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# Characterization of virus-like particles derived from a GII.3 norovirus strain distantly related with current dominating strains

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**Abstract** Genogroup II, genotype 3 noroviruses (GII.3 NoVs) are secondary to GII.4 NoVs in causing acute non-bacterial gastroenteritis worldwide. In our previous study, we found that virus-like particles (VLPs) derived from a GII.3 NoV strain exhibited no binding activity to any salivary and synthetic histo-blood group antigens (HBGAs) tested. In this study, the nucleotide sequence encoding the major capsid protein of another documented GII.3 NoV strain was codon-optimized and synthesized, and the major capsid protein was expressed using recombinant baculovirus virus expression system. The assembly of VLPs was verified by electron microscopy, and the binding profiles of the assembled VLPs to salivary HBGAs were determined, and in vitro VLP-salivary HBGAs binding blockade assay was used to test the cross-blocking effects of hyperimmune sera produced against different genotypes (GI.2, GII.3, and GII.4). The expression of the major capsid proteins led to the successful assembly of VLPs, and in vitro VLP-salivary HBGAs binding assay indicated that the assembled VLPs bound to salivary HBGAs from blood type A, B, AB, and O individuals, with the highest binding capacity to type A salivary HBGAs. In vitro VLP-salivary HBGAs binding blockade assay demonstrated the absence of blocking activities for hyperimmune sera produced against GI.2and GII.4 VLPs and the presence of blocking activity for that against GII.3 VLPs. Our results suggest the absence of cross-blocking activities among different genotypes and the presence of blocking activities between GII.3 NoVs from different clusters, which might have implications for the design of multivalent NoV vaccines.

**Keywords** Noroviruses · Histo-blood group antigens · Cross-blocking · GII.3 NoV virus-like particles

## Introduction

Noroviruses (NoVs) are the leading cause of acute non-bacterial gastroenteritis worldwide [1]. It can be divided into at least six genogroups (GI–GVI) based on nucleotide sequences of the major capsid protein, with GI, GII, and GIV infecting humans. GI and GII NoVs can be further divided into at least nine GI (GI.1–GI.9) and 18 GII genotypes (GII.1–GII.18), with GII.4 causing most epidemics and sporadic cases worldwide [1]. NoVs are single-stranded, positive polarity RNA viruses with polyadenylated tails and a genome size of approximately 7.5–7.7 kb. NoVs can infect B cells in vitro and in vivo, but proper small animal models are still lacking which delayed our understanding of the biological life cycles of NoVs when compared with other enteric viruses [2]. Epidemiology studies indicate that GII.4 NoV variant, Sydney 2012, is currently the epidemic strain, accounting for approximately 70 % of the identified cases, while GII.3 NoVs is ranked in the second place, accounting for approximately 20 % of the identified cases [3, 4]. In some countries, the incidence rate of the GII.3 NoVs-related cases is as high as more than 50 % [5].

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GII.3 NoVs are mostly isolated from children under 5 years of age presenting acute gastroenteritis. Evolutionary analysis of GII.3 NoV strains isolated from a period spanning more than 30 years indicates that it evolves at a similar rate with the prevalent GII.4 NoVs. Amino acid sequence analysis of major capsid proteins from distant strains shows a change of approximately 30 %, less than that of GII.4 NoVs (67.1 %) [6].

Histo-blood group antigens (HBGAs) are carbohydrates covalently linked to lipids or proteins anchored on red blood cells or epithelial cells or existing freely in body fluids such as saliva. The linkage of HBGA recognition with NoVs infection was first demonstrated by a human volunteer challenge study as secretor-negative volunteers were not infected by Norwalk virus even at high doses [7]. Further studies using in vitro receptor binding assay demonstrate that virus-like particles (VLPs) derived from the capsid protein of Sydney 2012 strain exhibit broad binding patterns against HBGAs, while those from GII.3 strains show weak binding activity to certain HBGAs or no binding activity to any HBGAs tested [6, 8, 9]. The discovery of new receptors or factors that promote the binding of GII.3 NoV VLPs to HBGAs is a key for vaccine development and vaccine efficacy evaluation. In our previous study, a GII.3 NoV strain was isolated and its capsid protein was expressed using recombinant baculovirus expression system which led to successful assembly of VLPs. In vitro VLP-HBGAs binding assay indicates that it shows no binding signal to all HBGAs tested.

