* value were strongly correlated.

Statistical analysis

Continuous clinical data were analyzed using the Wilcoxon test, and binary data were analyzed using the chi-squared test. All statistical analyses were performed using SAS software v. 9.4 (SAS Institute, Inc., Cary, NC, USA), and a *P*-value with $\alpha = 0.05$ was accepted as the threshold for statistical significance.

Results

Clinical characteristics and viral load detection

The clinical and laboratory manifestations of all 58 admitted patients with NoV infection admitted to Chang Gung Memorial Hospital between 2017 and 2018 were analyzed. The average age of the patients was 23.5 ± 17.5 months (mean ± standard deviation). The shedding periods ranged from 1 day to 19 days. The demographic data of the 58 confirmed NoV cases are shown in Table 1. The NoV GII.4 Sydney strain was the main variant determined from the outbreak in winter and spring. We analyzed the viral load of the identified strains according to the copy number quantification methods established above and found increases in viral load varying from Day 3 to Day 8; thus, the curve over this period showed rough plateau-like features without peaks (Fig. 2). After Day 8, the viral load gradually declined, and fecal viral shedding decreased to less than 5 copy numbers/g feces on Day 15. In terms of viral copy number following disease onset, the mean log value of viral loads in 1 g of feces on days 3, 4, 5, 6, 7, and 8 were 7.6, 5.4, 7.0, 6.9, 5.3, and 6.9, respectively. Cases in which shedding copy numbers were greater than 10^6 in 1 g of feces on days 3, 4, 5, 6, 7, and 8 respectively represented 75%, 62%, 100%, 50%, 60%, and 100% of the total number of cases. The detection rates based on copy number cut-off values of

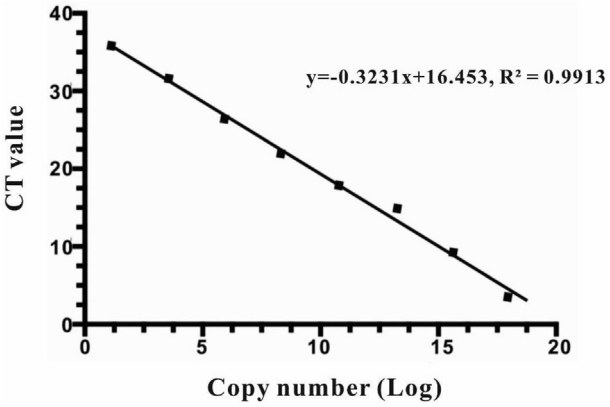

Figure. 1. Standard curve determined from the copy numbers of the defined cloned RdRp plasmid. The lines were obtained by the linear regression of points referring to the serial dilution of RdRp cDNA.

Table 1 Demographic data of the 58 confirmed norovirus (NoV) infection cases.

Patients' characters	Number (%) Age, severity (mean, interquartile range)
Age (months) (mean ± SD[a])	23.5 ± 17.5
Sex	Male, 35; female 23
Season	Spring 21 (36.2%), summer 5 (8.6%), autumn 15 (25.9%), winter 17 (29.3%)
Severity (median ± interquartile range)	9 ± 3[b]
Clinical setting	Sporadic 18, outbreak 40

[a] SD: standard deviation.
[b] Assessed by modified Vesikari Score.

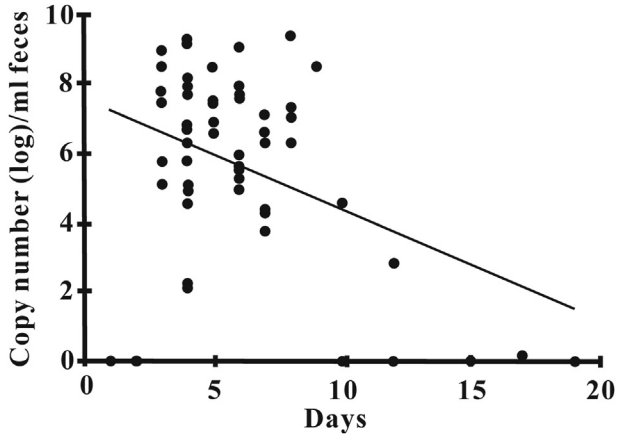

Figure. 2. Trend of viral shedding in children with norovirus (NoV) gastroenteritis as determined by real-time quantitative RT-PCR using SYBR® Green. Viral loads in the feces of the patients are indicated by solid circles. The trend line is generated by GraphPad Prism 6.0 (GraphPad Software, Inc.).

greater than 10^5 and 10^6 in 1 g of feces were 72% and 52%, respectively.

