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Viral shedding in gastroenteritis in children caused by variants and novel recombinant norovirus infections

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Abstract

Human norovirus (NoV) is the leading cause of acute gastroenteritis and the rapid transmission of NoV renders infection control problematic. Our study aimed to investigate viral shedding in gastroenteritis in children caused by variants of emerging norovirus strains infections.

We used RNA-dependent RNA polymerase (RdRp) sequencing to measure NoV genome copies in stool to understand the relationship between the clinical manifestations and viral shedding in hospitalized patients. The near full-length NoV genome sequence was amplified via reverse transcription-polymerase chain reaction (RT-PCR) and NoV recombination was analyzed using the Recombination Analysis Tool (RAT).

From January 2015 to March 2018, 77 fecal specimens were collected from hospitalized pediatric patients with confirmed NoV gastroenteritis. The NoV genotypes were GII.4 (n = 22), non-GII.4 (n = 14), GII.4 Sydney (n = 21), and GII.P16–GII.2 (n = 20). Viral load increased from days 2 to 9 from the illness onset, resulting in an irregular plateau without peaks. After day 9, the viral load declined gradually and most viral shedding in feces ceased by day 15. The average viral load was highest in GII.4 Sydney followed by GII.P16–GII.2 infections and lowest in non-GII.4 infections. GII.4 unclassified infections showed the longest viral shedding time, followed by GII.4 Sydney infections, GII.P16–GII.2 recombinant infection resulted in the shortest duration. NoVs evolved to form a group of GII.P16–GII.2 variants during the 2017 to 2018 period.

The viral load and shedding period and was different in variants of NoV infections in children. High mutation rate of emerging and re-emerging variants was observed to an enhanced epidemic risk rendering continuous surveillance.

Abbreviations: AGE = acute gastroenteritis, NCBI = National Center for Biotechnology Information, NoV = norovirus, ORFs = open reading frames, RAT = Recombination Analysis Tool, RdRp = RNA-dependent RNA polymerase, RT-PCR = reverse transcription-polymerase chain reaction.

Keywords: acute gastroenteritis, children, norovirus infections, viral shedding

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H-YC and C-CL contributed equally to this work.

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The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request. All data generated or analyzed during this study are included in this published article [and its supplementary information files]. The datasets generated during and/or analyzed during the current study are publicly available.

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1. Introduction

Norovirus (NoV) is the major agent of infectious gastroenteritis and an important cause of epidemics. The rapid person-to-person transmission of NoV renders infection control problematic. NoV shedding is influenced by multiple factors, including age and viral copy number. Although real-time reverse transcriptase-polymerase chain reaction (RT-PCR) enables the identification of NoV and monitoring of its transmission, quantitative RT-PCR assay for NoV RNA is limited. The NoV genome contains 3 open reading frames (ORFs): ORF1 to ORF3. ORF1 encodes nonstructural proteins, and ORF2 and ORF3 encode capsid proteins VP1 and VP2, respectively.[1] NoV is a diverse virus that can be genetically classified into 10 genogroups (GI–GX), but only genogroups GI, GII, GIV, GVIII, and GIX are associated with human disease, with the GII genogroup being the most prevalent.[2] Each genogroup can be subdivided into multiple genotypes based on the sequence diversity of their VP1 proteins.[3] With recombination frequently occurring in the ORF1 and ORF2, genotyping of both RNA-dependent RNA polymerase (RdRp) in ORF1 and VP1 in ORF2 is necessary to establish a recombinant identity for the virus.[4]

NoV infects at a very low viral concentration and this can lead to outbreaks affecting many patients.[5,6] Most are caused by contact contamination with feces and vomitus, although there are also outbreaks of foodborne diseases.[7] Acute gastroenteritis occurs approximately 24 to 48 hours after NoV infection. The main symptoms are vomiting, diarrhea, abdominal pain, and nausea. In Taiwan, GII.4 Sydney strains and Novel GII.17 NoV variants have been reported to cause acute gastroenteritis (AGE) outbreaks in children in 2014 and 2015 individually.[8–10] Since 2016, the uncommon NoV GII.P16–GII.2 caused AGE epidemic in Taiwan and China.[11,12]

In this study, we investigated variants of NoV infection and to evaluate the clinical impact of NoV shedding in the feces, including that of emerging genetics recombinant strains of NoV GII.P16–GII.2.