To characterize further the actual binding profiles of GII.3 NoVs to salivary HBGAs, we selected a documented GII.3 strain (GenBank accession No. AGI17594) of which the major capsid protein had a similarity of 91.8 % in amino acid sequence with previously isolated GII.3 strain [10]. The nucleotide sequence encoding the major capsid protein of the documented GII.3 isolate was codon-optimized, synthesized, and inserted into recombinant baculovirus transfer vector to express the major capsid protein. Subsequently, in vitro VLP-salivary HBGAs binding and binding blockade assays were used to fully characterize the binding profiles of produced VLPs and cross-blocking effects of hyperimmune sera against different genotypes, respectively.

## Materials and methods

### Generation of recombinant baculoviruses and purification of VLPs

The nucleotide sequence encoding the major capsid protein of the selected GII.3 NoV isolate was codon-optimized based on the codon usage frequency of *Spodoptera* (*S.*) *frugiperda* cells, synthesized by GenScript company, and ligated into pBacPAK9 transfer vector with BamHI and NotI restriction enzyme sites at its 5′ and 3′ sites, respectively. The construct was sequenced to be correct and used to transfect sf9 cells as described previously [11]. Briefly, purified pBacPAK9 vector containing the target gene (2 μg) was first mixed with 5 μl Bsu36 I-digested viral DNA, and then 4 μl Bacfectin was added. The mixture was vortexed and incubated at room temperature for 20 min before being transferred to wells preseeded with sf9 cells in a 6-well plate. The medium containing recombinant baculovirus was generally harvested 5–7 days later for storage or for infecting more cells. Expression of target protein was verified by Western blotting; protein purification was performed using methods as described previously [11]. The integrity of CsCl purified VLPs was negatively stained and observed under electron microscope.

### GII.3 and GII.4 NoV VLP positive serum sample screening

Serum samples were collected from outpatients undergoing routine blood test. Anti-GII.3 and GII.4 (GenBank accession No. KF306214) NoV VLP IgG-positive serum samples were determined using enzyme-linked immunosorbent assay (ELISA). Briefly, purified GII.3 and GII.4 VLPs were coated onto a 96-well plate at 0.2 μg/ml (100 μl/well). Human serum samples diluted 1:100 in PBS supplemented with 0.05 % Tween-20 (PBS-T) and 1 % BSA were added to VLP-coated wells, and the plate was incubated at 37 °C for 1 h. After washing for five times with PBS-T, HRP-conjugated goat anti-human IgG diluted 1:20,000 in PBS-T containing 1 % BSA was added. The plate was incubated at 37 °C for 30 min and then washed five times with PBS-T. Final product was detected by the addition of HRP substrates, followed by measurement of Optical Density values at 450 nm ($OD_{450}$). Serum samples giving high $OD_{450}$ values against both VLPs were used as detection antibody in in vitro VLP-salivary HBGAs binding and binding blockade assays.

### Phylogenetic analysis

The full-length major capsid protein (VP1) coding nucleotide sequence used in this study, together with 20 VP1 coding nucleotide sequences from GII.3 strains available in the GenBank database, was used for evolutionary analysis. Sequence alignments were performed with Clustal W, and parameter values for the best-fit model of nucleotide substitution were determined to be TN93 +G (Tamura-Nei, gamma distribution shape parameter). A phylogenetic tree was inferred by maximum-likelihood reconstruction based on the nucleotide alignment of full-length VP1 nucleotide sequences as implemented in the Mega software. The statistical

significance of constructed phylogenetic tree was estimated by bootstrap analysis with 1000 pseudoreplicates datasets. The GenBank accession numbers of complete VP1 nucleotide sequence used for phylogenetic analysis are as follows: KJ499443, KJ499444, KC464324, KC464325, JQ743333, KC464326, KC464327, HM072045, HM072046, HM07 2044, HM072042, HM072041, HM072040, JX984948, JN699040, JN699039, KF306213, KC597140, AB385626, AB385642, and GQ849127.

