



Letter

Genetic characterization of rarely reported GII.3[P25] norovirus strain detected in patients with acute gastroenteritis in Huzhou, China, 2021

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Dear Editor,

Noroviruses (NoVs) are now recognized as the leading cause of sporadic and epidemic nonbacterial acute gastroenteritis (AGE) in children and adults worldwide (Glass et al., 2009). NoVs possess a positive-sense, single-stranded RNA genome about 7.6 kb in length, which consists of three open reading frames (ORFs) (Prasad et al., 1999). ORF1 encodes non-structural proteins required for replication, including RNA-dependent RNA polymerase (RdRp), while ORF2 and ORF3 encode the major capsid protein VP1 and minor capsid protein VP2, respectively. The major capsid protein VP1 can be structurally divided into a shell (S) domain and a protruding (P) domain, with the P domain further divided into P1 and P2 subdomains (Vongpunsawad et al., 2013; Thorne and Goodfellow, 2014). P2 subdomain is the hypervariable region of the capsid and associated with the receptor-binding function and viral immunogenicity.

NoVs are genetically diverse and can be classified into at least ten genogroups (GI–GX), of which viruses from GI, GII, GIV, GVIII, and GIX infect humans (Kroneman et al., 2013; Chhabra et al., 2019). Each genogroup could be further divided into various genotypes based on either VP1 (capsid genotypes) or partial RdRp (P-types) sequences. Viruses in these ten genogroups can be further divided into 49 confirmed capsid genotypes and 60 confirmed P-types. Despite the genetic diversity, a single genotype (GII.4) has been regarded as the most important cause of NoVs associated AGE outbreaks for almost two decades (Bok et al., 2009; Hoa Tran et al., 2013; Ai et al., 2021). As a major driving force of viral evolution, natural recombination is common between NoV strains. Recombination often occurs within or near the ORF1/ORF2 overlap region, producing strains with differing VP1 and RdRp genotypes, further increasing genetic diversity (Bull et al. 2005, 2007). Indeed, the vast majority of contemporary non-GII.4 NoVs are recombinant viruses, and some have been widely reported as the common cause of outbreaks and sporadic infections in recent years, such as GII.6 [P7], GII.3 [P12], GII.4 [P31] and GII.2 [P16] (White, 2014; Ao et al., 2017; Niendorf et al., 2017; Li et al., 2023).

Our previous study also indicate that NoVs circulating in Huzhou City were predominantly GII.4 variants and non-GII.4 recombinants. From July 2014 to June 2018, at least eight genotypes were identified in outbreaks in Huzhou, including GI.6 [P11], GI.5 [P4], GII.2 [P2], GII.6 [P7], GII.4 Sydney [P31], GII.17 [P17], GII.3 [P12], and GII.2 [P16], and 81.1% of the genotyped outbreaks were caused by recombinant strains (Chen et al., 2020). In March 2021, an AGE outbreak caused by rarely detected GII.3 [P25] NoV was reported in Huzhou, China. To better understand the genetic background and molecular characteristics of this recombinant, samples that tested positive for NoV by RT-qPCR were selected for sequencing and analyzed for evolution, recombination, and antigenic mutations using bio-analysis software (Supplementary Material). Nucleotide sequences obtained in this study were submitted to the GenBank database with the accession numbers OL451532, and OL444826–OL444832.

On March 24, 2021, a junior high school in Wuxing District, Huzhou City, China, reported an AGE outbreak to the Wuxing Center for Disease Prevention and Control. During the outbreak, a total of 17 cases (aged 13–55 years old, with 10 males and 7 females, including 15 students and 2 teachers) fit the definition of the probable case between March 23 and March 25. The 15 student cases were distributed across 3 grades and 6 classes, ranging in age from 13 to 17 years. Diarrhea (82.3%, 14/17) was the most common symptom, and 76.5% (13/17) of cases had vomiting, 11.8% (2/17) of cases had abdominal pain, 5.9% (1/17) of cases had fever. All cases were mild and no hospitalization, severe illness, or deaths occurred.

