SHORT COMMUNICATION

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# The endemic GII.4 norovirus-like-particle induced-antibody lacks of cross-reactivity against the epidemic GII.17 strain

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## Abstract

Norovirus-like particle (VLP) vaccine is promising against human norovirus infection. Unfortunately, genetic diversity of norovirus hindered the development of this vaccine. In this study, the immunogenicity of norovirus VLPs induced by the endemic GII.4 and the epidemic GII.17 genotypes, and the cross-reactivity between them as well as GI.1 and GII.3 VLPs were evaluated in mice by using serum IgG and histo-blood group antigen (HBGA) blocking antibodies as index. Results showed well immunogenicity of both GII.4 and GII.17 VLPs in mice. Serum IgG GMT (Geometric Mean Titer) were 3.63 (GII.4) and 3.88 (GII.17) respectively, and sustained to the 15th week. The HBGA blocking antibodies were 130 (GII.4) and 360 (GII.17) respectively at the end of the 4th week. Additionally, there was a dramatically statistical difference found in the cross-reactivity within genogroup (GII.3, GII.4 and GII.17) ($p < .001$), and also showed similar difference between genogroups (GI.1 vs. GII.3, GII.4 and GII.17) ($p < .001$). Summarized the pPICZa *pichi pichia* expression system showed a potential to be the alternative for expression of norovirus VLPs in secretion form, and the little cross-reactivity found between the endemic strain and the epidemic strain provides an evident for the consideration of selecting candidates of norovirus vaccine strains.

**KEYWORDS**
cross-reactivity, HBGA, norovirus, vaccine, virus-like particles

## 1  |  INTRODUCTION

Norovirus is one of the major pathogens which cause the global nonbacterial acute gastroenteritis epidemic and brought enormous disease burden to the society.[1] Additionally, the World Health Organization (WHO) found that norovirus infection is the most common cause of death in food-borne gastroenteritis in the "global estimate of the burden of foodborne disease".[2]

The limitations in vitro cell culture and animal models for norovirus have seriously hindered the vaccine development. Fortunately, virus-like particle (VLP) is of great significance in the development of norovirus vaccine. Norovirus VLP has similar immunogenicity and antigenicity as the natural virus particles, which can induce B cell and T cell responses in the host, and stimulate the development of innate immune and adaptive immune responses.[3] Currently, several candidate human norovirus VLP vaccines are in preclinical and/or clinical trials,[4] but no one is licensed till now.

Both genetic diversity and lack of effective heterologous protection result in the challenge for vaccine development.[5] The norovirus genotypes circulated in human beings influenced by immunotypes, which constituted of certain genotypes and their evolutionary patterns.[6] Besides, the genotype-specific herd immunity exerted influence on the

endemic norovirus genotype in the next season and resulted in the serial infections with multiple genotypes in children.[7] In addition, many of antibodies access to the identified broadly cross-reactive epitopes between GI and GII or within GII strains do not bind intact particles. As documented, GII strains cause ~90% of outbreaks, while 50%–70% caused by GII.4 strains.[8] To be noted, during the period of 2014–2015, the prevalence rate of norovirus GII.17 in Asia suddenly elevated, especially in China, and then transformed to be endemic.[9]

The question is does the vaccine provide protection against non-epidemic strains when that happened? In this study, we attempt to explore the immunogenicity of norovirus VLPs induced by the endemic GII.4 and the epidemic GII.17 genotypes, and the cross-reactivity between them as well as GI.1 and GII.3 VLPs in mice by using serum IgG and histo-blood group antigen (HBGA) blocking antibodies as index.

## 2 | MATERIALS AND METHODS

### 2.1 | Recombinant proteins preparation

The recombinant GII.4 and GII.17 VLPs (3 µg/dose) were prepared by adjuvant adsorption with 200 µg Al(OH)$_3$ per dose (totally 500 µl/dose). Both the yeast whole protein lysis (3 µg/dose) and phosphorate buffered salt (PBS), also adjuvanted with 200 µg Al(OH)$_3$ per dose, were used as negative control groups. Additionally, VLPs of GI.1 and GII.3 genotypes were kindly provided by Anhui Zhifei Longcom Biopharmaceutical Co., Ltd and used for test the cross-reactivity.