### In vitro VLP-salivary HBGAs binding assay

In vitro VLP-salivary HBGAs binding assay was performed as described previously with minor modifications [12]. Briefly, purified GII.3 VLPs diluted in PBS-T plus 1 % BSA at 2 μg/ml were added to wells coated with blood type A, B, AB, and O saliva samples in 96-well plates and incubated at 37 °C for 1 h. Wells with PBS-T containing 1 % BSA buffer only served as negative control. Human anti-GII.3 and anti-GII.4 VLP IgG-positive sera diluted 1:1000 in PBS-T plus 1 % BSA were added to indicated wells and incubated at 37 °C for 1 h. Subsequently, HRP-conjugated goat anti-human IgG diluted 1:20,000 in PBS-T plus 1 % BSA was added and incubated at 37 °C for 30 min. The final product was detected by the addition of TMB and peroxide urea. Five washes with PBS-T were performed between incubations. The $OD_{450}$ was read by a Multiscan reader.

### In vitro VLP-salivary HBGAs binding blockade assay

In vitro VLP-salivary HBGAs binding blockade assay was performed basically in the same manner as in vitro VLP-salivary HBGAs binding assay except the preincubation of GI.2, GII.3, and GII.4 VLPs (2 μg/ml) with rabbit anti-genotype specific hyperimmune sera (GI.2, GII.3 and GII.4) or rabbit serum at 37 °C for 30 min before transferring to blood type A and O saliva sample-coated wells. For the detection of blocking activities of hyperimmune serum against the binding of GI.2 VLPs to salivary HBGAs, hyperimmune serum specific for GI.2 VLP was used as detecting antibody. Duplicate wells added with PBS-T only served as positive control. The blocking index was calculated in % as (mean OD without sera-mean OD with sera)/mean OD without sera × 100 %.

## Results

### Expression of major capsid protein led to assembly of VLPs

The full-length of the expressed capsid protein of the selected GII.3 isolate was 549 amino acids (aa), one aa longer than those of most and our previously reported GII.3 strains (548 aa) [10]. The inserted aa (T) was located between aa305 and aa306 compared with those of other GII.3 strains. Recombinant baculovirus containing the codon-optimized capsid protein coding sequence was generated and target proteins were purified by CsCl density gradient centrifugation. The visible band was collected, ultracentrifuged, dissolved in filtered PBS, and examined under TEM after negative staining with phosphotungstic acid. As shown in Fig. 1a, expressed capsid proteins were successfully assembled into VLPs. The intactness of expressed capsid proteins were analyzed by Western blot, showing a single band (Fig. 1b,) rather than a doublet band that is commonly seen for most expressed capsid proteins from genogroup II NoVs [11, 13].

### The selected GII.3 NoV isolate did not cluster with known isolated strains

The full-length nucleotide sequence of the major capsid protein of the selected GII.3 isolate, along with those of other 20 GII.3 strains available in the GenBank database, was used for phylogenetic analysis to map out the evolutions of GII.3 NoVs (Fig. 2). Two distinct clusters were identified, with cluster I comprising earlier isolated strains (1975–1999) and cluster II comprising recent isolates (2001–2014). Surprisingly, the strain used in this study did not fit into any clusters, but seems more closely related with cluster II. Of note, in cluster II, two subclusters can be clearly identified.

### Assembled GII.3 VLPs bound to all types of salivary HBGAs

In vitro VLP-salivary HBGAs binding assay was used to characterize the binding patterns of the prepared VLPs. HBGAs present in all saliva samples used in this study have been determined by HBGA epitope-specific monoclonal antibodies (submitted data). As shown in Fig. 3, GII.3 VLPs bound to all saliva samples tested, demonstrating the highest binding capacity to blood type A salivary HBGAs, the lowest to blood type B and AB salivary HBGAs, and the moderate to blood type O HBGAs.