A total of 23 samples were collected for NoV detection by real-time RT-PCR (Supplementary Material), including 17 rectal swab samples from cases, 2 environmental swab samples (from desk and doorknob) and 4 drinking water samples. About 10 rectal swab samples from cases (8 students and 2 teachers) and 1 environmental swab sample were tested positive for NoV GII, whereas all drinking water samples were negative. Epidemiological studies suggested that NoV was transmitted

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from person to person and not via contaminated food or drinking water in this outbreak.

Using overlapping primers MON431/G2SKR, partial *RdRp* and *VP1* sequences were successfully obtained from seven rectal swab samples. Analysis revealed that sequences of the seven strains share higher degree nucleotide identity (99.6%–100%). All sequences were genotyped as NoV GII.3 [P25] using the online NoV genotyping tool (<http://www.rivm.nl/mpf/norovirus/typingtool>). Phylogenetic analysis based on partial *RdRp* and *VP1* sequences (Fig. 1A and B) showed that GII.3 [P25] strains in this study clustered together with the GII.3 [P25] strain identified in 2022 in USA (CA-RGDS-1158/2022/US), and formed a relatively independent subcluster (designated as GII.3 [P25] 2021–2022 subcluster).

To better understand the molecular characteristics of these rarely detected GII.3 [P25] NoV strains, the complete nucleotide sequence of one GII.3 [P25] strain N21051 was sequenced by next-generation sequencing (NGS). The genome of N21051 consists of 7584 nucleotides (nt), excluding the poly(A) tail. It contained three open reading frames (ORFs), ORF1, ORF2, and ORF3, with lengths of 5,136, 1,647, and 765 nt, respectively. ORF1 and ORF2 had an overlap of 20 nucleotides (nt 5121–5140), whereas ORF2 and ORF3 had a single nucleotide overlap (“A” at position 6767). A BLAST search of GenBank with the complete N21051 genome sequence showed that highest identity with CA-RGDS-1158/2022/US strain (GenBank accession number OP690505) was observed (Query cover = 99% and Identity = 99.75%). Simplot analysis using GII.3 [P3] (U02030/TV24/1991/CA) and GII.25 [P25] (MG495083/Dhaka 1928/2012/U) as subtype references revealed that N21051 was GII.3 [P25] recombinant strain with a potential recombination breakpoint (nt 5161) located near the ORF1/2 junction (Fig. 1D).

The full-length *VP1*-coding nucleotide sequences of N21051, together with 86 GII.3 *VP1* sequences available in the GenBank database from 1972 to 2022, were used for evolutionary analysis. GII.3 [P25] strains from Japan in 2000 (Hokkaido/34B/2000/JP) and 2002 (T66eGII/02/JP), of which the full-length sequence were not available in GenBank, were excluded from the evolutionary analysis. Phylogenetic analysis showed that the GII.3 *VP1* sequences could be divided into three clusters (Cluster I–III). Cluster I contained three *RdRp* genotypes (GII.P41, GII.P16, and GII.P3) detected during the years from 1972 to 2014, which were further divided into two subclusters (subcluster Ia and subcluster Ib) and N21051 fell in cluster Ia (Fig. 1C). GII.3 [P25] strain N21051 in this study was clustered with GII.3 [P25] strain detected in 2022 in USA (CA-RGDS-1158/2022/US), and was most closely related to GII.3 strain identified in Japan in 2006 (DQ093063.1336/2006/JP).

To identify potential antigenic mutations, the *VP1* amino acid sequence from 28 GII.3 NoVs representative of cluster I (spanning 1972 to 2022), including N21051 sequence from this study, were aligned. Compared with the earliest GII.3 strain (ShippensburgC2/1972/US) available in GenBank, 19 unique amino acid substitutions were identified in the *VP1* region of GII.3 [P25] 2021–2022 strains, with 89.47% (17/19) of these substitutions were located in the P domain (Supplementary Fig. S1).