2. Materials and methods

2.1. Patients and specimens

Fecal specimens were collected from paediatric patients with NoV gastroenteritis who were admitted to Chang Gung Children's Hospital (CGCH), a pediatric referal center in northern Taiwan. Informed consent was obtained from a legal guardian of each subject involved in the study. This study was approved by the Institutional Review Board of Chang Gung Memorial Hospital (CGMH103-5084A3 and CGMH104-9820A3). We also analyzed the clinical characteristics of these patients. All methods were carried out in accordance with relevant guidelines and regulations.

2.2. Nucleic acid isolation and genotyping

Viral nucleic acid was extracted from fecal samples 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). cDNA synthesis and PCR were performed according to the manufacturer's recommendations (SuperScript III First-Strand Synthesis System; Invitrogen). The PCR primers and conditions used to determine norovirus genotypes were described previously.[13] NoV sequences were identified using the norovirus typing tool website (RIVM) and uploaded to the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov).

2.3. Analytical sensitivity of viral shedding

Primer sequences were designed for NoV RdRp gene sequencing. The reactions used 10-fold serial dilutions of norovirus GII.4 DNA as positive controls at starting concentrations of 10^8 DNA copies/mL. To evaluate the amplification efficiency of the real-time RT-PCR assays, standard curves were generated for NoV GII.4 DNA copy numbers (10^7, 10^6, 10^5, 10^4, and 10^3 DNA copies/mL) versus Cq values were generated.[14] The coefficient of determination (R^2) in the linear regression analysis was 0.99, indicating a strong correlation between the copy number and the C_q value. The viral shedding period was the interval from the peak viral load to the virus becoming undetectable. The Fisher exact test was used to evaluate the significance of differences in clinical features. The significance of a difference between 2 independent samples was analyzed using the nonparametric Mann–Whitney U test.

2.4. Phylogenetic and recombination analysis

The near full-length NoV genome sequence was amplified via RT-PCR using primers and conditions described previously.[15] The NoV sequences were aligned using Clustal W with the default parameters, and a phylogenetic tree was constructed with MEGA-X software using the neighbor-joining method.[16] The evolutionary distances were computed using the p-distance method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was shown. NoV recombination was analyzed using the Recombination Analysis Tool (RAT).[17]

3. Results

3.1. Clinical samples and viral load

From January 2015 to March 2018, 77 fecal specimens were collected from hospitalized pediatric patients (age, 1 month to 5 years) with confirmed NoV gastroenteritis. The samples were collected from day 1 to day 20 following symptoms onset. To analyze the viral load growth curve, we divided the samples into 4 genogroups using the norovirus typing tool: GII.4 (n = 22), non-GII.4 (n = 14), GII.4 Sydney (n = 21), and GII.P16–GII.2 (n = 20) strains. Plotting the viral load and shedding time, to set up the curve, it shows that viral load increased from day 2 to 9, resulting in an irregular plateau with no peaks. After day 9, the viral load declined gradually, and most viral shedding in feces had ceased by day 15 (Fig. 1).

Clinically, AGE in children is caused by different genotypes and variants. GII.4 Sydney caused the longest duration of diarrhea (6.32 ± 5.48 days) (GII.4 Sydney vs non-GII.4 Sydney, $P = .001$), followed by non-GII.4 infections, while GII.4 unclassified and GII.P16–GII.2 infections NoV genotypes infections, but was less common in GII.P16–GII.2 infections (13 of 20, 65%). Fever was very common, with the highest frequency in non-GII.4 and GII.P16–GII.2 infections, while it was less common in GII.4 Sydney infections (Table 1). The average viral load was highest in GII.4 Sydney infections (GII.4 Sydney vs non-