### 2.2 | Animal immunization

Specific pathogen free (SPF) BALB/c mice were intraperitoneally immunized with the first dose at 6 weeks of age and boosted with an interval of 21 days. Four groups were setup with eight mice in each group. Two groups were immunized with GII.4 and GII.17 VLP respectively, while the other two groups were immunized with yeast whole protein lysis and PBS as negative controls. At Week 4, 6, 11, and 15 post the first dose, the retro-orbital blood was sampled for analyzing norovirus specific IgG and HBGA blocking antibodies. The animal experiment was approved by the Ethical Committee of the Chinese National Institutes for Food and Drug Control (NIFDC), and all the procedures were conducted according to the Animal Experiment Guidelines of the NIFDC. The research was supported by the 13th 5-year National Science & Technology Major Project (2018ZX09739002-002) sponsored by the Ministry of Science and Technology of the People's Republic of China.

### 2.3 | Serum IgG antibodies detection

Serum IgG against type-specific and heterologous VLPs were detected by enzyme-linked immunosor-bent assay (ELISA). Briefly, 96-well MaxiSorp plates (Thermo Fisher Scientific) were coated with GII.4 and GII.17 VLPs (1 µg/ml). Serum samples were added as 2-fold dilution series starting from 1:40 dilution. Biotin-labeled goat anti-mouse IgG antibody was used to detect the bound antibodies, when reacting with o-Phenylenediamine dihy-drochloride (OPD)-substrate (Sigma-Aldrich). Absorbance (optical density [OD]) was measured at 490 nm/620 nm in a microplate reader (EON, BioTek). OD ($A_{490nm}$–$A_{620nm}$) versus dilution curve was plotted. The OD + 2∗standard deviation (SD) of the negative blood of 32 mice that were not treated was used as the Cut-off value. The value to be tested >cut-off value was positive, and the value to be tested ≤cut-off value was negative. Results are expressed as the end-point titer of the individual mouse sera and defined as the highest dilution giving an OD reading above the Cut-off value. Results are compared in the form of logarithmic conversion based on 10.

### 2.4 | HBGA blocking antibodies detection

The binding ability of GII.4/GII.17 VLPs and HBGAs (Le a, Le a (H type 1), H (type 3), Blood A (tri), and Blood B (tri)) (Glycotech) was determined. Briefly, the 96-well MaxiSorp plates (Thermo Fisher Scientific) were coated with 10 µg/ml streptomycin to bind the biotinylated HBGAs (5 µg/ml). Then GII.4/GII.17 VLPs (3 µg/ml) were added to bind each kind of HBGAs. The norovirus genotype specific mouse monoclonal antibody (1:2000 dilutions) and horseradish peroxidase (HRP)-labeled goat antimouse IgG antibody was used as detection antibodies. The staining and OD reading was similar as that used in serum IgG assay.

The HBGA-blocking assay was performed as below. The coating of 96-well microplates with streptomycin was as mentioned above, followed by adding the biotinylated H3 HBGA (Glycotech) (2.5 µg/ml). Meanwhile two-fold serial dilutions of serum (starting from 1:20 dilution) were inculated with each VLP (GII.4, GII.17, GII.3, and GI.1) at 37°C. Then the mixture was reacted with the coated HBGA to detect the unneutralized VLPs. Norovirus genotype specific rabbit polyclonal antisera (anti-GI.1, anti-GII.3, anti-GII.4 and anti-GII.17) were used to bind each type of VLPs. Goat antirabbit IgG-HRP was used to detect bound rabbit antibody. The staining and OD reading was similar as above. Blocking index was calculated as follows: ([VLP control absorbance−test serum absorbance]/VLP control absorbance) × 100%. Results are expressed as the fifty-percent-blocking titer (BT50), a serum titer blocking ≥50% of the VLPs binding to the HBGAs. The minimum BT50 value of the serum to be tested was 25, and the value was designated to be 12.5 when lower than 25, and comparison between groups was performed. Results are compared in the form of logarithmic conversion based on 10.

