



Research Article

Epidemiology and genetic diversity of norovirus GII genogroups among children in Hubei, China, 2017–2019

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ABSTRACT

Norovirus (NoV) is an important cause of viral acute gastroenteritis (AGE). To gain insights into the epidemiological characteristics and genetic diversity of NoV among children in Hubei, 1216 stool samples from children (≤ 5 years) obtained under AGE surveillance from January 2017 to December 2019 were analyzed. The results showed that NoV was responsible for 14.64% of AGE cases, with the highest detection rate in children aged 7–12 months (19.76%). Statistically significant differences were found between male and female infection rates ($\chi^2 = 8.108$, $P = 0.004$). Genetic analysis of *RdRp* and *VP1* sequences showed that NoV GII genotypes were GII.4 Sydney [P31] (34.35%), GII.3 [P12] (25.95%), GII.2 [P16] (22.90%), GII.4 Sydney [P16] (12.98%), GII.17 [P17] (2.29%), GII.6 [P7] and GII.3 [P16] (each at 0.76%). GII.17 [P17] variants were divided into the Kawasaki323-like lineage and the Kawasaki308-like lineage. A unique recombination event was detected between strains of GII.4 Sydney 2012 and GII.4 Sydney 2016. Significantly, all GII.P16 sequences associated with GII.4/GII.2 obtained in Hubei were correlated with novel GII.2 [P16] variants that re-emerged in Germany in 2016. Antigenic site analysis of complete *VP1* sequences from all GII.4 variants from Hubei identified notable variable residues of antibody epitopes. Genotyping under continuous AGE surveillance and observation of the antigenic sites of *VP1* are important monitoring strategies for emerging NoV strains.

1. Introduction

Acute diarrhea is a major global public health problem caused by bacteria, viruses, or parasites. Diarrheal diseases are one of the leading causes of illness and death in children under five years of age, accounting for more than 500,000 deaths worldwide each year (Collaborators, 2017). Norovirus (NoV) is an important cause of pediatric acute gastroenteritis, community-acquired diarrhea, diarrheal outbreaks, and food-borne diseases globally (Mans, 2019). It is an unenveloped single-stranded plus-stranded RNA virus, belongs to the family *Caliciviridae*, with a genome of 7.5–7.7 kb containing three open reading frames (ORFs) encoding structural and non-structural proteins (Xi et al., 1990). ORF1 encodes an NTPase, protease, and RNA-dependent RNA polymerase (RdRp). ORF2 and ORF3 encode the major capsid protein VP1 and the minor capsid protein VP2, respectively (Kobayashi et al., 2016). VP1 has domains known as shell (S), which surrounds the viral RNA and protruding (P). In the P domain, the P2 sub-domain is highly variable and contains major neutralization epitopes associated with

histo-blood group antigens (HBGAs) (Lochridge et al., 2005; Cao et al., 2007; Chen et al., 2006).

Noroviruses are genetically diverse and can be phylogenetically segregated into various genogroups (G) and P-groups based on the amino acid (aa) diversity of the VP1 and nucleotide (nt) diversity of the RdRp. At present, noroviruses are divided into ten genogroups (GI–GX) and eight P-groups as well as two tentative genogroups (GNA1 and GNA2) and two tentative P-groups (GNA1.P and GNA2.P) (Chhabra et al., 2019). GI, GII, and GIV can infect humans, among which GII is the main genogroup causing acute diarrhea in children. Currently, GII noroviruses are separated into 37 P-types along with nine tentative P-types (GII.P-NA1–GII.P-NA9), and 26 genotypes (GII.1–GII.27) (excluding GII.15, which now represents a novel genogroup) along with two tentative GII genotypes (GII.NA1 and GII.NA2) (Vinjé, 2015; Chhabra et al., 2019).

A variety of recombinant genotypes containing various ORF1/ORF2 combinations have developed, increasing the genetic diversity of noroviruses (Bull et al., 2005). GII.4 genotypes are the most prevalent worldwide, and new variants of GII.4 emerge regularly, causing

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pandemics and replacing the previous dominant variants (van Beek et al., 2018). Since the mid-1990s, GII.4 variants such as Grimsby 1996, Farmington Hills 2002, Hunter 2004, Den Haag 2006, New Orleans 2009, and Sydney 2012 have emerged and caused worldwide epidemics (Parra, 2019; van Beek et al., 2018). Since 2014, newly emerged non-GII.4 variants have tended to cause disease epidemics (Tohma et al., 2018). New GII.17 [P17] variants have been reported as the main causes of outbreaks in East Asia (Chen et al., 2015; Matsushima et al., 2015; Xue et al., 2016), America (Degiuseppe et al., 2017), and Europe (Dinu et al., 2016; Giammanco et al., 2017). In winter 2016, a novel recombinant of GII.2 [P16] emerged in Germany (Niendorf et al., 2017) and became prevalent globally. Between 2016 and 2017, a novel dominant GII.4 variant of the Sydney 2012 capsid with a GII.P16 RdRp, was detected in South Korea, Japan, France, and Italy (Matsushima et al., 2016; Bidalot et al., 2017; Medici et al., 2018; Choi et al., 2017).

Surveillance of viral diarrhea in Hubei Province began in 2013, with the purpose of mastering the pathogen spectrum characteristics, epidemic trend, genotype and variation characteristics of major pathogens of viral diarrhea. In early surveillance, the main genotype of noroviruses were GII.4 Sydney 2012, GII.17 and GII.3. GII.2 was detected during surveillance of the 2016 norovirus outbreak (Li et al., 2017, 2018). In this study, we assessed the epidemiological features and genetic diversity of norovirus GII variants among children under five years old with acute gastroenteritis in Hubei Province of China from 2017 to 2019.

2. Materials and methods

2.1. Sample collection

A viral acute diarrhea surveillance program was conducted in Hubei Province (Xiangyang City, Yichang City and Hanchuan City) from January 2017 to December 2019. According to the definition of “diarrhea cases”, fecal samples were obtained from patients who defecated >3 times a day with significant changes in fecal characteristics (including watery, egg-like, mucilaginous, and thin paste). A total of 1216 fecal samples were collected from children ≤ 5 years of age with acute gastroenteritis. Each sample was approximately 5–10 mL and was collected in a clean and dry sterile stool sampling cup. Samples were frozen at −20 °C for later use.

2.2. Nucleic acid extraction

From each sample, approximately 200 µL (solid about the size of a pea) was collected and placed in a 1.5-mL centrifuge tube with phosphate-buffered saline (pH 7.4) to prepare a 10% fecal suspension. The suspension was vibrated violently with a vortex three times and centrifuged at 5000 ×g for 5 min. The supernatant of the fecal suspension was frozen at −70 °C for nucleic acid extraction. Nucleic acid extraction reagent (EX-RNA/DNA) from Xi'an Tianlong Company was used (Xi'an, China). DNA and RNA were extracted simultaneously and the extracted nucleic acids were stored at −80 °C for later use.

2.3. RT-PCR for viral detection

The nucleic acids of norovirus were detected using RT-PCR. The AgPath-ID One-Step RT-PCR Kit (Life Technologies, USA) was used with a real-time PCR platform (Applied Biosystems 7500). The primers and probes used to detect viral nucleic acid associated with norovirus have been described previously (Vega et al., 2011). The primer set Cog 1F/Cog 1R was used with the TaqMan probe Ring 1C and the primer set Cog 2F/Cog 1R was used with TaqMan probe Ring 2 for amplification of NoV GI and NoV GII, respectively. The reactions were carried out in a final volume of 25 µL consisting of 0.4 µmol/L of each primer, 0.2 µmol/L of each TaqMan Probe, 4 µL 5 × RT buffer, 1 µL enzyme mix, and 5 µL extracted RNA. The reaction conditions were as follows: a reverse transcription step at 45 °C for 10 min, a denaturation step at 95 °C for 10 min,

and 40 cycles of amplification (denaturation at 95 °C for 15 s and a combination of annealing and extension at 60 °C for 1 min). Then, NoV GII-positive samples with threshold cycle (CT) values of <30 detected through real-time RT-PCR were genotyped.