### Binding of GII.3 VLPs to type A and O salivary HBGAs was unable to be blocked by rabbit hyperimmune sera specific for GI.2 and GII.4 VLPs

To determine if the binding activity of GII.3 VLPs to salivary HBGAs can be blocked by anti-genotype-specific hyperimmune sera, in vitro VLP-salivary HBGA binding blockade assay was performed. Type A and O salivary HBGAs were used as they gave higher OD values during

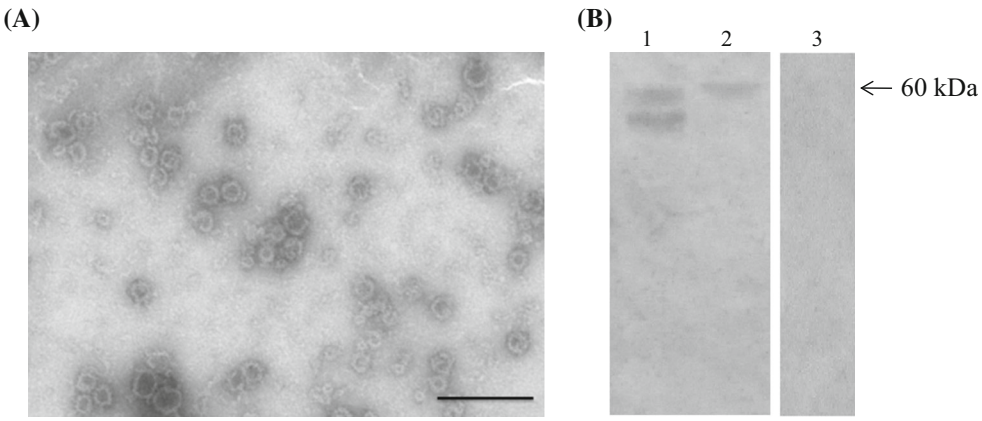


**Fig. 1** EM (**a**) and Western blot (**b**) analyses of purified GII.3 VLPs. In (**b**), *lane 1* was loaded with purified GII.3 VLPs as reported in our previous study as control [9], *lane 2* with harvested sf9 cell medium infected with recombinant baculovirus constructed in this study, and *lane 3* with supernatant from sf9 cells infected with wild-type baculovirus. *Scale bar*, 200 nm