### 2.5 | Statistical analysis

Statistical analyses were done by using GraphPad Prism 8.0.1 and IBM SPSS Statistics software version 19.0. Fisher's exact test was used to

determine the intergroup differences in IgG and HBGA blocking antibody titers. Statistical significance was defined as a $p < .05$.

## 3 | RESULTS

The serum IgG antibodies induced by the GII.4 and GII.17 norovirus VLPs produced in this study were detected and compared statistically at Week 4, 6, 11 and 15 post the first dose, with both yeast proteins and adjuant as the controls. Results showed that serum IgG antibodies were effectively induced by the endemic GII.4 and epidemic GII.17 norovirus VLPs in BALB/c mice, which expressed and self-assembled in secretion form by using *pichi pichia* expression system. Only 1 week post the second dose, both the VLPs induced the production of anti-GII.4 or anti-GII.17 IgG antibodies quickly, with 3.63 GMT (Geometric Mean Titer) and 3.88 GMT respectively, which showed significant statistical difference when compared to both the two negative control groups ($p < .05$) (both the GMTs of negative controls less than 2.0) (Figure 1A,B). As time goes on, the peak of serum IgG antibodies found in the 6th weeks post the first dose in both two experimental groups. Subsequently it has experienced small fluctuations till the end of this study, and the sustaination of serum IgG antibodies were also detected till to the 15th weeks post the first dose (Figure 1C,D). The cross-reactivity between anti-GII.4 IgG and other three VLPs, also between anti-GII.17 IgG and other three VLPs were assayed and compared. Results showed that the serum IgG antibodies were genogroup and genotype specific. There was no significant cross-reactive found between anti-GII.4 IgG and GI.1, GII.3, GII.17 VLPs ($p < .05$), also between anti-GII.17 IgG and GI.1, GII.3, GII.4 VLPs ($p < .05$) (Figure 1E,F).