Among all NoV genotypes, GII.3 is one of the most common genotypes associated with NoV infection, particularly in infants and young children (Saito et al., 2020; Yi et al., 2021). Phylogenetic analyses suggested that the evolution of GII.3 NoV strains is particularly driven by intergenic recombination and the acquisition of new *RdRp* genotypes (Boon et al., 2011; Mahar et al., 2013). Acquisition of new *RdRps* may lead to a faster mutation rate and increased genetic diversity, improving overall GII.3 fitness. According to previous reports, GII.3 has been associated with at least nine different *RdRp* genotypes since 1975, such as GII.3 [P4], GII.3 [P12], GII.3 [P19], GII.3 [Pa], GII.3 [Pb], GII.3 [Pd], GII.3 [Pg], GII.3 [P16] and GII.3 [Pe] (Bull et al., 2005; Mathijs et al., 2011; Medici et al., 2014). Online BLAST searches indicated that the recombinant GII 3

[P25] had been detected in AGE cases in Japan in 2000 (Hokkaido/34B/2000/JP) and 2002 (T66eGII/02/JP). After that, no sequence of related type was submitted to the GenBank database. Nearly 20 years later, this rare recombinant re-emerged in China (this study) and the United States (CA-RGDS-1158/2022/US). As far as we know, GII 3 [P25] recombinant has not been reported in the literature before in China. Phylogenetic analyses indicated that the *VP1* genes of GII.3 [P25] 2021–2022 strains in this study were separated from Japan 2000–2002 strains, and genetically closely related to GII.3 strain identified in Japan in 2006.