2.4. Norovirus genotyping and obtaining the complete VP1 sequence

For NoV genotyping, partial RdRp sequences in ORF1 (240 bp) and VP1 sequences (278 bp) in ORF2 were amplified using conventional RT-PCR using a PrimeScript One Step RT-PCR Kit (Takara, Dalian, China). In this study, we mainly aimed to characterize the genotypes of NoV GII. The 25-µL reaction system consisted of 5 µL RNA template, 12.5 µL 2 × RT-PCR buffer, 1.0 µL PrimeScript enzyme mix, and 0.5 µL each of 10 µmol/L MON431(+) and G2SKR(−) primers (Cannon et al., 2017) for GII genotype amplification. All of the PCR products obtained were sequenced directly (Shanghai Sheng Gong, China). RNA was reverse transcribed for 30 min at 50 °C followed by a denaturation step at 94 °C for 3 min and 40 cycles of amplification (denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 1 min).

To obtain the complete VP1 genes of NoV GII genogroup viruses, cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Vilnius, Lithuania) with the primer VN3T20 (5'-GAGTGACCGCGGCCGCT20-3') (Wang et al., 2005). The complete VP1 gene of NoV GII was amplified using the Ex Taq kit (Takara) with an oligonucleotide primer pair (NVFP: 5'-ATG TTY MGR TGG ATG AGR TTY-3', NVPR: 5'-TGN AYC ATN TCR TTY TCA T-3') targeting the complete genome of strain GII.4/Sydney/NSW0514/2012/AU (JX459908), which was 5012–6900 bp in length. PCR cycling conditions were as follows: denaturation for 3 min at 94 °C; then 35 cycles of 94 °C (30 s), 50 °C (30 s), and 72 °C (2 min); followed by a final extension for 7 min at 72 °C. PCR products were visualized on a 1% agarose gel and sequenced directly (Shanghai Sheng Gong, China).

2.5. Phylogenetic analysis

Sequences of NoV GII variants obtained in this study were compared to GenBank reference strains using the Basic Local Alignment Search Tool (BLASTn). The genotype of norovirus was first determined using the web-based norovirus typing tool v.1.0 (<http://www.rivm.nl/mpf/norovirus/typingtool>). Identity analysis between NoV strains was conducted using MegAlign in Lasergene 7.0 (<http://www.dnastar.com>). Phylogenetic trees were constructed using MEGA 7.0 and supported by bootstrapping with 1000 replicates using the substitution model identified as the best model for branch support based on the Bayesian information criterion for analysis, as suggested by the MEGA 7.0 model test.

2.6. Recombination analysis

Reference sequences for recombination analysis were downloaded from GenBank. Multiple alignments were conducted using MegAlign in Lasergene 7.0 (<http://www.dnastar.com>). Gaps were removed prior to further analysis. Analysis of potential recombinant sequences was performed using the region spanning the ORF1/ORF2 overlap, which included the RdRp and the VP1 gene. The most frequent sites for recombination were identified using similarity plots with SimPlot software (Lole et al., 1999).

2.7. Tertiary structure and antigenic variation in complete VP1 sequences of GII.4 variants

Full-length GII.4 VP1 protein sequences were aligned and compared with reference sequences obtained from GenBank. Informative variable sites were identified and analyzed using the MegAlign tool in Lasergene 7.0 (<http://www.dnastar.com>). Structural similarity models representing the VP1 protein P domains of GII.4 Sydney variants were generated using Swiss-Model and visualized via Visual

Molecular Dynamics (VMD) software version 1.9.3. The VP1 aa sequences of norovirus GII.4 Sydney variants were uploaded to Swiss-Model (<https://swissmodel.expasy.org/interactive>) to obtain their three-dimensional (3D) crystal structures. The appropriate template for the capsid protein was selected as that with the highest identity score for the 3D crystal structure of norovirus capsid (Protein Data Bank accession number PDB 5J35. A). Similarity-based 3D structures of the norovirus GII.4 Sydney variants were visualized using VMD (Wu et al., 2020).

2.8. Statistical analysis

Data were analyzed using SPSS 20.0 software and statistical analysis was performed using the chi-square test. A *P*-value below 0.05 was considered statistically significant.

2.9. Nucleotide sequence accession numbers

Nucleotide sequences of these samples were submitted to the GenBank database under the following accession numbers: OP432545–OP432579, OP432620–OP432681, OP432685–OP432688, OP433084–OP433112, and OP433444 (Supplementary Table S1). The accession numbers of six complete VP1 sequences of GII.4 variants from Hubei are OP595531–OP595539 (Supplementary Table S2).

3. Results

3.1. Epidemiological features of NoV

The viral acute diarrhea surveillance program was conducted from January 2017 to December 2019 in Xiangyang City, Yichang City and Hanchuan City of Hubei Province. In total, 1216 fecal samples were collected from children ≤ 5 years old with acute gastroenteritis. About 178 positive samples for NoV were detected, resulting in a positive rate of 14.64%. There was a statistically significant difference in the detection rate of NoV among years ($\chi^2 = 6.062, P = 0.048$) (Table 1). Among NoV-positive samples, the majority fell into the GII genotype (97.19%, 173/178).

The NoV-positive rate of male children was 12.24% (88/719), while that of female children was 18.11% (90/497). The positive rate of NoV differed significantly between male and female populations among children ≤ 5 years old ($\chi^2 = 8.108, P = 0.004$). The highest infection rate of NoV was observed in children from 7 months to 12 months of age, with a positive rate of 19.76%, followed by the group from 13 months to 24 months (16.67%) and the group from 25 months to 36 months (15.11%). The positive rate of NoV differed significantly among age groups ($\chi^2 = 35.437, P = 0.000, P < 0.05$) (Table 1).

The temporal prevalence of NoV is shown in Fig. 1. The difference among positive rates of NoV in each month was statistically significant

Table 1
Prevalence of norovirus infected cases.

Distribution	Total	No. of NoV positives (%)	χ^2	<i>P</i>
Total	1216	178 (14.64)		
Year of collection				
2017	294	56 (19.05)	6.062	0.048
2018	424	57 (13.44)		
2019	498	65 (13.05)		
Gender				
Male	719	88 (12.24)	8.108	0.004
Female	497	90 (18.11)		
Age distributions				
0–6 months	242	9 (3.72)	35.437	0.000
7–12 months	501	99 (19.76)		
13–24 months	198	33 (16.67)		
25–36 months	139	21 (15.11)		
37–48 months	85	11 (12.94)		
49–60 months	51	5 (9.80)		

($\chi^2 = 223.464, P = 0.000, P < 0.05$). Pandemic seasonality was apparent for NoV infection. Among children ≤ 5 years old in Hubei, NoV had two distinct peaks in prevalence per year. The higher epidemic peak occurred from October to November, while the lower epidemic peak occurred from March to April (Fig. 1).