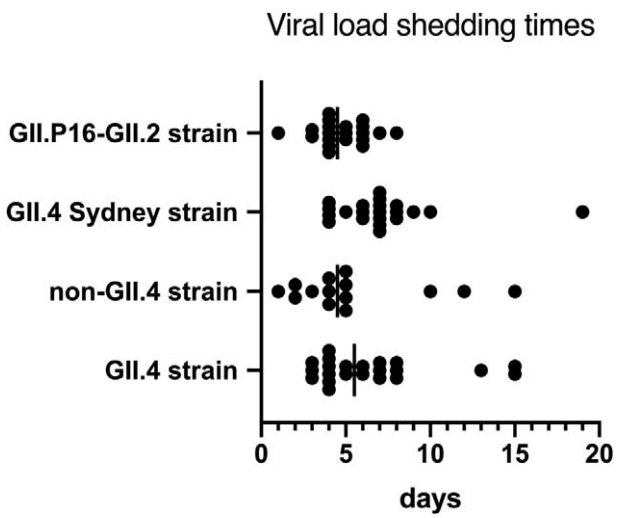


Figure 1. Norovirus viral load in fecal samples in children with NoV gastroenteritis was determined by real-time quantitative RT-PCR by using SYBR green. The viral load is shown by solid markers.

GII.4 Sydney, $P=.030$), followed by GII.P16–GII.2 infections, and was lowest in GII.4 unclassified and non-GII.4 infections. The longest viral shedding time was found in the GII.4 unclassified group (GII.4 unclassified vs other strains, $P=.015$), followed by GII.4 Sydney infections, while the shortest was in GII.P16–GII.2 infection patients. The GII.4 Sydney 2012 (n=21, 27.2%) and GII.P16–GII.2 (n=20, 26.0%) strains were the predominant epidemic genotypes among the 77 NoV strains analyzed. There were 7 outbreaks of GII.P16–GII.2 recombinant strains in late 2017 and early 2018; their clinical presentations are listed in Table 2. Their major manifestations were vomiting and diarrhea, similar to those of AGE caused by other NoVs.

3.2. Circulating NoV GII.P16–GII.2 recombinant variants

Seven NoV outbreaks (CGMH-1406, -1407, -1413, -1418, -1419, -1420, and -1422) involved recombinant GII.P16–GII.2 NoV strains. CGMH-1406 and -1420 had identical nucleotide sequences, as did CGMH-1407, -1413, -1418, and -1419. All confirmed NoV sequences were deposited in NCBI GenBank (accession numbers: MK898953 for CGMH1407, MH979229 for CGMH1420, and MK864096 for CGMH1422).

Phylogenetic analysis of GII NoV sequences showed that the 7 GII.P16–GII.2 NoV sequences isolated in 2017 to 2018 were evolutionarily close to those found in Changhua and Hsinchu in 2016 (i.e., KY457733 and KY457736), and in China in 2017 (i.e., MG746043; Fig. 2A and B). When the VP1 amino-acid sequences of GII.P16–GII.2 NoVs were analyzed, those isolated in 2017 to 2018 were grouped in a separate branch (Fig. 2C). Forty-one variants (41/452, 7.6%) of the VP1 amino-acid sequence were found, 13 (13/41, 31.7%) of which were unique to 2017 to 2018 CGMH variant NoV sequences (Fig. 3A and B). In addition, 12 and 10 (12/13, 92.3%; 10/13, 76.9%) variants were found in the P domain and P2 subdomain, respectively.

4. Discussion

Noroviruses are a major cause of outbreaks of AGE worldwide. Norovirus has more than 20 genotypes, which belong to the GI and GII genogroups. NoV has high gene diversity in the host,

Table 1

Clinical characteristics of children with norovirus infection with different genotypes.