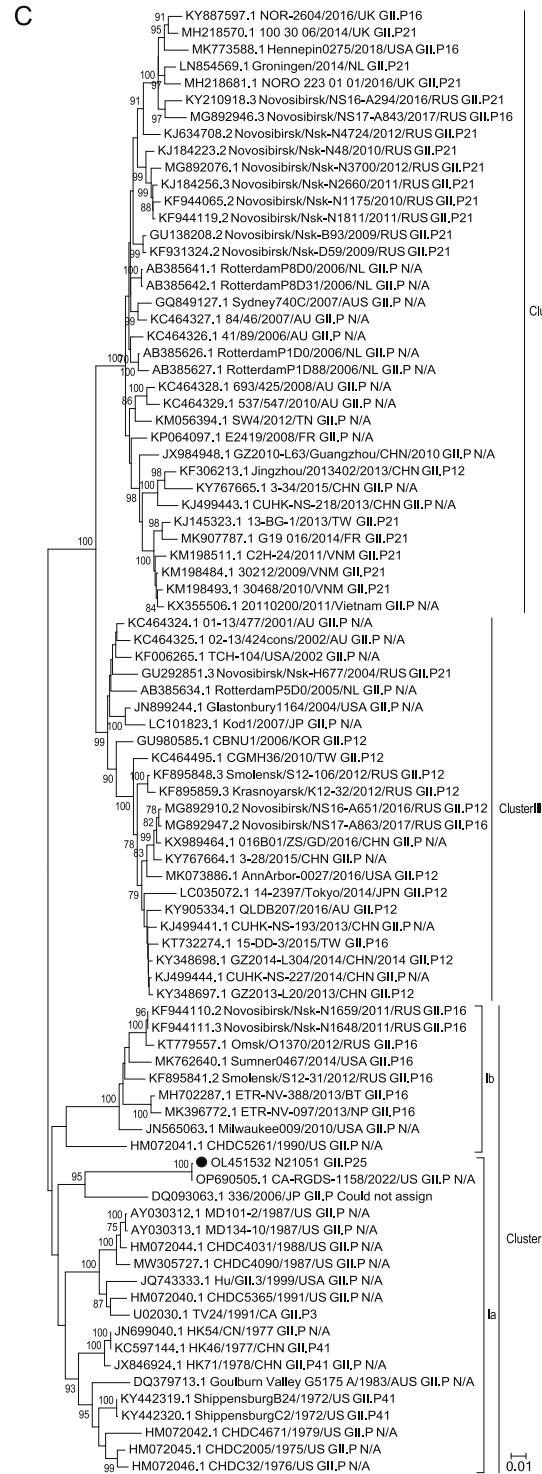
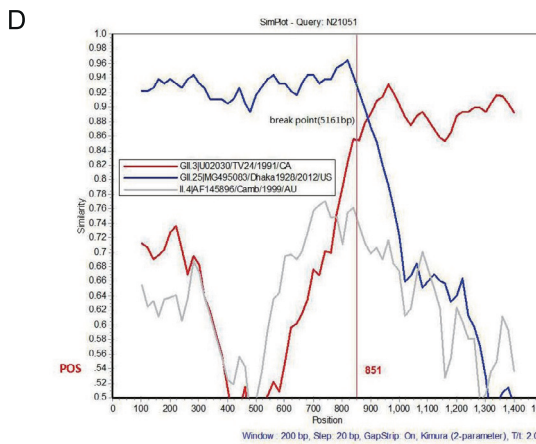
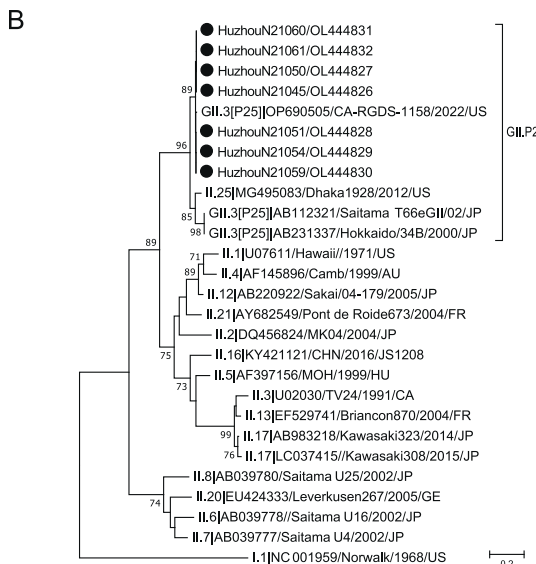
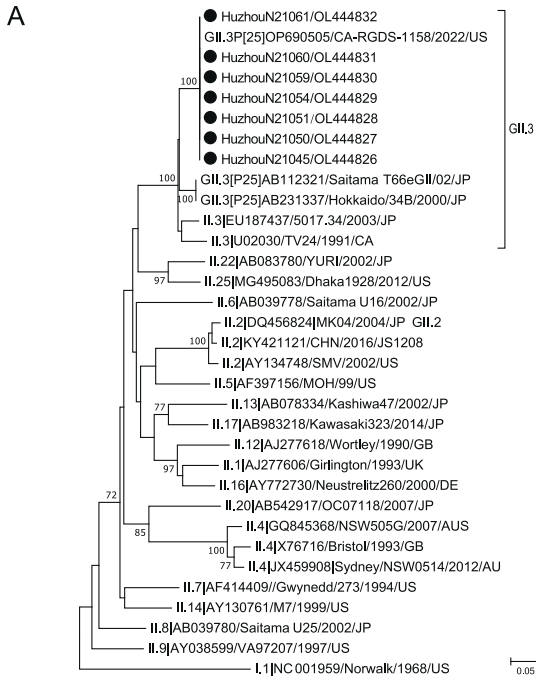
GII.3 NoVs undergo constant evolution, intergenic recombination was not the only force driving GII.3 NoVs evolution, with evidence of antigenic variations and selective (Boon et al., 2011; Mahar et al., 2013; Saito et al., 2020). In 2020, Saito et al. performed an evolutionary analysis of 239 full-length GII.3 *VP1* sequences detected in various countries from 1972 through 2019 to map out the evolution of GII.3 NoVs over time (Saito et al., 2020). Time-scaled phylogenetic tree showed that three distinct clusters (I, II, and III) have emerged over time, which was the same as that in our study (Fig. 1C). Previous studies also showed that GII.3 NoVs could be divided into GII.3a, 3b and 3c based on partial *VP1* sequences (region C nt 5058–5401 and region D nt 6432–5684, positions based on ×86557) as used by CaliciNet surveillance network (Vinje, 2015), in which GII.3a and GII.3c were compatible with the present cluster I and cluster II. Compared with the earliest GII.3 strain of cluster I (ShippensburgC2/1972/US), GII.3 [P25] 2021–2022 strains identified 19 unique amino acid substitutions in their *VP1* gene. Just recently, Yufang Yi et al. experimentally identified two GII.3-specific blockade antibodies 8C7 and 8D1, and mapped the epitopes of 8C7 and 8D1 to residues 385–400 and 401–420 of the *VP1* capsid protein, respectively (Yi et al., 2021). In addition, another structural study has defined the HBGA-binding site (HBS) on the GII.3 strain TV24 (cluster I) P domain, which is shaped primarily by eight residues including T357, R358, K363, D386, D388, S449, G451, and R452 (Yang et al., 2019). It is worth pointing out that among these unique amino acid substitutions carried by GII.3 [P25] 2021–2022 strains, seven substitutions (Q391M, N404T, E405D, A406E, S412V, D415R and F420V) were found in blockade antibody epitopes area and 1 substitution (R452T) was found in HBS III. Taken together, we postulate that such antigenic variation may have contributed to the re-emerge of GII.3 [P25] strains.