3.2. Genotype distribution of NoV GII genogroups

A total of 131 positive samples for NoV GII were genotyped (Supplementary Table S1). The NoV GII genotypes based on the partial RdRp (about 240 bp) and VP1 (about 278 bp) sequences are shown in Fig. 2. The dominant VP1 genotype and RdRp type were GII.4 at 47.33% and GII.P16 at 36.64%, respectively. The GII.4 VP1 genotypes were identified as GII.4 Sydney variants. According to the dual-typing methods combining the RdRp type and VP1 genotype, the main recombinant was GII.4 Sydney [P31] at 34.35% (45/131), followed by GII.3 [P12] (25.95%), GII.2 [P16] (22.90%), GII.4 Sydney [P16] (12.98%), GII.17 [P17] (2.29%), and GII.6 [P7] and GII.3 [P16] (each at 0.76%). A wider variety of recombinant variants were detected in the age groups of 7–12 months and 25–36 months (Fig. 3A). The temporal distribution of NoV GII variants is illustrated in Fig. 3B. From 2017 to 2019 in Hubei, the main circulating NoV GII recombinants varied monthly. GII.2 [P16] was first detected and was dominant at the beginning of 2017, but was replaced by the GII.4 Sydney [P31] genotype at the end of 2017. In 2018, GII.2 [P16] and GII.3 [P12] became high-frequency genotypes, while GII.3 [P16] and GII.6 [P7] were observed sporadically. In 2019, the main genotypes shifted to GII.4 Sydney 2012 [P31] and GII.4 Sydney [P16].

3.3. Novel GII.4 [P16] recombinant and GII.4 [P31] recombinant

In our study, GII.4 variants were divided into two recombinant forms, designated GII.4 Sydney 2012 [P31] (*n* = 45) and GII.4 Sydney [P16] (*n* = 17) (Table 2). Most of the GII.4 VP1 genotypes harboring the GII.P31 RdRp type were similar and clustered with a reference sequence of the GII.4 Sydney 2012 variant (JX459908) from Australia (97.5%–98.9% nt identity) (Fig. 4). While most GII.4 variants harboring the GII.P16 RdRp type fell into the clade with a novel GII.4 Sydney 2012 variant (98.2%–98.6% nt identity) (Kawasaki194, GenBank ID LC175468) reported in 2016 from Japan (Fig. 4). Only one GII.4 [P16] strain in our study (strain Hubei-014-2019), detected in 2019, clustered with GII.4 Sydney 2012 (JX459908) in the phylogenetic tree based on partial VP1 sequences; this strain shared 98.6% and 96.1% nt identity with the GII.4 Sydney 2012 variant (JX459908) and Kawasaki194 (LC175468), respectively. Based on partial VP1 sequences, this GII.4 [P16] strain (Hubei-014-2019) had 95.7%–96.1% nt identity with other GII.4 strains harboring GII.P16 from Hubei, and 96.1%–97.5% nt identity with other GII.4 strains harboring GII.P31 RdRp from Hubei. Aside from this unique GII.4 [P16] strain, all other GII.4 strains harboring GII.P16 from Hubei had 93.9%–95.7% nt identity with other GII.4 strains harboring GII.P31 from Hubei.

3.4. Non-GII.4 recombinant genotypes of NoV GII genogroups

The GII.2 [P16] recombinant was detected in an outbreak for the first time in Hubei in early 2017 (Li et al., 2018). This novel recombinant was also detected in acute gastroenteritis surveillance among children. Based on partial VP1 sequences, all GII.2 [P16] strains from Hubei fell into a single cluster with strain 16-G0831 (KY357449), which re-emerged in 2016 from Germany (Niendorf et al., 2017) (Fig. 5). Most GII.2 strains collected in Hubei had 97.8%–99.3% nt identity with strain 16-G0831 and 98.2%–99.6% nt identity with strain GII.2 [P16] (MH068791) from an outbreak in Hubei in early 2017.

GII.3 variants made up a large percentage of NoV VP1 genotypes among children in this study. Two recombinant GII.3 forms were observed harboring GII.P12 and GII.P16 P-types, namely GII.3 [P12] (*n* = 34) and GII.3 [P16] (*n* = 1), respectively. The phylogenetic analysis showed all GII.3 VP1 sequences clustered closely (Fig. 5). The partial VP1

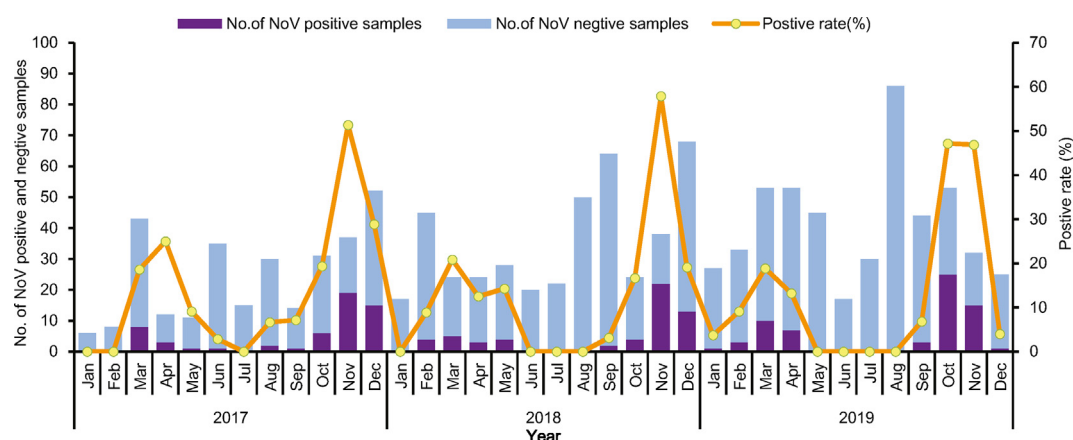


Fig. 1. Temporal distribution of norovirus infections in children ≤ 5 years of age with acute gastroenteritis in Hubei, 2017–2019.

sequences of GII.3 harboring GII.P12 had 98.6%–100.0% nt identity with strain SZ16334 (MT678734) collected in Shenzhen, China, in 2016 and 97.8%–99.3% nt identity with strain CA-RGDS-1098 from the United States in 2019 (MT755733). The GII.3 [P16] variant in this study had 96.1% nt identity with the GII.3 [P16] strain NS17-A947 (MG892951) collected in Russia in 2017 based on partial *VP1* sequences.

GII.17 [P17] variants were observed sporadically in Hubei and were detected only in 2017 ($n = 2$) and 2019 ($n = 1$). Novel GII.17 [P17] variants first emerged in 2015 in China (Chen et al., 2015; Xue et al., 2016) and were replaced by GII.2 [P16] in 2016–2017 (Ao et al., 2017, 2018). From 2017 to 2019 in Hubei, the frequency of the GII.17 [P17] variant was low. According to our phylogenetic analysis, the GII.17 [P17] variants from Hubei could be divided into Kawasaki323-like and Kawasaki308-like sub-clusters, respectively (Fig. 5). The GII.17 variants clustered with strain Kawasaki323 shared approximately 98.9% nt identity. By contrast, the GII.17 strain collected in 2019 clustered with strain Kawasaki308, sharing 99.3% nt identity. The GII.6 [P7] variant was rarely detected in NoV infections in Hubei prior to this study. From 2017 to 2019, only one strain of the GII.6 [P7] genotype was observed among children in 2018. The GII.6 [P7] strain from Hubei had 94.6% nt identity of the partial *VP1* gene with strain 016Q04 collected in 2016 from Guangdong (KY407216), China, and fell into the same clade in the phylogenetic tree (Fig. 5).