The binding patterns of five types of HBGAs with GII.4 (A) and GII.17 (B) VLPs were analyzed. H (type 3) HBGA showed the optimal binding activity with both the GII.4 and GII.17 VLPs, followed by Blood B (tri), Le a (H type 1), Blood A (tri), and Le a (Figure 2A,B). Based on
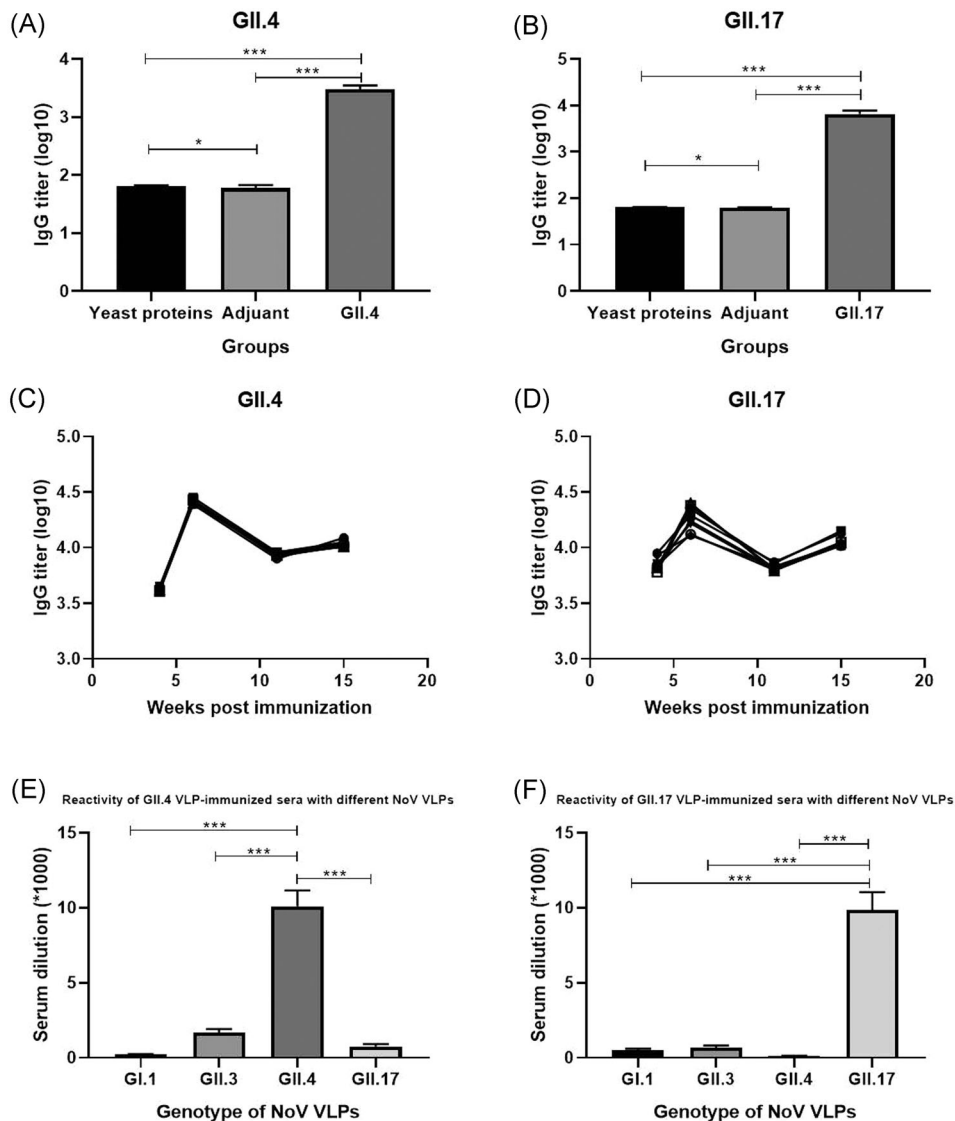


