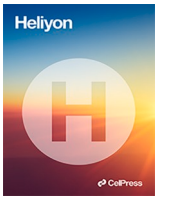
Heliyon 10 (2024) e26567

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

The variation of antigenic and histo-blood group binding sites synergistically drive the evolution among chronologically emerging GII.4 noroviruses

Xiaojing Hong [a,b], Liang Xue [b,**], Yingwen Cao [a], Ruiquan Xu [a], Jingmin Wang [a], Junshan Gao [b], Shuidi Miao [b], Yueting Jiang [c,***], Xiaoxia Kou [a,*]

[a] *Guangzhou Key Laboratory for Clinical Rapid Diagnosis and Early Warning of Infectious Diseases, KingMed School of Laboratory Medicine, Guangzhou Medical University, Guangzhou, China*

[b] *Guangdong Provincial Key Laboratory of Microbial Safety and Health, State Key Laboratory of Applied Microbiology Southern China, Institute of Microbiology, Guangdong Academy of Science, China*

[c] *Department of Laboratory Medicine, First Affiliated Hospital of Guangzhou Medical University, Guangzhou, 510120, China*

ARTICLE INFO

Keywords:
Norovirus
GII.4
Capsid protein
Evolution mechanism

ABSTRACT

Norovirus, commonly found on shellfish and vegetables, is a foodborne virus with GII.4 as the dominant genotype responsible for widespread outbreaks since 1995. Continuous variation of major capsid protein VP1 can lead to changes in the immunogenicity and host receptor binding ability of norovirus, which is an important evolutionary mechanism. Therefore, analyzing the immunogenicity of VP1 and its binding ability to various HBGAs in GII.4 variants could improve our understanding of the persistent prevalence of GII.4. Here, the results suggest that GII.4 has gradually enhanced its HBGAs binding ability over time for various types of receptors. Variants exhibit significantly stronger immune response to homologous mouse antiserum than heterologous ones, highlighting the importance of variation of antigenic and histo-blood group binding sites in driving the evolution of GII.4. These synergistic forces constantly lead to antigenic drift and changes in receptor binding, resulting in continuous emergence of new variant strains and sustained prevalence.

1. Introduction

Viruses are the most uncontrollable threats to global public health, among which norovirus has been one of the most prominent pathogens causing foodborne viral gastroenteritis, bringing huge health burdens [1,2]. Norovirus, commonly found on shellfish and vegetables, is a highly contagious foodborne virus that can cause diarrhea, nausea and stomach cramps. The illness caused by norovirus can affect individuals across all age groups, and typically, it is self-limiting [3]. Nevertheless, individuals who are young, elderly, or have compromised immune systems may be confronted with grave perils to their lives as a consequence of this [4,5].

As an RNA virus, norovirus has a size of approximately 40 nm and the following genome features: it has a total length of 7.5–7.8 kb

* Corresponding author.
** Corresponding author.
*** Corresponding author.
E-mail addresses: xueliang@gdim.cn (L. Xue), jyting8899@126.com (Y. Jiang), kouxiaoxia163@163.com (X. Kou).

https://doi.org/10.1016/j.heliyon.2024.e26567
Received 22 October 2023; Received in revised form 17 December 2023; Accepted 15 February 2024
Available online 24 February 2024

and contains three open reading frames (ORFs). Non-structural proteins, encoded by ORF1 and with a size of approximately 200 kDa, can undergo cleavage to produce six distinct non-structural proteins. ORF2 is responsible for the synthesis of VP1, the major capsid protein with a size of 58 kDa and can assemble into an icosahedral VLP (virus-like particle) that antigenically resembles wild virions. The minor capsid protein, VP2, approximately 29 kDa, is encoded by ORF3 [6]. According to the variations observed in ORF2 gene sequence, norovirus can currently be classified into 48 genotypes, with an extremely rich genetic diversity [7,8]. However, GII.4 is responsible for about 80% of norovirus outbreaks worldwide, and is the dominant genotype that has caused widespread foodborne norovirus outbreaks since 1995 [9,10]. The sustained prevalence of GII.4 is associated with the continual emergence of novel epidemic variants. However, the evolutionary mechanisms and factors underlying intra-genotypic variants of norovirus genotypes, as well as their impact on evolution, are still poorly understood at present.

The constant selective pressures from herd immunity leads to the substitution of amino acids accumulated by the norovirus VP1, which is a primary mechanism driving virus evolution [11,12]. Besides, histo-blood group antigens (HBGAs), which served as crucial ligand during the norovirus infection process, have been considered one of the key factors influencing the evolution of norovirus [13,14]. During the biosynthesis process of HBGAs, the FUT2 gene encodes an α1,2-fucosyltransferase that facilitates the formation of the H-type HBGAs. These H-type structures can subsequently form the A type, B type, and O type, while those unable to produce H-type HBGAs are termed as non-secretory type [15]. Many researchers have found that the HBGAs binding ability of different norovirus genotypes is different [16–18]. It has also been shown that GII.4 has a broader range of HBGAs binding than other norovirus genotypes [19,20]. Hence, the investigation of the disparity in the associations between GII.4 variants and HBGAs based on the chronological emergence is helpful to better understand its evolution.