Fever was observed in 22 (38.5%) patients, including those infected by NoV GII.4 (15 in 35, 42.9%) and non-GII.4 (7 in 23, 30.4%) while without statistically significant difference of prevalence in the 2 groups. Febrile patients showed longer shedding periods (6.8 ± 3.1 days after disease onset) in comparison with 36 afebrile patients (5.7 ± 3.4 days after disease onset) ($p = 0.03$). A significantly longer shedding period was observed in patients infected by the NoV GII.4 Sydney strain (21 cases; 6.9 ± 3.1 days after disease onset) compared with those infected by non-GII.4 Sydney strains (37 cases; 5.7 ± 3.7 days after disease onset) ($p < 0.01$).

This study population included a 2-year-old severe combined immunodeficiency male child infected by NoV who presented with protracted diarrhea for over 3 months and received BMT thereafter. Prior to BMT, the viral load of the patient was as high as 1.4×10^{11} during hospitalization. We collected the feces of this patient at different time points and analyzed the viral load of the samples. Following BMT on Day 38 after admission, the viral load of the patient significantly decreased from 1.0×10^{10} to 5.9×10^{2}. The shedding trend and its association with BMT are shown in Fig. 3; interestingly, the patient demonstrated a shedding period of over 100 days (days 38–140) even after BMT. We tracked this patient, analyzed the NoV gene, and found that the NoV GII.P16-GII.2 new strain has the same gene sequence for 30 days. The gene sequence alignment of this strain differed from those of NoV GII.P16-GII2 strains identified in other countries and shared only 90%–98% identity. This finding indicates that further mutation occurs in immunodeficient patients.

Norovirus genotype analysis

The two predominant epidemic genotypes among the 58 NoV strains observed in this study were GII.4 Sydney 2012 (36.2%) and GII.P16-GII.2 (32.8%) (Table 2). Phylogenetic

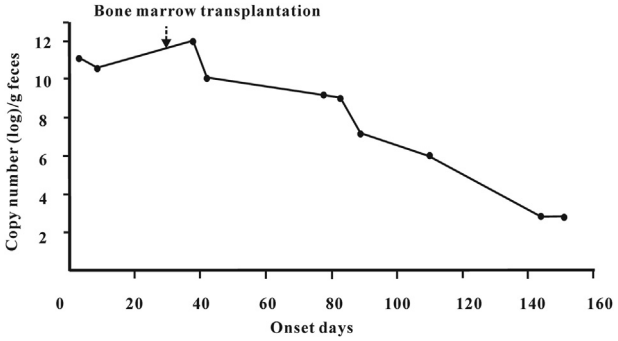

Figure. 3. Norovirus (NoV) viral loads before and after the bone marrow transplantation (BMT) of a patient with immune deficiency. The red line represents the curve of NoV viral shedding in feces. The dotted line shows the time of BMT after NoV infection.

analysis of the whole gene showed that GII.P16-GII.2 could be sub-divided into two clusters: GII.2a and GII.2b. The GII.2a cluster includes strains from Hong Kong, USA, China, and Taiwan. The Taiwan strains of GII.P16-GII.2 were detected in 2018. The other GII.P16-GII.2 strains were grouped into the GII.2b cluster, which was primarily detected in Japan in 2010 (Fig. 4A). Similarly, the GII.4 Sydney strains could be sub-divided into the GII.4a and GII.4b clusters. The GII.4a cluster includes Asian strains, such as those from Korea, Japan, Thailand, and Taiwan. The GII.4 Sydney 2012 Taiwan strain detected in 2018 belonged to the GII.4a cluster (Fig. 4B). The GII.4b cluster includes strains from the USA, Australia, Canada, and British. The GII.4 Sydney 2012 strain was the main genotype circulating in Taiwan.11 These results indicate that the NoV GII.P16-GII.2 and GII.4 Sydney 2012 strains are the major epidemic strains in Asia.

Discussion

This study analyzed 58 samples collected from patients with NoV-associated AGE in northern Taiwan in 2017–2018.

Table 2 Distribution of Norovirus genotypes and sub-genotypes identified from patients admitted to the hospital in 2017–2018 (n = 58).