FIGURE 1 Serum IgG antibodies induced by the endemic GII.4 and epidemic GII.17 norovirus VLPs in BALB/c mice, and cross-reactivity between and within genogroups. *$p < .05$; **$p < .01$; ***$p < .001$. VLP, virus-like particle
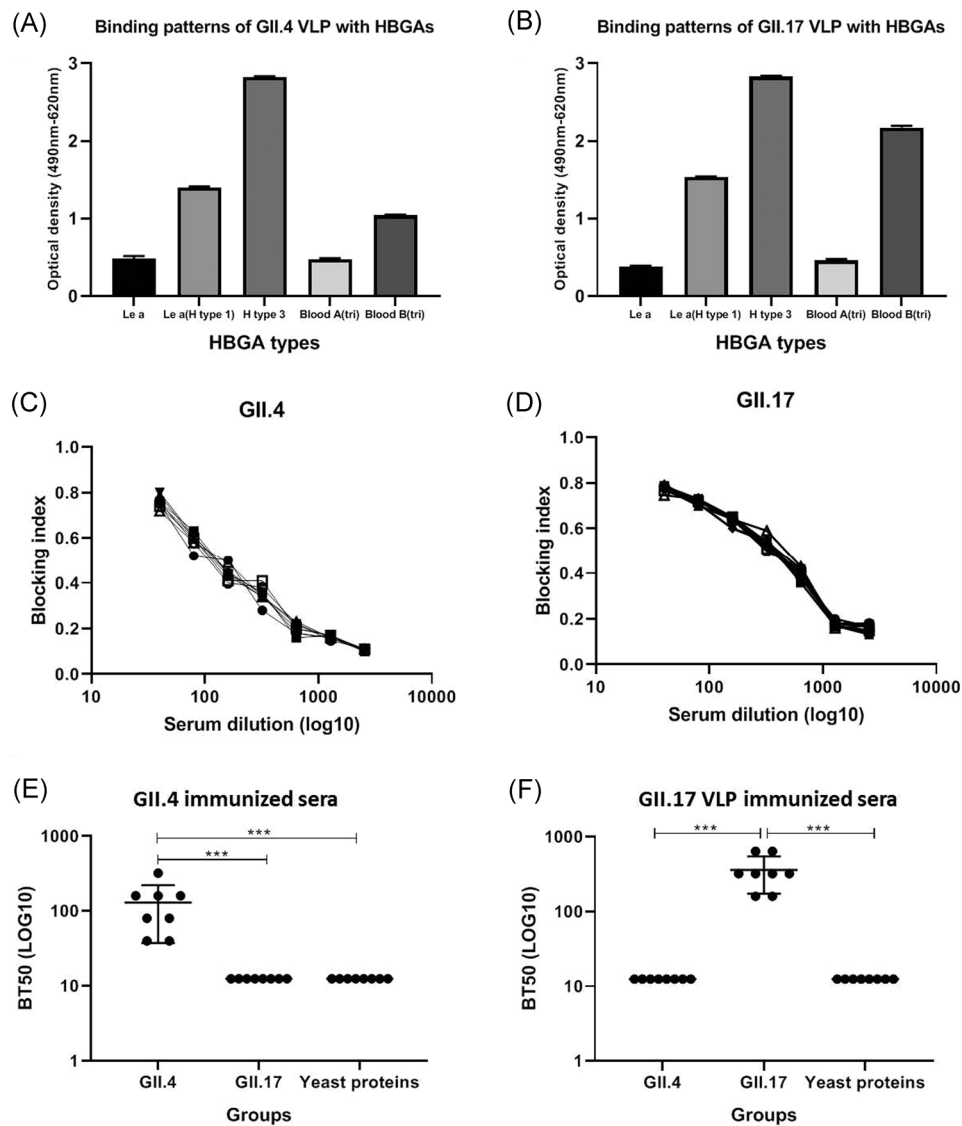
**FIGURE 2** The binding activities of HBGA types with GII.4 and GII.17 VLPs, and HBGA blocking antibodies induced by both VLPs in mice. *$p$ < .05; **$p$ < .01; ***$p$ < .001. HBGA, histo-blood group antigen; VLP, virus-like particle

H (type 3) HBGA, the blockade index declined with the serial dilution of the serum, which showed a good linear relationship (Figure 2C,D). The BT50 results showed that both GII.4 and GII.17 VLPs induced HBGA blocking antibodies in BALB/c mice only 1 week post the second dose, which showed significant statistical difference when compared to the negative control group ($p$ < .05). Although the average BT50 values (~130 for GII.4 and ~360 for GII.17) were not as high as that reported in other study,[10] the GII.4 VLP immunized serum showed no cross-reactivity with GII.17 VLP and vice versa (Figure 2E,F).

## 4 | DISCUSSION

As expected, both norovirus GII.4_Sydney (GenBank Accession No.: KX354007) and GII.17 (GenBank Accession No.: KX024652) VLPs, which expressed in pPICZa *pichi pichia* expression system in

secretion form, can induce genotype specific IgG and HBGA blocking antibodies in mice, as described by Tomé-Amat J who first performed the secreted production of assembled norovirus GII.4 (GenBank Accession No.: AF080551) VLPs from Bg11 *pichia pastoris* strain with pJ912 expression vector.[11] The results re-indicated that it is technically feasible for secretion expression of VP1 proteins and self-assemble of norovirus VLPs in *pichi pichia* expression system, and the VLPs showed similar immunogenicity as those produced with other expression systems.