The absence of conventional animal models and cell culture systems poses a challenge in norovirus research, especially in the culture of the virus. At present, the VLPs (virus-like particles), which are similar to nature virions structurally and antigenically, could circumvent this obstacle [21]. In addition, the blockade assay has been employed as an alternative method for assessing neutralization, with pig gastric mucin (PGM) type III serving as a substrate [22]. In this study, VLPs of chronologically emergent GII.4 variants have been produced. Antiserums were then prepared respectively to assess the differences in HBGAs binding capacity, antibody response levels, and cross-blocking activity among different GII.4 variants.

2. Methods

2.1. Evolutionary and spatio-temporal analysis

The VP1 gene sequences of GII.4 were obtained from the GenBank, along with accompanying details such as accession number, collection date and geographical region. Norovirus Typing Tool Version 2.0 (http://www.rivm.nl/mpf/typingtool/norovirus/) was used to subtype the sequences mentioned above. After collating of data, excel was used to analyze the genetic diversity and constituent, as well as the spatio-temporal analysis. The data was also visualized in Excel and Python software. Accession numbers of sequences for analysis were listed in Supplementary File 1.

To keep the phylogenetic tree accurate but also concise, CD-HIT software was employed with a threshold of 0.99 to reduce the redundancy in sequence similarity. Subsequently, the data were aligned using MAFFT software [23,24]. The optimal models for the selected VP1 genes of GII.4 sequences were identified by evaluating the Bayesian Information Criterion (BIC) values through jModelTest. The selected sequences underwent Markov Chain Monte Carlo (MCMC) simulations, with chain lengths of 2,000,000 steps, and samples were collected at intervals of 1000 steps. The tree was visualized in the International Tree Of Life (iTOL, http://itol.embl.de/).

2.2. Ethical statement

The animal protocols conducted in this study received ethical approval from the Laboratory Animal Ethics Committee of Guangdong Institute of Microbiology(permission number GT-IACUC202207131).

2.3. Production and morphological observation of the VLPs for the GII.4 norovirus strains

VP1 genes of selected GII.4 strains (Ancestral, US95_96, Farmington Hills, Hunter, Den Haag 2006b, New Orleans, and Sydney) were subjected to amplification using PrimeSTAR polymerase (Takara, Beijing, China). Subsequently, the PCR fragments were inserted into linearized pFastBac1 vectors to obtain recombinant pFastBac1-VP1. Positive clones were screened by PCR. The recombinant bacmid was obtained by transferring pFastBac1-VP1 into DH10Bac, through transposition between the mini-attTn7 site on bacmid in DH10Bac and the mini-Tn7 site on the pFastBac. This transposition will lead to the destruction of LacZ gene on bacmid. Therefore, DH10Bac colonies containing recombinant bacmid can be identified by blue-white screening on the bacterial culture plate. After infecting Sf9 cells, recombinant baculovirus can be produced to initiate the expression of VP1 proteins.

The expressed VP1 proteins were purified by ultrafast centrifugation. The Sf9 cells were infected for 96 h and subsequently harvested through centrifugation at 7000×*g* for 10 min. After resuspending with PBS, the collected cells were crushed for 3 min by using the ultrasonic crusher (35% W), during which the temperature was consistently kept within the range of 2-8 °C. 7% PEG6000 (w/v) and 2% (w/v) NaCl were supplemented to the above crushing supernatant, and settled overnight at 4 °C to obtain the precipitation. After resuspending with PBS, cesium chloride (1.4 g/ml) was added and then centrifuged at 288,000×*g*, 24 h, at a temperature was 8 °C. The visible strips were collected and suspended with PBS, then centrifuged (141,000×*g*, 3 h, 8 °C) to obtain VLPs. To validate the

successful assembly of the VLPs, the transmission electron microscope (TEM) was used to visualize the morphology of the particles after negative staining.

2.4. Mice immunization

Female BALB/c mice purchased from the Guangdong Sijia Jingda Biotechnology Co., LTD were inoculated with 30 μg protein at 0, 2, 4 and 5 weeks, respectively. As a control, an equivalent volume of PBS was injected. VLPs were mixed with Freund's complete adjuvant during the initial injection, while Freund's incomplete adjuvant was used for the subsequent three injections. Serum samples were obtained at various time points, including before immunization and week 2, 4, 5, and 6, both stored at −20 °C.