Genotypes	n (%)
GII.4 Sydney	
GII.4 Sydney 2012	21 (36.2%)
Non-GII.4 Sydney	
GII.2	1 (1.7%)
GII.4	1 (1.7%)
GII.4 2006b	6 (10.3%)
GII.4 2009	1 (1.7%)
GII.4 2012b	6 (10.3%)
GII.6	1 (1.7%)
GII.14	1 (1.7%)
GII.17	1 (1.7%)
GII.P16-GII.2	19 (32.8%)

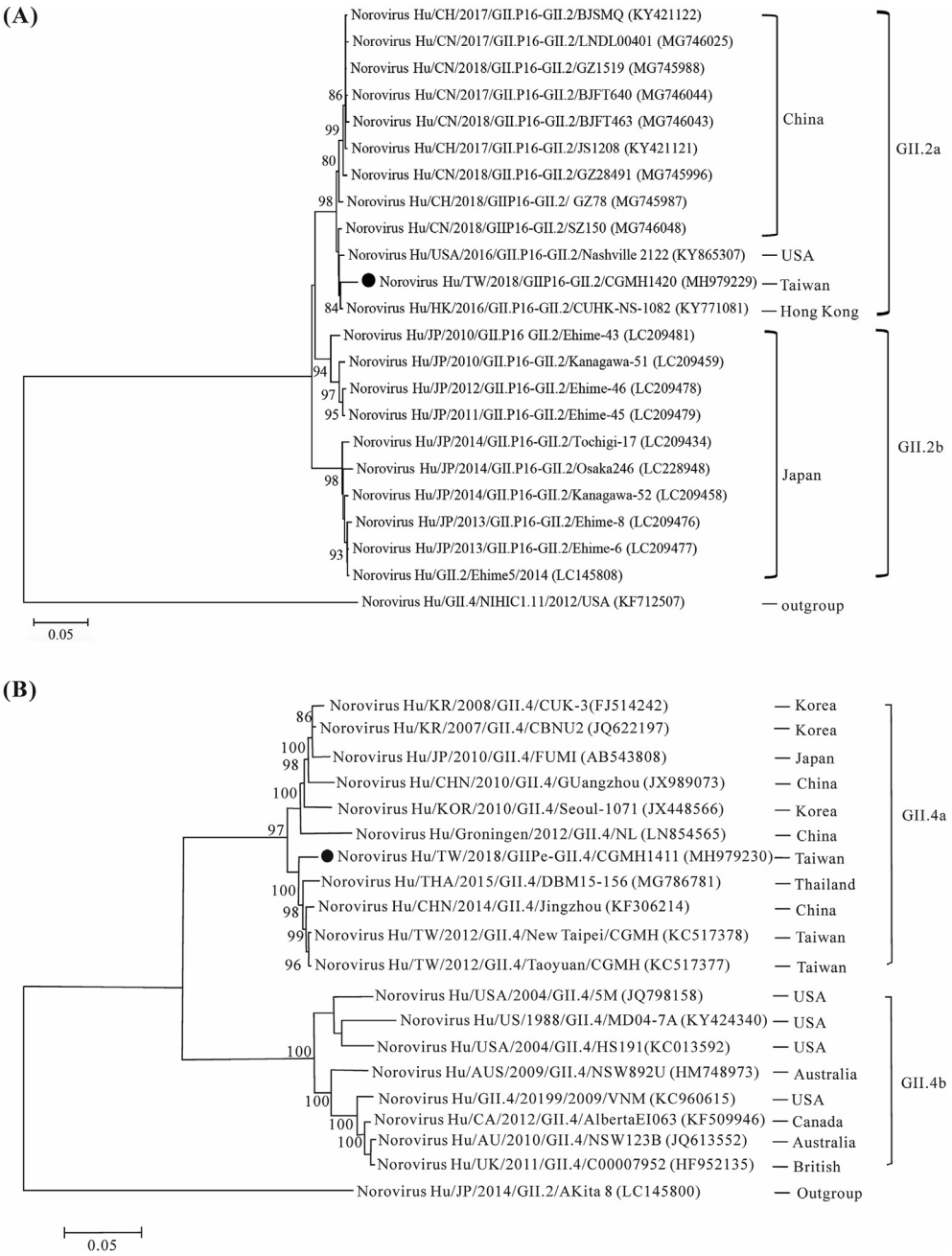

Figure. 4. Phylogenetic tree of norovirus (NoV) genotypes. Genotypes GII.2 (A) and GII.4 Sydney (B) were analyzed according to the full nucleotide sequence of the VP1 gene. The samples are marked with black circles. The scale bar indicates the number of mutations per genotype sequence position. The number at each node represents the percentage of 1000 bootstrap resamplings. Reference sequences were selected according to previous reports and are presented as accession numbers. The scale bar indicates the number of changes inferred as having occurred along each branch. Outgroup used for tests of phylogenetic signal and predictive modeling.