The stem cell-derived enterocytes supported the cultivation of multiple human norovirus strains,[12] but the difficulty of this technique limited its application even though it provide tools for the assessment of methods to prevent and treat human norovirus infections. Up to data, the HBGA blocking antibody still serves as a surrogate neutralization assay, as shown in both norovirus infected chimpanzees and norovirus challenged human beings.[13,14] It was

further supported by the highly correlation between serum neutralizing antibody levels assayed in human intestinal enteroids and HBGA blocking antibodies.[15] HBGAs are formed by the combination of several neutral monosaccharides with different types of glycosidic bonds. Different norovirus variants have different susceptibility to HBGAs.[16] In this study, both the endemic GII.4_Sydney and epidemic GII.17 VLPs preferred binding H (type 3) HBGA to others. Previous study showed that GII.4 Sydney VLPs showed a broad binding pattern but the GII.17 Kawasaki308 VLPs only weakly associated with long-chain saccharides containing H type.[17] It has been proved that P2 subdomain of norovirus VP1 protein act as key attachment factors that facilitate infection.[18,19] Additionally the distally located P2 subdomain and sequence variation in and around the second carbohydrate-stabilizing domain of ORF2 alters VLP structure and modulates HBGA binding patterns even within a genotype.[20] Therefore, this difference can be partly explained by the sequence variation in key regions. Meanwhile the affect of protein expression system on the formation and conformation of VLPs should also be taken into account, which needs further evaluation.

Heterotypic HBGA-blocking activity has been observed in several studies between or within genogroups in human beings. However, the cross-reactivity seemed a bit limited. For example, the fold increases and peak titers of heterotypic antibody (GI.4, GI.7 and GII.4) were more modest than that reactive with the challenge antigen (GI.1), and also varied individually.[21] Similarly, we also found there was little cross-reactivity between the endemic GII.4 and the epidemic GII.17 strains, as well as strains between (GI.1) and within (GII.3/GII.4/GII.17) genogroup by using intracellular expressed VLPs as immunogen and serum IgG and/or HBGA blocking antibodies as indicators for humoral immunity. Coincidentally, the limited cross-immune reactivity by the ELISA and little reactivity by the HBGA blocking assay between GII.4 norovirus and the new GII.17 variant were found among natural infected patients in 2014–2016 in China.[6] That is, the norovirus vaccine only containing the endemic strains would not provide sufficient protection against the emerging strains.

Unfortunately, the HBGA-blocking antibodies measured in this study were not ideal, which were lower than those reported in previous studies,[5] especially the GII.4. As documented, the structural integrity and stability of VLP particles or P particles play an important role in the immune effect of proteins and in the binding ability and blocking ability of HBGA. For example, the dimer and pentamer forms of VP1 are less sensitive to HBGA than the VLP form,[22] as well as P particles are more immunogenic than P monomer and dimer, and the blocking ability of HBGA is significantly enhanced.[23] It is also to be noted that statistic difference also found on serum IgG antibodies between the yeast protein and adjuvant groups when testing the GII.17 VLPs, but not found when testing GII.4 VLPs. It is most likely that the number of mice (eight in each group) was not sufficient to discriminate this little difference. However, when taken orally, the yeast lysate containing VLPs induced a high level of immune response, and the mice did not have an immune response against the yeast protein in the experiment without any side effects.[24]

In conclusion, the pPICZa *pichi pichia* expression system showed a potential to be the alternative for expression of norovirus VLPs in secretion form. However, no cross-reactivity found between the endemic strain and the epidemic strain demonstrated provides an evident for the consideration of selecting candidates of norovirus vaccine strains.

## CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

## AUTHOR CONTRIBUTIONS

Study design: Jialiang Du, Qiong Gu, Qiming Li, and Tai Guo. Study performance: Jialiang Du, Qiong Gu, and Yan Liu. Data analysis: Jialiang Du, Qiong Gu, and Yan Liu. Draft: Jialiang Du and Qiong Gu. Revision: Jialiang Du, Qiong Gu, Yan Liu, Qiming Li, Tai Guo, and Yan Liu.

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