3.5. GII.P16 RdRp type associated with GII.4/2/3 *VP1* genotypes

Partial *RdRp* sequences of GII.P16 in our study equipped with GII.4/2/3 *VP1* genotypes were subjected to phylogenetic analysis. Notably, all GII.P16 sequences harboring GII.4/2/3 *VP1* genotypes in our study were correlated and clustered closely, but could be clearly divided into different clusters (Fig. 6). All GII.P16 sequences harboring GII.2 were grouped with the GII.2 [P16] strain (KY357449) from Germany in 2016, with 98.8%–100.0% nt identity. The GII.P16 sequence harboring GII.3 from Hubei differed distinctly from previously reported GII.3 [P16] strains and grouped with GII.P16 sequences harboring GII.2 that emerged after 2016. The previously reported GII.P16 sequences harboring GII.3 and GII.13 were located in distinctly separate clades. All GII.P16 sequences harboring GII.4 clustered with the Kawasaki194 strain (LC175468) from Japan in 2016 (97.5%–99.2% nt identity), the NOR-2565 strain (KY887601) (97.5%–99.2% nt identity) from the United Kingdom in 2016. These GII.P16 sequences also grouped with the reference GII.3 [P16] strains, the Hennepin0275 strain (MK773588) from the United States in 2018 (96.3%–97.1% nt identity), and the NOR-2598 strain (KY887606) from the United Kingdom in 2016 (96.7%–98.3% nt identity). The results of genetic analysis indicated that all GII.P16 sequences associated with GII.4 Sydney, GII.2, and GII.3 obtained in Hubei

were correlated with novel GII.2 [P16] variants that re-emerged in Germany.

3.6. Recombination analysis of the norovirus GII genogroup

Based on the results of genotyping using a web-based norovirus typing tool, 36 positive samples were selected for recombination breakpoint analysis using SimPlot software and the RDP4 program. Recombinant events among these strains were detected with P -values ranging from 5.556×10^{-11} to 7.556×10^{-2} (Table 2). Recombination events were found in the patterns of GII.2 [P16], GII.3 [P12], GII.3 [P16], GII.4 [P16], GII.4 [P31], and GII.6 [P7] while no evidence of recombination was detected in GII.17 [P17]. We selected the above recombinant strains for breakpoints analysis, and the nucleotide recombination sites of different recombinant strains were located in ORF1, ORF2 or the junction of ORF1/ORF2 which were shown in Table 2. Similarity plots of the selected GII.2 [P16], GII.3 [P16], GII.3 [P12], GII.4 [P16], GII.4 [P31], and GII.6 [P7] strains are shown in Fig. 7A–F, respectively. Meanwhile, a novel recombination pattern was found in the GII.4 Sydney strain Hubei-Nov-014-2019 between the GII.4 Sydney 2012 strain (JX459908) and the GII.4 Sydney 2016 strain Kawasaki194 (LC175468) led to the phylogenetic relationship with the GII.4 Sydney 2012 strain (JX459908) in the *VP1* sequence. The recombination breakpoint of this strain was located at nucleotide position 5059 in ORF1 (Table 2). A similarity plot of this recombinant strain (Hubei-Nov-014-2019) is shown in Fig. 7G.

3.7. *VP1* variation of recombinant GII.4 sydney viruses

Studies have shown that GII.4 variant differences are associated with monoclonal antibodies mapped to major variable antigenic sites in the P domain of the *VP1* protein (Tohma et al., 2022). Three complete *VP1* sequences from GII.4 Sydney 2012 [P16] strains and six from GII.4 Sydney 2012 [P31] strains were obtained in this study. We compared the positions of the main tertiary structures (helices and sheets) as well as the distribution of basic and acidic amino acids in the tertiary structure of the P domain between the NoV GII.4 strains from Hubei and GII.4 Sydney 2012 [P31] strain (JX459908) and GII.4 Sydney 2012 [P16] strain (LC175468) (Supplementary Fig. S1A). Despite the presence of non-synonymous mutations at important antigenic sites (residues 297, 372, and 373 in epitope A; 333 in epitope B; 393 in epitope D; 414 in epitope E; and 309 in epitope H), no significant differences were found in the tertiary structure of the P domain.

Furthermore, consensus aa sequences of the *VP1* gene were created from the complete GII.4 *VP1* sequences to compare aa substitutions in the S and P domain, particularly in the P2 subdomain with previous GII.4