Clinical presentations	GII.4 unclassified (N=22)	Non-GII.4 (N=14)	GII.4 Sydney 2012 (N=21)	GII.P16–GII.2 (N=20)	P
Diarrhea duration (IQR), d	1.8 (0.5–3.5)	3.4 (1.2–5.8)	6.3 (0.8–10)	1.9 (0.4–3.5)	.001*
Vomiting, N (%)	17 (77)	11 (78)	17 (80)	13 (65)	.825†
Fever N (%)	14 (64)	11 (79)	8 (38)	15 (75)	.320‡
Average viral load, genome (copies/mL) (IQR)	6.02 (2.8–9.2)	5.7 (1.4–10.0)	7.25 (4.6–9.8)	6.8 (5.2–8.5)	.030§
Viral shedding time (IQR), d	7.7 (1.2–15)	5.5 (1.5–9.6)	6.6 (3.1–10)	(3.2–6.3)	.015‖

IQR = interquartile range.
* Statistical results of comparing GII.4 Sydney 2012 norovirus infection to other infections.
† Statistical results of comparing GII.4 Sydney 2012 norovirus infection to other infections.
‡ Statistical results of non-GII.4 norovirus infection to other infections.
§ Statistical results of comparing GII.4 Sydney 2012 norovirus infection to other infections.
‖ Statistical results of comparing GII.4 (unclassified group) norovirus infection to other infections.

Table 2

Clinical findings in 7 children with acute gastroenteritis caused by norovirus recombinant strain GII.16–GII.2.

Age in years (Y) and months (M), and gender	Symptoms	Clinical findings	Outbreak time	Norovirus strain
5Y7M, F	Vomiting and diarrhea, mild fever	Marked leukocytosis	Aug. 2017	GII.p16–GII.2
9M, M	Vomiting and diarrhea	Skin rash	Nov. 2017	GII.p16–GII.2
4Y5M, F	Vomiting	N	Nov. 2017	GII.p16–GII.2
2Y, M	Diarrhea, mild fever	CVID, protracted diarrhea	Nov. 2017	GII.p16–GII.2
15Y, F	Diarrhea, high fever*	Low abdominal pain, marked leukocytosis	Dec. 2017	GII.p16–GII.2
8Y1M, M	Vomiting and diarrhea	Blood in stool	Feb. 2018	GII.p16–GII.2
8Y, M	Vomiting and diarrhea, high fever	None	Feb. 2018	GII.p16–GII.2

CVID = common variable immunodeficiency disorder, F = female, M = male.
* High fever: body temperature higher than 39°C.