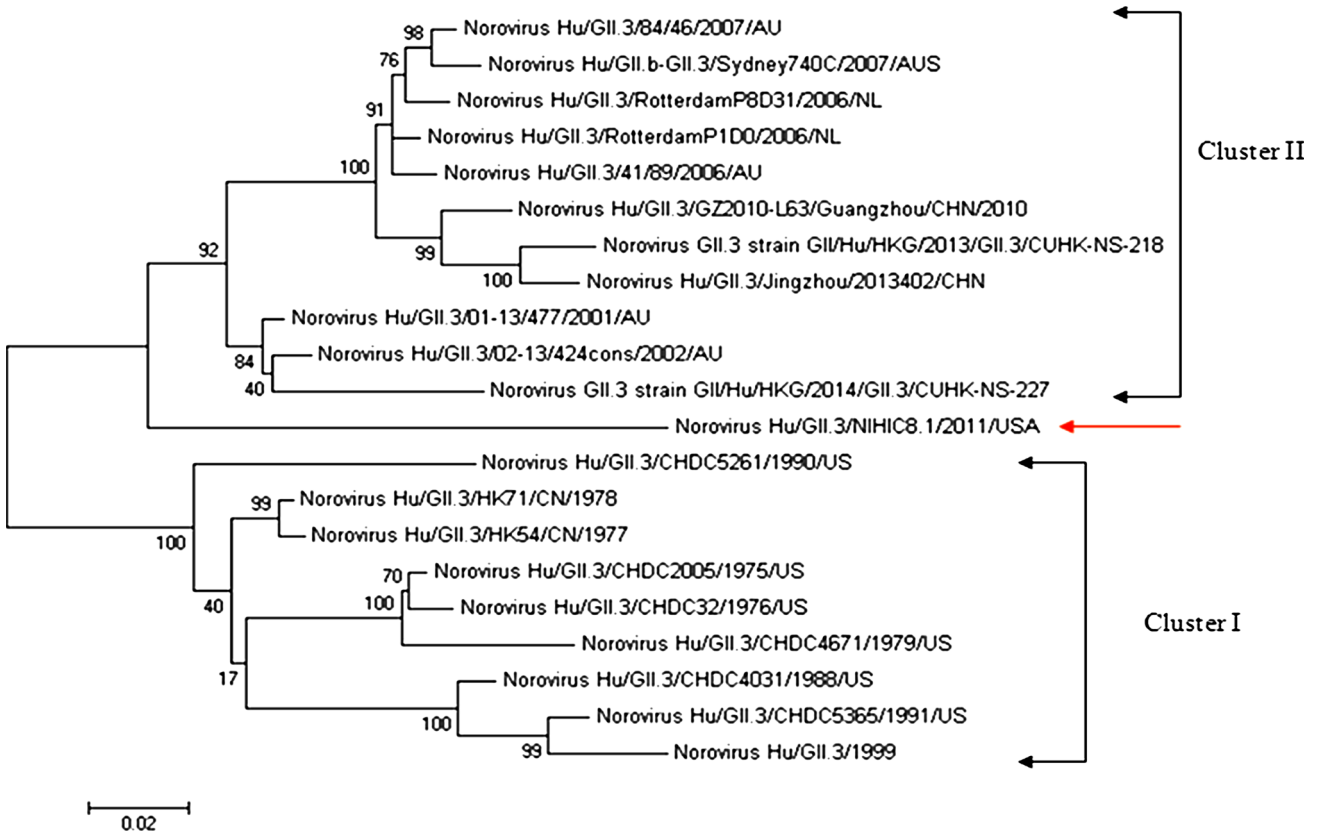


**Fig. 2** Phylogenetic analysis of 21 GII.3 NoVs based on complete ORF2 nucleotide sequences. Bootstrap values are shown on the corresponding branches and *scale bar* represents the units for the expected numbers of substitution per site. The strain used for VLP preparation in this study is marked by *red arrow*

VLP-salivary HBGAs binding assay. As expected, rabbit anti-GI.2 and GII.4 VLP hyperimmune sera did not show blocking activities against the binding of GII.3 VLPs to blood type A and O salivary HBGAs ,while previously prepared anti-GII.3 NoV VLP hyperimmune serum blocked the binding of GII.3 VLPs to blood type A and O salivary HBGAs (Fig. 4a, b).

## Discussion

Epidemiological studies indicate that GII.3 NoVs are secondary to the most isolated GII.4 NoVs, as the etiology agent for acute non-bacterial gastroenteritis. In our ongoing epidemiological study of NoVs in Zhengzhou city, Henan province, China, 19 GII.3 strains were isolated, accounting

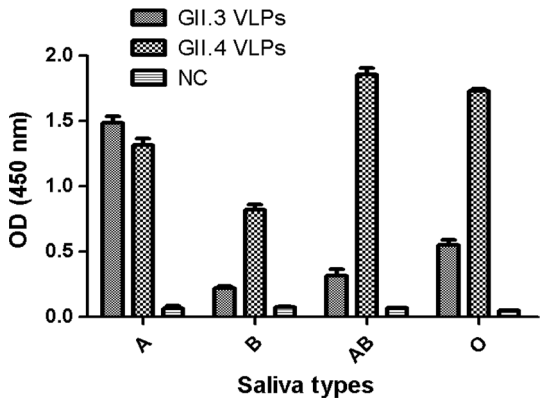


**Fig. 3** Saliva-VLPs binding assay. Binding of GII.3 and GII.4 VLPs (2 μg/ml) to salivary HBGAs was detected using anti-GII.3 and GII.4 IgG-positive human serum, followed by the addition of HRP-conjugated goat anti-human IgG