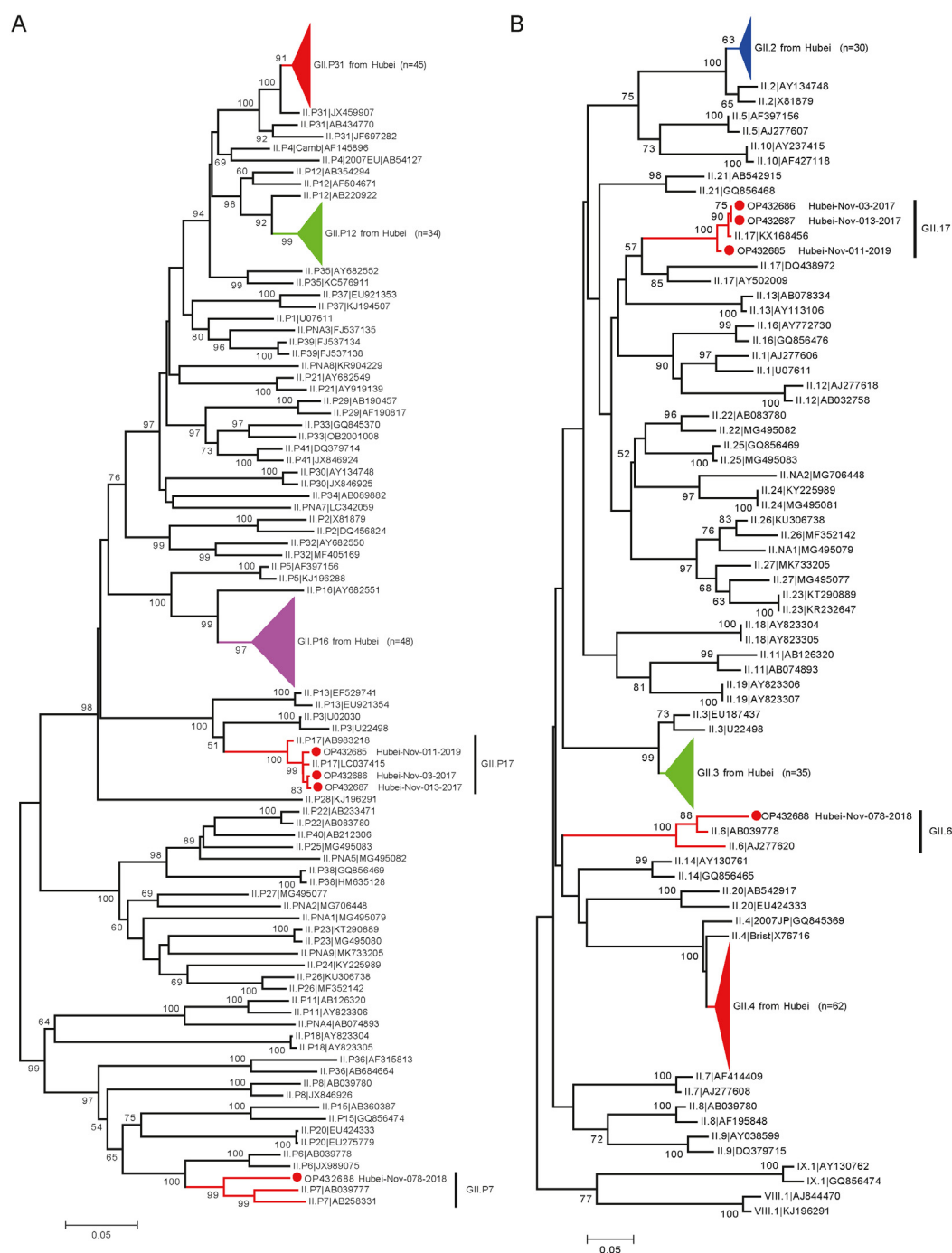


Fig. 2. Phylogenetic analysis of the NoV GII genogroup based on partial nucleotide sequences of the polymerase (*RdRp*) and *VP1* regions. **A** Phylogenetic trees based on the partial *RdRp* sequence (240 bp). **B** Phylogenetic trees based on the partial *VP1* gene sequence (278 bp). NoV strains obtained from children ≤ 5 years old in Hubei in 2017–2019 are indicated with red circles. The trees were constructed using MEGA 7.0 with the neighbor-joining method using the Kimura 2-parameter model. Bootstrap values (1000 replicates) are indicated on the phylogenetic tree.

variants as reference viruses (Supplementary Fig. S1B). Although the S domain is highly conserved (Tohma et al., 2019; Chen et al., 2006), differences of A8V, V119I, V145I, S171L, N172S, P174S, and D193E were observed in the S domain among the GII.4 strains, as illustrated in Supplementary Fig. S1B. In epitopes A–H and the NERK and EHNQ motifs, no substitutions were detected between Hubei GII.4 [P16] strains and the reference GII.4 [P16] strain. Notably, variable substitutions of residues were present in the VP1 amino acid sequences among all GII.4 Sydney [P31] strains from Hubei and reference strains (Supplementary

Fig. S1B). Substitutions occurred in all GII.4 Sydney 2012 [P31] strains from Hubei of R297H, D372N, and R373H/N in epitope A, V333M in epitope B, H414P/L in epitope E and N309S and D310N in epitope H. In our study, the epitopes G (residues 352, 355, 356, 357, 359, and 364) (Tohma et al., 2019) and F (residues 327 and 404) (Mallory et al., 2019), motifs NERK (N310, E316, R484, and K493) and EHNQ (E488, H501, N522, and Q523) (Lindesmith et al., 2014) were also conserved and remained unchanged among all GII.4 Sydney [P16] and GII.4 Sydney [P31] strains from Hubei.

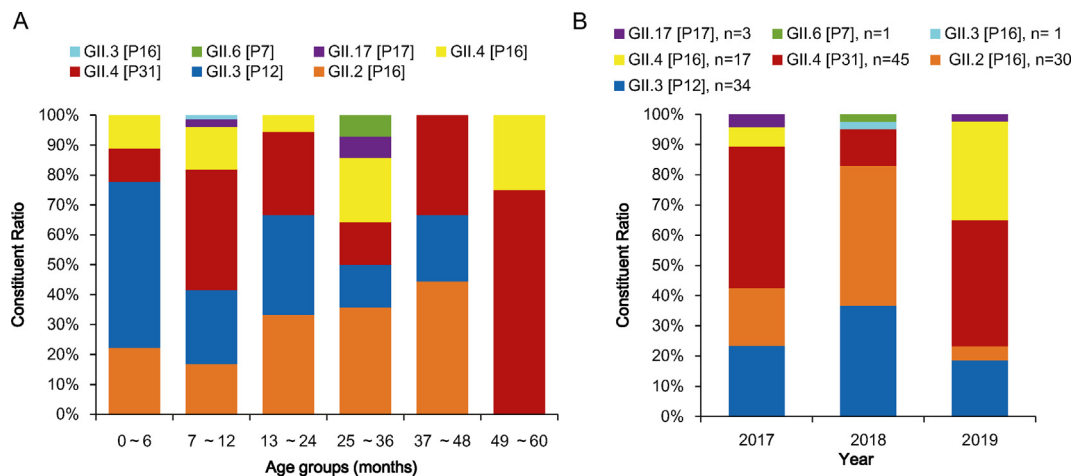


Fig. 3. A Constituents of norovirus GII recombination genotypes obtained from various age groups of children (≤ 5 years) with acute gastroenteritis in Hubei, 2017–2019. B Temporal distribution of norovirus GII genotypes in children ≤ 5 years of age with acute gastroenteritis in Hubei, 2017–2019.

4. Discussion

Through sentinel surveillance of acute gastroenteritis among children ≤ 5 years old, we obtained the epidemiological characteristics and genetic diversity of NoV GII strains from Hubei Province in central

China from 2017 to 2019. Over three consecutive years of NoV monitoring, the detection rate of NoV was 14.64%. The detection rate of NoV showed a statistically significant difference between male and female populations among children ≤ 5 years old in this study, although some studies have found no significant difference in the NoV detection rates

Table 2

Analysis of breakpoints in recombinant strains of norovirus GII genotype in Hubei from 2017 to 2019.

Samples	Sample genotype	Reference strains for breakpoints analysis	Reference strain genotype	Predicted breakpoint nt position	Breakpoint on ORF	Bootscan P-value
Hubei-Nov-025-2018	GII.2[P16]	AY772730 Neustrelitz260/2000/DE	GII.16[P16]	5087	ORF1/2	1.089×10^{-5}
Hubei-Nov-042-2017				5073	ORF1	1.214×10^{-5}
Hubei-Nov-07-2018				5092	ORF1/2	2.036×10^{-4}
Hubei-Nov-016-2018				5092	ORF1/2	1.356×10^{-5}
Hubei-Nov-010-2019				5092	ORF1/2	1.356×10^{-5}
Hubei-Nov-028-2019	GII.3[P16]	AY772730 Neustrelitz260/2000/DE	GII.16[P16]	5087	ORF1/2	4.466×10^{-6}
Hubei-Nov-038-2017				5092	ORF1/2	1.356×10^{-5}
Hubei-Nov-030-2018				5084	ORF2	2.175×10^{-8}
Hubei-Nov-029-2017				5083	ORF2	5.556×10^{-11}
Hubei-Nov-045-2017				5083	ORF2	1.567×10^{-10}
Hubei-Nov-02-2018	GII.3[P12]	AB220922 Sakai/04-179/2005/JP	GII.4[P12]	5083	ORF2	7.225×10^{-10}
Hubei-Nov-06-2018				5083	ORF2	3.693×10^{-9}
Hubei-Nov-010-2018				5083	ORF2	6.865×10^{-9}
Hubei-Nov-017-2018				5083	ORF2	5.387×10^{-9}
Hubei-Nov-026-2018				5083	ORF2	1.982×10^{-8}
Hubei-Nov-027-2018	GII.4[P16]	AY772730 Neustrelitz260/2000/DE	GII.16[P16]	5083	ORF2	8.032×10^{-10}
Hubei-Nov-029-2018				5083	ORF2	8.756×10^{-7}
Hubei-Nov-039-2019				5083	ORF2	5.387×10^{-9}
Hubei-Nov-026-2017				5083	ORF2	5.556×10^{-11}
Hubei-Nov-014-2019				5083	ORF1/2	3.016×10^{-8}
Hubei-Nov-017-2019	GII.4[P31]	GU445325 New_Orleans2009/USA	GII.4[P4]	5083	ORF1/2	4.016×10^{-8}
Hubei-Nov-026-2019				5083	ORF1/2	4.016×10^{-8}
Hubei-Nov-055-2017				5083	ORF1/2	4.016×10^{-8}
Hubei-Nov-063-2017				5083	ORF1/2	3.856×10^{-8}
Hubei-Nov-036-2019				5034	ORF1	1.085×10^{-4}
Hubei-Nov-051-2017	GII.6[P7]	KJ196295 JP/2010	GII.4[P4]	5034	ORF1	1.057×10^{-4}
Hubei-Nov-061-2017				5034	ORF1	5.007×10^{-5}
Hubei-Nov-031-2018				5034	ORF1	1.496×10^{-2}
Hubei-Nov-076-2018				5034	ORF1	7.669×10^{-4}
Hubei-Nov-025-2019				5034	ORF1	9.037×10^{-4}
Hubei-Nov-027-2019	GII.4[P16]	LC175468 Kawasaki194	GII.4[P4]	5034	ORF1	9.107×10^{-3}
Hubei-Nov-06-2017				5034	ORF1	1.057×10^{-4}
Hubei-Nov-07-2017				5034	ORF1	2.205×10^{-4}
Hubei-Nov-030-2017				5034	ORF1	1.127×10^{-3}
Hubei-Nov-031-2019				5034	ORF1	9.107×10^{-3}
Hubei-Nov-078-2018	GII.17[P17]	LC037415_Kawasaki308	GII.7[P7]	5013	ORF1	2.027×10^{-3}
Hubei-Nov-014-2019				5059	ORF1	7.556×10^{-2}
Hubei-Nov-03-2017				No evidences of recombination		
Hubei-Nov-013-2017						
Hubei-Nov-011-2019						