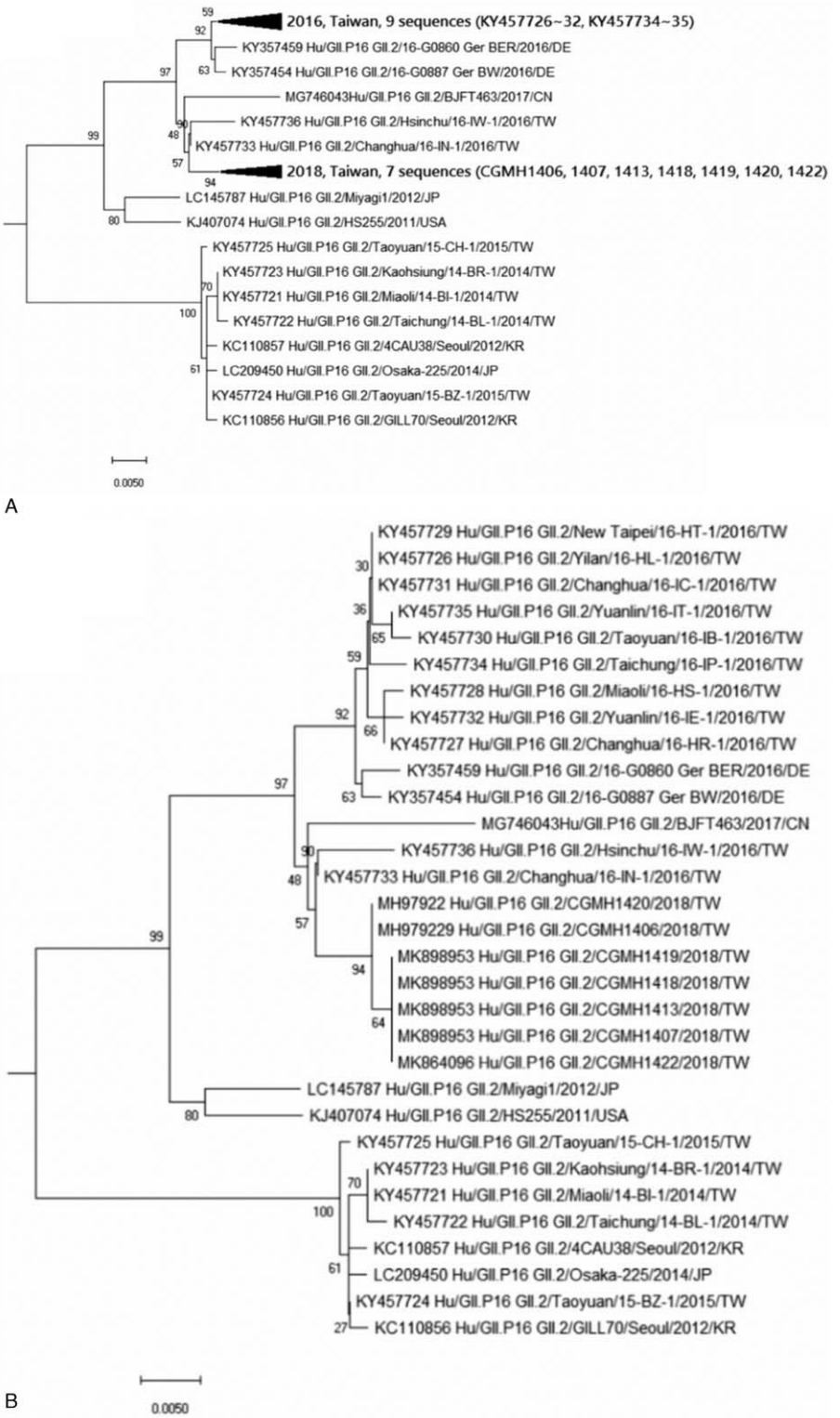


Figure 2. Phylogeny analysis of human GII.P16–GII.2 VP1. (A) The compressed region of GII.P16–GII.2 and GII.P4_GII.4 have 31 and 7 norovirus sequences within it, respectively. (B) The compressed tree region of GII.P16–GII.2. This region contains 31 GII.P16–GII.2 norovirus sequences. (C) Totally 24 GII.P16–GII.2 VP1 amino-acid sequences were selected for the construction of phylogenetic tree using neighbor-joining method. The evolutionary distances were computed using the *p*-distance method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. All GII.P16–GII.2 human noroviruses found in CGMH in 2018 were located in same lineage.