for 27 % (19/70) of total isolates. The endemic status of GII.3 NoVs in children suggests that infection might confer long-term immunity in later life. HBGA binding–blocking antibodies in serum have been suggested to be effective indicators of protection against NoV infection [14–16]. Detailed HBGA binding–blocking activities of anti-GII.4 sera have been characterized, and sera collected from human volunteers immunized with consensus GII.4 VLPs exhibited blocking activities against broad GII.4 variants, including novel variants not circulating at the time of vaccination [17]. Contrary to GII.4 strains, immunogenicity of GII.3 NoVs has not been studied in human volunteers. Due to weak binding capacity or absence of binding capacity of GII.3 NoV VLPs to synthetic and salivary HBGAs, the homotypic and heterotypic cross-blocking activities of anti-NoV VLP hyperimmune sera cannot be performed [6, 9, 18]. In our previous study, assembled GII.3 VLPs were unable to bind to blood type A, B, AB, and O salivary HBGAs. Further study using more saliva samples (26 samples) from blood type A, B, AB, and O individuals indicated that GII.3 VLPs bound weakly to salivary HBGAs from several blood type A and B individuals (unpublished data). To characterize the binding profiles of GII.3 NoVs and test the cross-blocking activities of anti-genotype specific hyperimmune sera, the major capsid protein of a GII.3 NoV strain in circulation but distantly related to dominating GII.3 strains in aa sequence was expressed using the recombinant baculovirus expression system.

The expressed VP1 has 549 aa in length and a T insertion between aa305 and aa306 compared with those of dominating GII.3 strains. The inserted site is predicted to form a surface-exposed loop by homology modeling [19]. The similarity in aa sequence between the expressed VP1 and current dominating GII.3 isolates is approximately 92 %, with most varied aa located in the P2 subdomain. To improve protein expression level, the coding sequence for the major capsid protein was codon-optimized. The recombinant VP1 was expressed and purified; assembly of VLPs was verified by electron microscopy. SDS-PAGE and Western blotting analyses indicated the presence of a doublet band that is commonly seen for most GII NoV VP1. Of note, the uncleaved form of VP1 is dominant, while cleaved VP1 was dominant in previously prepared GII.4 and GII.3 VLPs [9, 11]. It is possible that cleavage depends on the N-terminal sequence of VP1 as the N-terminal sequence swapped with that of prototype Norwalk virus led to the abortion of cleavage. Further study by adding certain length of aa to the N-terminal of the previously expressed GII.4 VP1 showed that a 4-aa addition did not affect the cleavage, while an 11-aa addition totally aborted the cleavage (unpublished data).

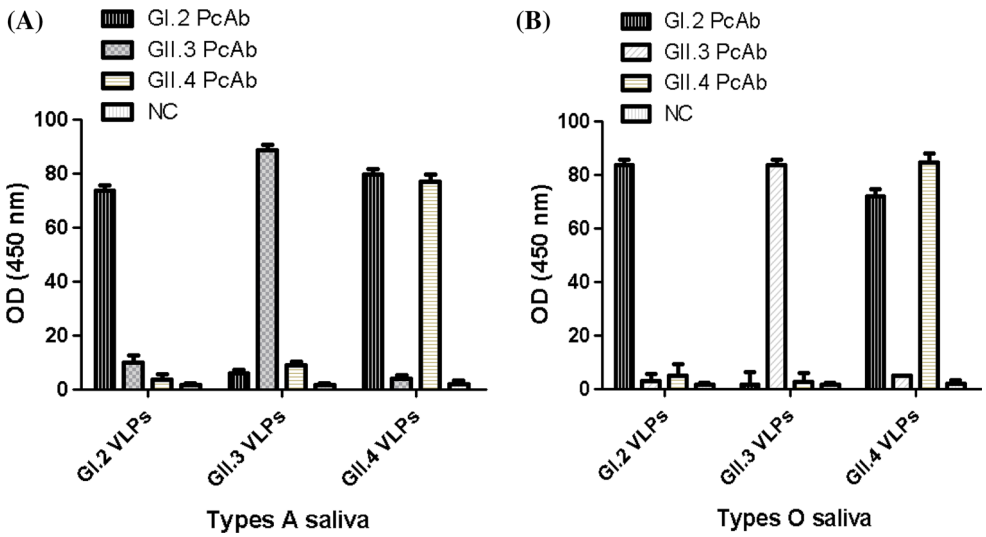


**Fig. 4** Saliva-VLPs binding blockade assay. Rabbit anti-GI.2., GII.3, and GII.4 VLPs hyperimmune sera were used at 1:200, and GI.2, GII.3, and GII.4 VLPs were used at 2 μg/ml