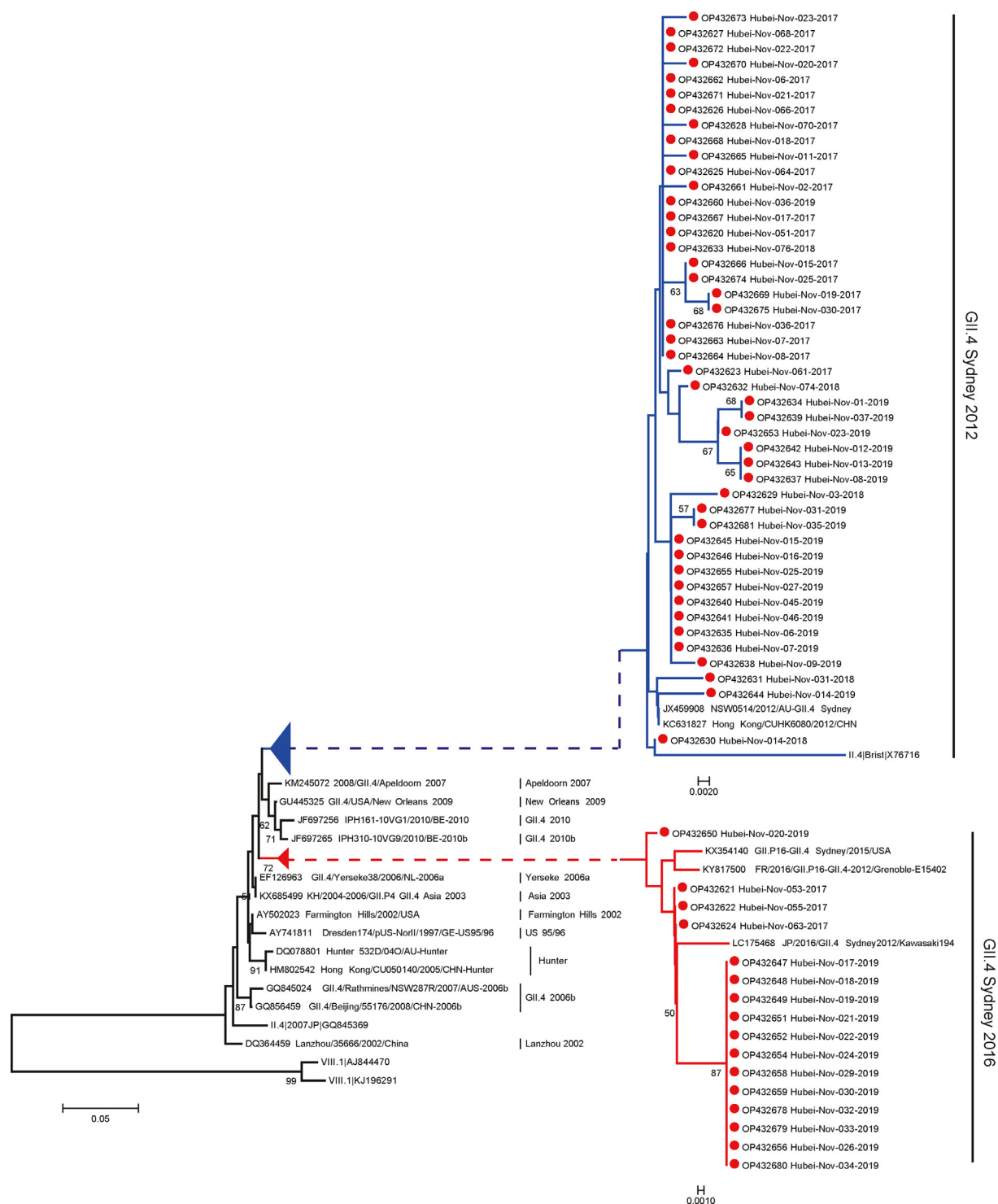


Fig. 4. Phylogenetic trees of partial VP1 gene sequences of norovirus GII.4 strains (278 bp). Solid red circles indicate variants collected in Hubei in 2017–2019. The phylogenetic tree was constructed using the neighbor-joining method and Kimura two-parameter model in MEGA 7.0. Bootstrap values (1000 replicates) are indicated on the phylogenetic tree.

for girls and boys (Lu et al., 2020; Zou et al., 2015). Children with NoV infection were mostly aged from 7 months to 12 months. This result was consistent with previous studies reporting that most children with NoV infection are under two years of age (Zou et al., 2015; Sai et al., 2013; Lu et al., 2019). NoV infection occurred mainly during the cold seasons of autumn and winter in other parts of China (Zou et al., 2015; Sai et al., 2013; Lu et al., 2019). However, in Hubei, two distinct prevalence peaks were observed per year, with the higher epidemic peak occurring

from October to November and the lower epidemic peak occurring from March to April.

GII.4 variants are the most common source of norovirus infections worldwide and are often associated with an increase in the number of outbreaks (Vinjé, 2015). Non-GII.4 NoV variants can also cause outbreaks and are dominant in the human population in specific locations and times, such as the recent predominance of GII.17 strains from 2014 to 2016 in Asia (Tohma et al., 2018; Lu et al., 2016) and GII.2 strains