Two group genotypes, namely, GII. 4 Sydney and non-GII. 4 Sydney were also compared (Table 1). Globally, GII.4 could be identified in 67.2% of all NoV infections, but GII.2 was responsible for only approximately 1.2% of all NoV infections from 2004 to 2012.[16] The GII.P16-GII.2 strain was previously reported to be an emerging new recombinant NoV strain[17]; this strain was detected sporadically and became the predominant strain in late 2016.[18] This uncommon GII.P16-GII.2 variant provoked a prompt increase in sporadic AGE patients in Asia and Europe in the winter of 2016—2017; observations suggest that this variant could emerge as a widely distributed strain with the potential to provoke epidemic or pandemic conditions.[19] However, our results indicated that the NoV GII.4 Sydney strain was the major genotype while the GII.P16-GII.2 strain was the second predominant genotype in Taiwan in

2017—2018. Our phylogenetic tree analysis revealed that the GII.P16-GII.2 closely resembles the China clade (GII.2a) and is similar to the Japan clade (GII. 2b). This finding suggests that the Taiwan NoV GII. P16-GII.2 strain had genetically recombined with the China and Japan strains. Similar recombinations in polymerase genes between GII.P2 and GII.P16 strains have previously been reported.[20] The Taiwan GII.P16-GII.2 strain from 2018 represents a cluster that shares a close genetic distance (>99% similarity) with the China GII.P16-GII.2 strain, thus suggesting that these strains descended from a recent ancestor that may have been imported and spread within a short period of time.[21] The genotype of NoV GII.4 Sydney is identical to that of a strain that had been previously reported and has become the major endemic AGE pathogen in Taiwan in recent years.11 GII.4 Sydney was the predominant VP1 genotype spreading among children from 2012 to 2017 in Asia.[22,23] Differentiation of VP1 genes among the predominant genotypes determined by sequence-based typing methods may help improve the understanding on the epidemiology and evolution of NoVs.

This study is the first to report that febrile NoV patients could be correlated with longer shedding periods and higher viral loads. Viral infection with fever induces an immune response and inflammation processes that may increase the viral replication rate, resulting in a longer shedding time during the acute stage of infection.[24] We also found a longer shedding duration for the GII.4 Sydney strain compared with that for other NoV strains. In the last 10 years, new NoV GII.4 variants have emerged every 2 years; these variants may cause worldwide epidemics because new variants adapt to human populations or form as escape mutants from humoral and cellular immunity.[24—26] The longer shedding period of patients infected by the epidemic NoV GII.4 Sydney strain indicates that this strain has higher environmental transmissibility and may be a potential mutation indicator.[27]

The gene mutation of NoV may be a key component in prolonged viral shedding.[28] Patients infected by specific NoV strains with mutations at epitope A (279 nt) of the VP1 domain appeared to shed the virus for 1—2 months.[29] Mutations at epitopes A, C, D, and E of the P2 domain also allow the virus to shed from the feces for a longer time. The generation of such "escape mutants" is believed to be associated with host immunoselection.[30] Therefore, we collected the stools and traced the viral shedding of immunocompromised patients infected by the NoV GII.P16-GII.2 strain for a period of 30 days. We sequenced and aligned the genetic region of epitope A (279 nt) of the VP1 domain from patient DNA but found no A—G mutants in this region. In addition, BMT appeared to reduce NoV shedding. NoV has been reported to induce a vigorous T cell response to suppress the virus in children below the age of five.[30] However, NoV invasion could not be inhibited in the case of immunocompromised patients, which indicates that immunity was enhanced following BMT to inhibit the growth of NoV. This finding supports the belief that a poor immune system could result in longer viral shedding periods.[31,32]

In conclusion, we developed a sensitive SYBR® Green-based real-time qRT-PCR method to measure the NoV viral load in the feces of patients with NoV AGE. This method showed a sensitivity of as low as 50 RNA copies/ml, similar to that reported in previous studies.[33] Using this method, we found that febrile children and NoV GII. 4 Sydney strain could be associated with longer viral shedding. While some limitations, such as a small number of patients, inconsistencies in the timeline for sample collection, and incomplete clinical data, are present in our study, we found that significant viral shedding may last as long as 2 weeks. To the best of our knowledge, this study is the first to report a technique to monitor the prolonged NoV shedding time of immunocompromised patients. This improved method represents a tool that could evaluate outbreaks, monitor viral transmission, and help control NoV infection.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

Acknowledgements

We thank all the patients who participated in this study. This work was supported by grants from Chang Gung Memorial Hospital (CRRPG3F0084 and XMRRPG3H0991) and Ministry of Science and Technology (MOST 106-2314-B-038-097).

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