Evolutionary analysis based on polymerase and capsid protein regions is more helpful in understanding the driving forces behind the re-emergence of GII.3 [P25] strains. However, conducting a BLAST search on GenBank showed that there are few number of complete *RdRp* sequences available in the public databases for GII [P25] (only four complete *RdRp* sequences), making it impossible to conduct sufficient analysis of the evolution between the *RdRp* genes of GII [P25] strains.

In this study, the first genome sequence of rarely reported GII.3 [P25] recombinant strains in China, was determined and characterized by NGS in the spring of 2021 in Huzhou. It should be noted that we also observed a low detection of GII.3 [P25] in sporadic NoV cases in the winter of 2021 in Huzhou (data not show). Continuous surveillance is needed to monitor the epidemiology of this re-emerged recombinant and further studies are needed to explore the mechanisms behind the spread in population of this genotype.

Footnotes

This study was supported by grants from “Special support plan for local high-level talents in South Taihu Lake” of Huzhou. The authors declare that they have no conflicts of interest. This research was approved by the Ethics Committee of the Huzhou Center for Disease Control and Prevention. Each study participant (or their guardian) provided their informed consent for their information and samples to be used in this study.



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Fig. 1. A, B Phylogenetic analyses based on partial *VP1* sequences (A) and *RdRp* sequences (B) of GII.3 [P25] strains. The trees were generated using the neighbor-joining method, validated by 1000 bootstrap replicates. Bootstrap values $\geq 70\%$ are shown on the branch. GII.3 [P25] strains identified in this study are indicated by closed circles. The reference sequence names are formatted as genotype/GenBank accession number/sample name/isolate time/country. C Phylogenetic analyses based on complete *VP1* sequences of GII.3 strains. The trees were generated using the neighbor-joining method, validated by 1000 bootstrap replicates. Bootstrap values $\geq 70\%$ are shown on the branch. GII.3 [P25] strain identified in this study is indicated by closed circles. GII.P N/A represents that the polymerase sequence were not available. D SimPlot analysis of N21051 strains performed using a window size of 1500 nucleotides. Simplot analysis of N21051 using GII.3[P3] (U02030/TV24/1991/CA) and GII.25[P25] (MG495083/Dhaka1928/2012/U) as subtype references. The vertical line marks the beginning of ORF2 region.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.virs.2023.06.008>.

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