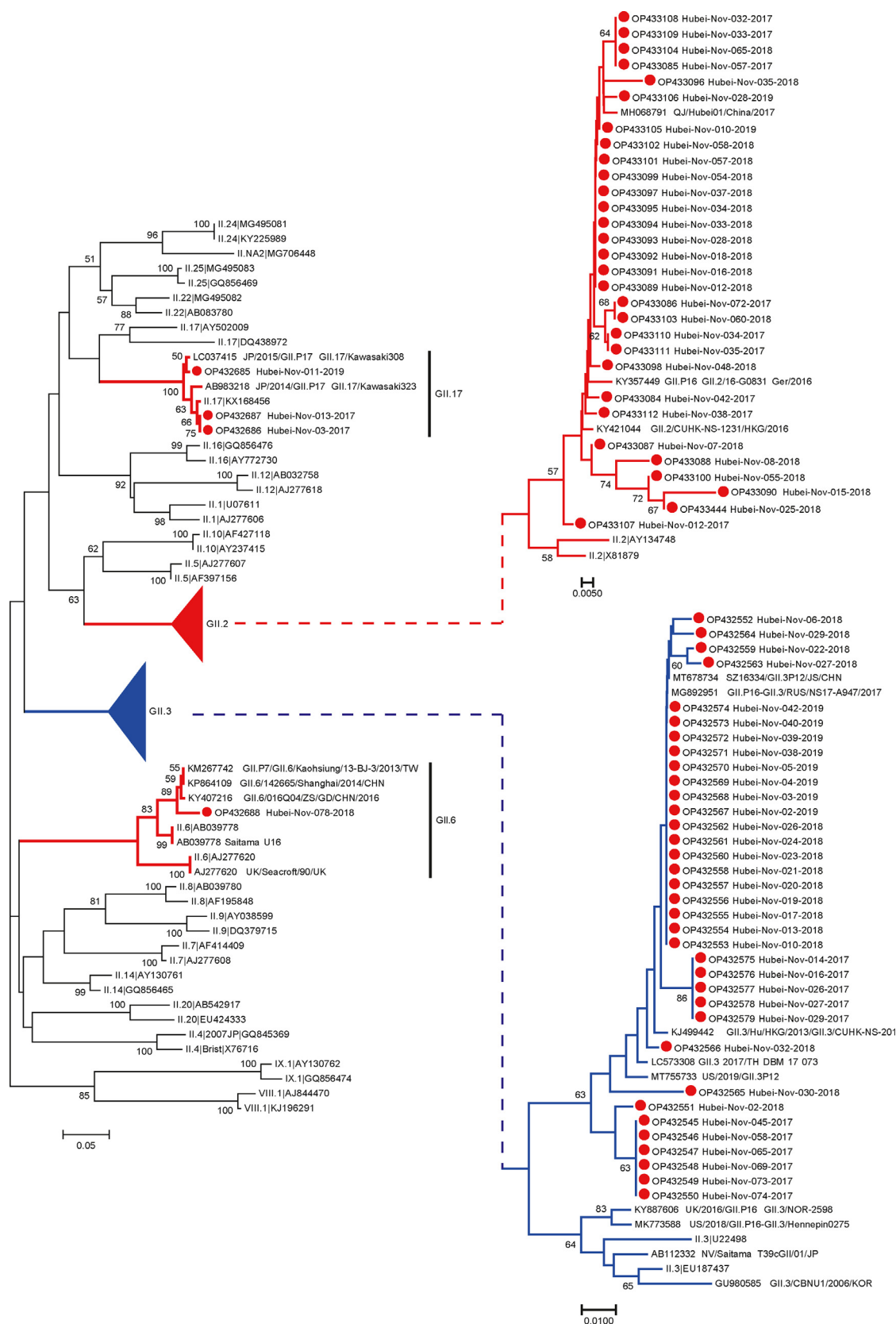


Fig. 5. Phylogenetic analysis of GII.2/3/17/6 genotypes based on partial nucleotide sequences of the VP1 gene (278 bp). NoV strains obtained from children ≤ 5 years of age in Hubei in 2017–2019 are indicated with red circles. The trees were constructed using MEGA 7.0 with the maximum-likelihood method and the Kimura 2-parameter model. Bootstrap values (1000 replicates) are indicated on the phylogenetic tree.



Fig. 6. Phylogenetic analysis of NoV GII.P16 RdRp types based on partial nucleotide sequences of the polymerase (*RdRp*) gene. NoV strains obtained from children ≤ 5 years of age in Hubei in 2017–2019 are indicated with red circles. The trees were constructed using MEGA 7.0 with the maximum-likelihood method and the Kimura 2-parameter model. Bootstrap values (1000 replicates) are indicated on the phylogenetic tree.

from 2016 to 2017 in Europe and Asia (Ao et al., 2017; Nagasawa et al., 2018; Niendorf et al., 2017). Before 2012, GII.4 variants harboring the GII.P4 RdRp type were dominant (Siebenga et al., 2009), and the GII.4 Sydney variant harboring the GII.P31 RdRp type was identified in 2012. The GII.4 Sydney 2012 [P31] variant was first reported in Australia in 2012 and became widespread since then (van Beek et al., 2013). In the winter of 2015, a novel GII.4 Sydney 2012 [P16] recombinant strain was first detected in the United States (Cannon et al., 2017) and became a dominant genotype worldwide that was detected in Japan, Korea, Brazil, Oceania, and European countries (Matsushima et al., 2016; Barreira et al., 2017; Bidalot et al., 2017; Medici et al., 2018; Lun et al., 2018; Choi et al., 2017) as well as other parts of China (Ai et al., 2021; Zheng et al., 2022). In Hubei, in addition to the GII.4 Sydney 2012 [P31] variants, novel GII.4 Sydney 2012 [P16] recombinant strains were detected, and the frequency increased sharply in 2019. A unique GII.4 Sydney 2012 [P16] strain (strain Hubei-014-2019) was found which arose from the

GII.4 Sydney 2012 [P31] strain (JX459908) and the GII.4 Sydney 2012 [P16] strain Kawasaki194 in 2016 (LC175468). Such unique recombination may have occurred as a result of co-circulation of strains GII.4 Sydney 2012 [P31] and GII.4 Sydney 2012 [P16] in Hubei.

In addition, several non-GII.4 variants played an important role in the circulation of NoV infections, including GII.2, GII.3, GII.17, and GII.6. In Hubei Province, GII.2 [P16] variants re-emerged and dominated in early 2017, but were replaced by the GII.4 Sydney [P31] genotype in late 2017. GII.3 [P12] variants had high detection rates in all years from 2017 to 2019, but GII.3 [P16] and GII.6 [P7] variant was observed sporadically in children. GII.17 noroviruses were sporadically prevalent around the world before 2012 (de Graaf et al., 2015). Since the winter of 2014–2015, GII.17 [P17] variants emerged as a novel recombinant genotype, and became the dominant genotype in Guangdong, Beijing, Shanghai, and other areas in southern China (Chen et al., 2015; Lu et al., 2015; Gao et al., 2015; Xue et al., 2016) as well as other Asian countries