Before conducting in vitro VLP-salivary HBGA binding and binding blockade assays, purified GII.3 and GII.4 VLPs were used in ELISA to screen for anti-GII.3 and GII.4 VLP IgG-positive human serum samples. The serum samples giving high OD values were used in subsequent VLP-salivary HBGA binding and binding blockade assays, as selected human serum samples gave higher sensitivity in both assays when compared with previously prepared rabbit anti-GII.3 VLP hyperimmune serum. Using previously characterized GII.4 NoV VLPs, the selected human serum samples showed the same results when compared with that of guinea pig anti-GII.4 VLP hyperimmune serum in both binding and binding blockade assays, indicating applicability of their uses in these assays. VLP-salivary HBGAs binding assay indicated that assembled GII.3 VLPs bound to all saliva samples with the highest binding capacity to blood type A saliva samples, moderate to blood type O saliva sample, and lowest to blood type AB and B saliva samples. Our results are consistent with previously reported MxV strain, a GII.3 NoV, which has been reported to bind to blood type A and B saliva samples, but not or weakly bind to blood type O saliva samples [8, 20]. The binding similarity indicates conservation of binding specificities to HBGAs for GII.3 NoVs as both VP1 share a similarity of 90.5 % in aa sequence.

Inter- and intra-genotypic immune cross-blocking activities against GII.3 VLPs have not been reported due to weak binding of GII.3 VLPs to synthetic and salivary HBGAs. In this study, GII.3 VLPs bound strongly to blood type A and O saliva samples which makes in vitro binding blockade assay feasible. Binding blockade assay using previously characterized rabbit anti-GI.2, GII.3, and GII.4 VLPs hyperimmune sera indicated the absence of intergenotype cross-blocking activities. Considering the presence of blocking activity of anti-GII.3 VLPs hyperimmune serum and distant relationship of the two strains as indicated by phylogenetic analysis, GII.3 strains might share a conserved HBGA binding interface. The broad binding profiles of this GII.3 VLP against salivary HBGAs and the fact that minimal numbers of isolates exhibit close sequence similarity suggests that HBGA binding spectrum might not be the only factor determining host susceptibility to GII.3 NoVs. In a phase I clinical study, it is found that certain placebo subjects without VLP-specific blocking antibodies prechallenge are not infected with GII.4 NoV inoculum [16]. The discoveries that enteric bacteria promotes NoV infection of B cells and that NoV binds to HBGA-like substance present on bacterial surface indicate that in vitro VLP-salivary HBGAs binding and binding blockade assays might not reflect actual susceptibility and protective status of an individual [2, 21]. We recently had an interesting finding that fecal pretreatment of previously expressed GII.3 VLPs promoted its binding capacity to blood type A, B, AB, and O salivary HBGAs. Boiling or addition of protease-cocktail inhibitors aborted or reduced such effect. SDS-PAGE analysis indicated that the capsid proteins were cleaved. The study of underlying mechanism of fecal extract enhancement is underway.

In summary, the absence of cross-blocking activities among anti-NoV VLP hyperimmune sera, including previously prepared anti-GII.3 VLP serum, suggests application of current clinical scheme used for GII.4 and GI.1 NoVs to study immunogenicity of GII.3 NoVs in human volunteers is required. To better characterize the binding profiles of newly isolated GII.3 strains, expression of VP1 of these strains is being planned. As matched saliva and serum samples were also collected in our epidemiological study, further study with these newly isolated GII.3 strains will definitely provide valuable information about immunogenicity and binding profiles of these viruses.

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**Compliance with ethical standards**

**Competing interests** The authors declare that they have no competing interests.

**Ethical approval** This article does not contain any study with human participants or animals performed by any of the authors.

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