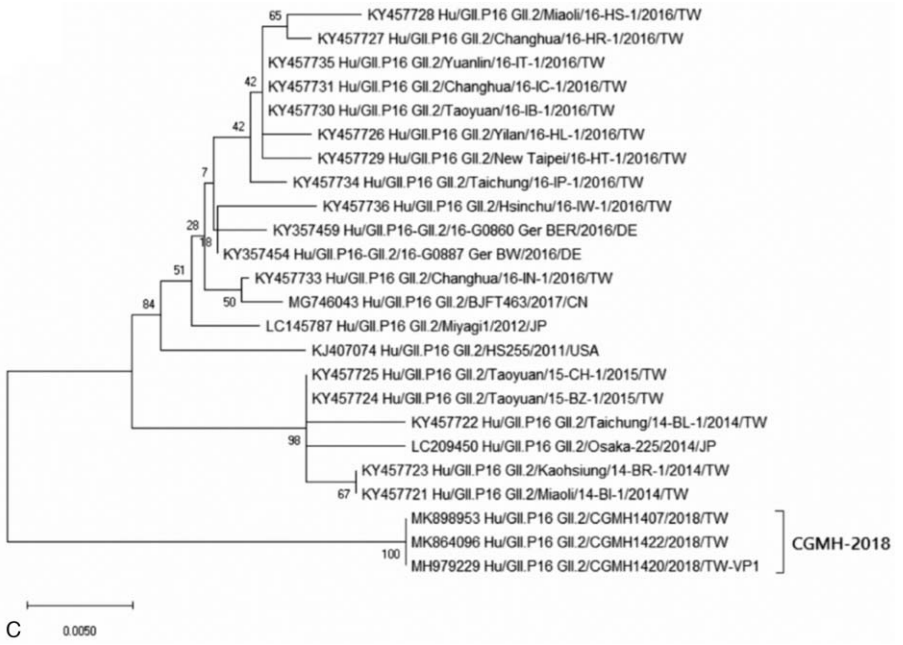
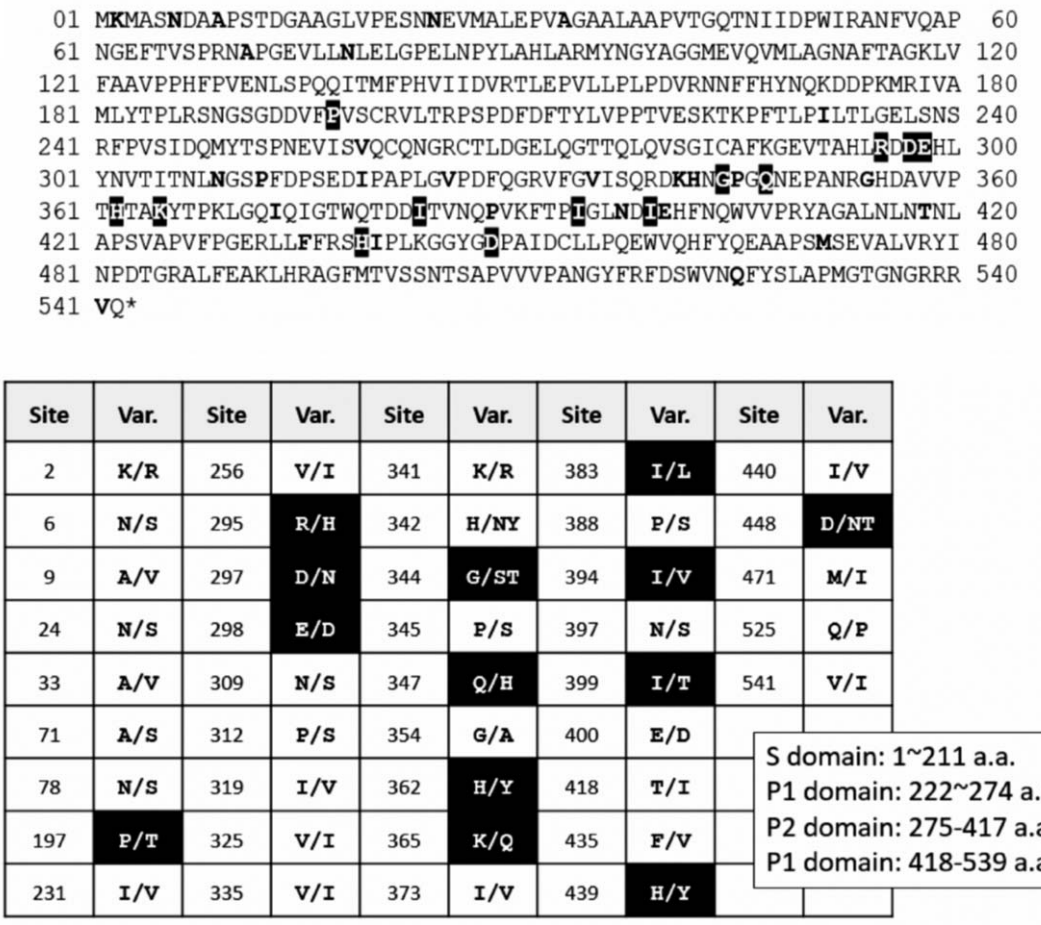


Figure 2. (Continued).



