**Virologica Sinica**

**Supplementary Data**

**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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**Materials and methods**

**AGE outbreak surveillance and Sample collection**

Cases of AGE were defined as patients with diarrhea (three or more loose stools) and/or vomiting (two or more episodes) within a 24 h period, possibly accompanied by abdominal pain, fever, and nausea. An outbreak was defined as to have three or more epidemiologically linked AGE cases within a period of three days. Outbreaks were reported to district-level Center for Disease Control and Prevention (CDC) for routine surveillance and outbreak investigations. Specimens were collected and sent to the Huzhou Municipal CDC for NoV detection. A confirmed NoV outbreak was defined as one from which at least two AGE cases tested positive for NoV.

**NoV detection**

Viral RNA was extracted from 200 μL fecal suspensions using a QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer’s instructions. The presence of GI and GII genogroup NoV was detected using a One Step PrimeScript® RT-PCR Kit (DRR064, TaKaRa, Dalian, China) and genogroup-specific primers and probes as described previously (Jothikumar et al., 2005; Ji et al., 2013).

**NoV genotyping**

Samples that tested positive for NoV by RT-qPCR were selected for genotyping. Sequences covering the overlap between ORF1 and ORF2 were amplified by RT-PCR using the primers MON431/G2SKR for GII viruses (Kojima et al., 2002; Vinjé et al., 2003). RT-PCR was carried out using a TAKARA One-Step RT-PCR Kit (RR057A, TaKaRa Biotechnology) with the following amplification conditions: RT at 50 °C for 30 min and denaturation at 94 °C for 2 min, followed by 40 cycles of 30 s at 94 °C, 35 s at 50 °C, and 1 min at 72 °C, and then a final extension at 72 °C for 10 min. All PCR products were purified and sent to the TaKaRa Biotechnology (Dalian, China) Company for Sanger sequencing. Preliminary genotypes were assigned by using the RIVM online NoV genotyping tool (<http://www.rivm.nl/mpf/norovirus/typingtool>) (Kroneman et al., 2013).

**Full-length genome amplification and sequencing**

One NoV-positive sample N21051 was selected for deep sequencing using the Illumina platform. The full genome was amplified by RT-PCR with a Multiple PCR Encirhment Kit for NoV (Shanghai Biogerm, China), performed in a GeneAmp PCR System 9700 (Thermo Fisher, USA). PCR products were purified using AMPure XP beads (Beckman Coulter, USA). The quantity of the purified nucleic acid sequences was examined using Qubit assay (Invitrogen, USA). Norovirus libraries were constructed using A Whole genome DNA Library Construction Kit (Shanghai Biogerm, China) according to the manufacturer’s instruction. Paired-end Illumina sequencing was performed on a Miseq platform by using a Miseq v2 Reagent Kit (Illumina, USA). The quality control and filtering of raw data were analyzed using Fastqc (v0.11.9) and Fastp (v0.23.2). Sequences were assembled using SPAdes (v3.14.1) software.

**Phylogenetic analysis**

All nucleotide and amino acid sequence alignments were performed using Bioedit and MEGA 7.0 software. Phylogenetic analysis was performed using MEGA software (version 7.0) (Kumar et al., 2016). The dendrograms were constructed using the neighbor-joining method validated by 1000 bootstrap replicates. Recombination analysis was conducted using the SimPlot program (version 3.5). The SimPlot analysis was performed by setting the window width and the step size to 200 bp and 20 bp, respectively.

**Reference**

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Fig. S1. Amino acid variation of the VP1 capsid region of GII.3 NoVs over time. The conserved HBGA-binding interfaces in GII.3 are indicated by empty rectangles. Blockade antibody epitopes sites defined for GII.3 strains are indicated in blue (8C7: sites 385–400; 8D1: sites 401–420). Compared with the earliest GII.3 strain (ShippensburgC2/1972/US), 19 unique amino acid substitutions carried by GII.3[P25] 2021–2022 strains are highlighted in orange.