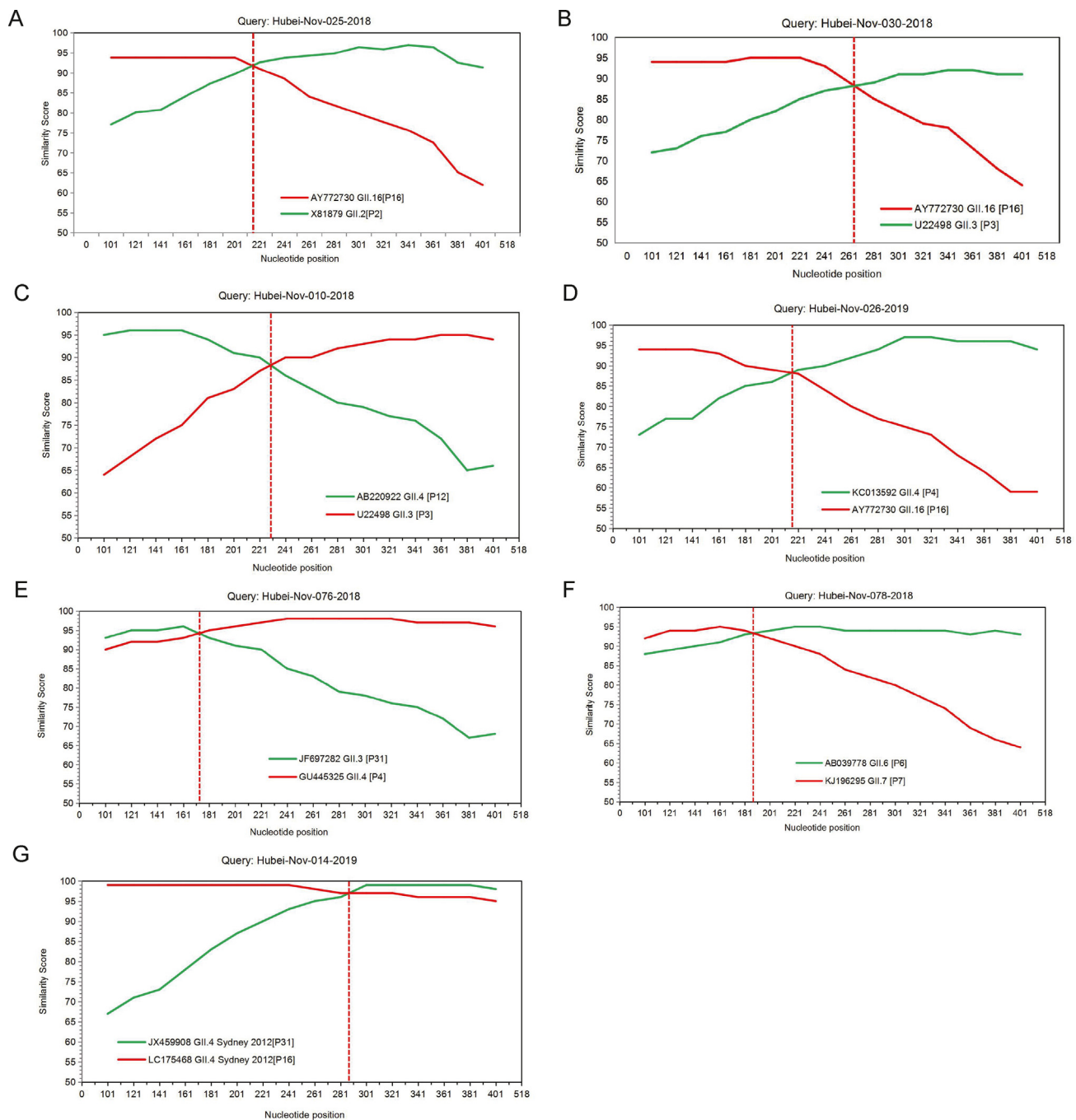


Fig. 7. Similarity plots of NoV GII recombination patterns detected in Hubei, China. The vertical axis indicates nucleotide sequence identity (%) between the query sequences of NoV recombinant strains in Hubei and reference strains. The red vertical line indicates the predicted recombination site.

(Matsushima et al., 2019; Koo et al., 2017). However, since the winter of 2014–2015, the variants of GII.17 [P17] is phylogenetically divided into two clusters (Chan et al., 2015): strains similar to Kawasaki323 (AB983218) from Japan, which circulated in 2013–2014, and strains similar to Kawasaki308 (LC037415), collected from Japan in 2015. The GII.17 variants in Hubei were also divided into the Kawasaki323-like and Kawasaki308-like sub-clusters, respectively. All GII.P16 sequences in our study associated with GII.4/2/3 capsid genotypes had correlations in their evolutionary origins, despite harboring different G genotypes.

In our study, no significant effect on tertiary structure in P domain was observed between two GII.4 capsid sequences harboring GII.P16 and GII.P31 despite aa substitutions at major antigenic sites in the P domain.

The capsid sequences of NoV GII.4 strains are undergoing epochal evolution, resulting in immune escape variants (Siebenga et al., 2007; Bok et al., 2009). Although the S domain (residues 1–221) forms the structural core and is highly conserved (Tohma et al., 2019; Chen et al., 2006), differences were observed among all GII.4 strains obtained in the present study. The P domain of the capsid monomer is highly variable and can be divided into two subdomains: the P1 subdomain (residues 226–278 and 406–520) forms a stalk from the central core and the P2 subdomain (residues 279–405), which is the most exposed region of the viral particle and is well positioned to interact with potential neutralizing antibodies and HBGA ligands (Chen et al., 2006; Tohma et al., 2022). Changes in epitopes are associated with escape from human antibody immunity

(Lindesmith et al., 2018a,b) which reduced the effect of interactions with human polyclonal sera and monoclonal antibodies (Lindesmith et al., 2013, 2018a,b; Shanker et al., 2011). In this study, according to our aa substitution analysis at major antigenic sites, frequent substitutions at epitope sites were observed including notable mutations of R297H, D372N, and R373H/N in epitope A; V333M in epitope B; G393S in epitope D; H414P/L in epitope E; and N309S in epitope H of GII.4 Sydney strains harboring GII.P16 and GII.P31. The substitution R373H in epitope A was reported to result in loss of the blockade of antibodies that anchor at 373 (Lindesmith et al., 2018a,b). Epitope D (residues 393–395) is a blockade epitope mediating the affinity for various HBGAs and ligand-binding patterns of GII.4 strains associated with escape from human immunity (Lindesmith et al., 2012a,b). Substitution at residue 393 indicated the differentiation in potential antibody affinity between the GII.4 Sydney 2012 [P16] variants and GII.4 Sydney 2012 [P31] strains. The NERK motif is a potential conformational determinant regulating antibody and the EHNQ motif was identified as a conserved region among GII noroviruses (Kolawole et al., 2017; Lindesmith et al., 2014). The EHNQ mutation caused the virus to lose its particle integrity and made it unable to bind carbohydrate ligands (Lindesmith et al., 2014). The NERK and EHNQ motifs were both conserved among all GII.4 Sydney 2012 [P16] and GII.4 Sydney 2012 [P31] strains collected from Hubei.

5. Conclusion

In conclusion, we demonstrated high diversity of NoV GII recombinant strains among children ≤ 5 years old with acute gastroenteritis in Hubei, China, from 2017 to 2019. Our study investigated the epidemiological features and genetic diversity of NoV GII recombinant strains circulating in Hubei based on continuous surveillance of NoV to elucidate the role of NoV recombinant strains in NoV infection dynamics. We found that the recently emerged variant GII.4 Sydney 2012 [P16] was present, and that a less frequently reported variant, GII.6 [P7], occurred sporadically in Hubei. Investigating the mechanics of polymerase switching and the mutations in antigenic sites of VP1 as part of further surveillance of NoV infections will be essential to clarify the circulation characteristics and genetic diversity of NoV recombinant strains.

Data availability

All the data generated or analyzed during the current study are included in this published article or available from the corresponding author upon request.

Ethics statement

Institutional review board approval was not requested, as this study was part of a routine laboratory-based investigation. No human experimentation was conducted. The only human materials used were stool samples that had been sent to our laboratory for routine surveillance of viral diarrhea. The results reported are associated with norovirus strains, with no patient information used.

Author contributions

Jing Li: conceptualization, investigation, data curation, writing original draft, visualization. Lingyao Zhang: data curation, review&editing draft, methodology, formal analysis. Wenjing Zou: investigation, methodology. Zhaohui Yang: investigation, methodology. Jianbo zhan: conceptualization, investigation, methodology, funding acquisition. Jing Cheng: conceptualization, methodology, funding acquisition.

Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.virs.2023.04.002>.

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