Site	Var.	Site	Var.	Site	Var.	Site	Var.	Site	Var.
2	K/R	256	V/I	341	K/R	383	I/L	440	I/V
6	N/S	295	R/H	342	H/NY	388	P/S	448	D/NT
9	A/V	297	D/N	344	G/ST	394	I/V	471	M/I
24	N/S	298	E/D	345	P/S	397	N/S	525	Q/P
33	A/V	309	N/S	347	Q/H	399	I/T	541	V/I
71	A/S	312	P/S	354	G/A	400	E/D		
78	N/S	319	I/V	362	H/Y	418	T/I		
197	P/T	325	V/I	365	K/Q	435	F/V		
231	I/V	335	V/I	373	I/V	439	H/Y		

Figure 3. The VP1 amino-acid sequence of 2017–2018 CGMH variants. (A) VP1 amino acids are varied in 41 sites (all bold and shade characters), among them 13 site (shade characters) are unique CGMH-isolated norovirus sequences (reference sequence is CGMH 1407). (B) Norovirus VP1 can be subdivided into 2 domains, the one is S domain (amino acid 12–288 in VP1) the other is P domain (amino acid 305–540 in VP1). Reference sequence is CGMH 1407 (MK898953).

caused by the recombination with variant genotypes, which affects the RNA virus evolution, epidemiological surveillance, and vaccine development.[18] RT-PCR sequencing and real-time PCR are basic tools for detecting and typing norovirus. The C_q values are calculated to evaluate the viral shedding time of NoV infection in AGE patients.[19] This is the first report of the NoV genome copies in stool and first analysis of the relationship between NoV genotype and viral shedding in hospitalized patients.

NoV infection can induce an immune response and inflammation and may drive viral replication, prolonging shedding during acute infection.[20] The GII.4 strain had a longer shedding duration than the other NoV strains we studied. A longer shedding period could be associated with gene mutations or recombination.[20,21] Hospitalized patients infected with NoV strains with mutations in the VP1 domain were observed to shed the virus for up to 2 months.[22] Mutations in P2 of the VP1 domain are also associated with prolonged shedding. But, the shedding time of NoV GII.P16–GII.2 strain is only 4 to 5 days and the sequence of the P2 domain is different from that of GII.4 stains. In our study, the P domain of NoV GII.4 Sydney is the same strain as previous reported by amino acid alignment. We speculate that protein structure of NoV variant strains may affect the shedding time.[23]

Clinical manifestations in our study showed the major manifestations were similar to those of AGE caused by other NoVs. Fever was uncommonly found, with the highest frequency in non-GII.4 and GII.P16–GII.2 infections. There was significantly longer diarrhea duration in GII.4 Sydney 2012 infected patients than others. Our results suggest that the 7 CGMH NoVs isolated in 2017 to 2018 evolved from 2 GII.P16–GII.2 NoV strains – 16-IN-1 and 16-IW-1 – identified in Taiwan in 2016.[11] There was no evidence that either was generated by recent recombination events. However, NoVs evolved to form a group of GII.P16–GII.2 variants during the 2017 to 2018 period (Fig. 3). This suggests that amino-acid sequence variants in the VP1 domain or P2 subdomain can arise due to selection by antibodies against previously circulating NoVs. In addition, a high frequency of missense mutations in the VP1 P2 domain may pose a threat to public health. Because the phylogenetic analysis was based on only approximately 3000 nucleotides, we were unable to confirm that new recombination had occurred. An analysis of the full genome is necessary to assess NoV evolution and recombination.

In conclusion, we used the real-time RT-PCR to quantify the genome copies of the NoV load in patients with acute gastroenteritis. The viral shedding time was 5 to 8 days in different strains after the illness started. Novel NoV GII.P16–GII.2 variants are circulating in Taiwan and causing AGE. A new recombination event was identified and explained the mutant of circulating NoV affect the shedding time by viral load calculation. The high mutation rate observed may be related to an enhanced epidemic risk, and so continuous surveillance is necessary.

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