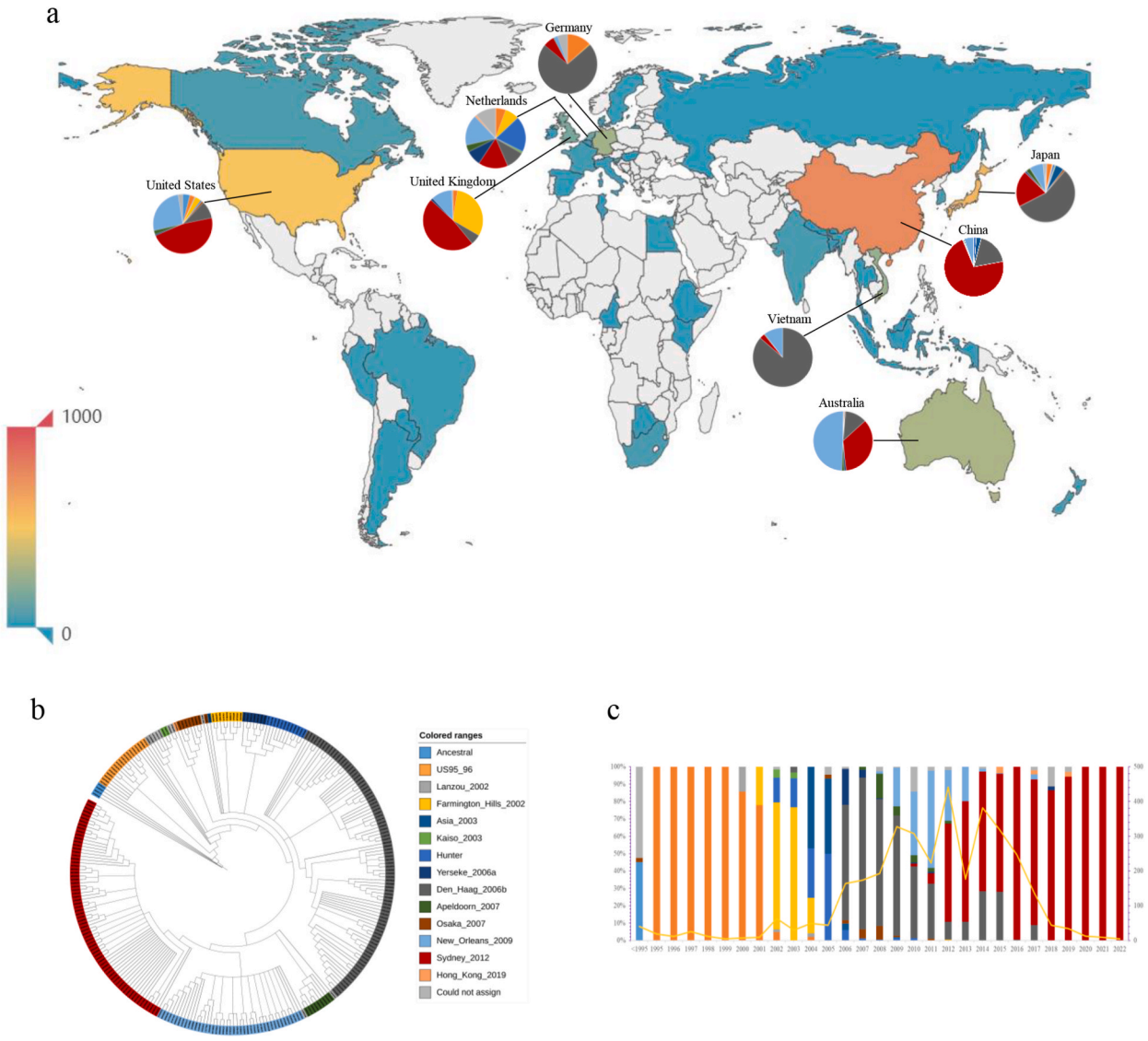


Fig. 1. Evolutionary and spatio-temporal analysis of the major capsid protein of GII.4 norovirus. (a) Geographic distribution of GII.4 norovirus. Colors from blue to red represented the number of GII.4 norovirus in different countries from less to more, as shown on the left legend. The variants of GII.4 are represented by pie charts, showing the top eight countries in terms of overall GII.4. The color of the variants in the pie chart is the same as the figure (b). (b) Phylogenetic tree of norovirus GII.4 variants. Different colors were used to distinguish clades based on the variant determination results from the online Norovirus Typing Tool (https://www.rivm.nl/mpf/typingtool/norovirus/). The phylogenetic tree was visualized in the International Tree Of Life (iTOL, http://itol.embl.de/). (c) Chronologically based prevalence of norovirus GII.4. The Y axis on the left indicated the proportion of different variants, corresponding to the bar chart. While the Y axis on the right corresponding to the line chart, indicating the number of GII.4. Different colors represent the major GII.4 variants within this period with the same color scheme from (b). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

2.5. Enzyme linked immunosorbent assay

The antibody responses, both antigen-specific and cross-reactive were assessed using an indirect enzyme-linked immunosorbent assay (ELISA). VLPs were diluted to 2 μg/ml with ELISA coating buffer, and coated onto the 96-well microtiter plates with 100 μl per well, during which the temperature was maintained at 4 °C overnight. Then the wells underwent 5 rounds of washing using 1 × PBST solution and were subsequently blocked with 5% skimmed milk at a temperature of 37 °C for a duration of 2 h. The serum samples underwent a 2-fold dilution series, commencing with a dilution of 1:200, and were then incubated at 37 °C for 1 h. After washing 5 times with 1 × PBST, the wells were subjected to a 30 min incubation at 37 °C with the secondary HRP-conjugated goat anti-mouse IgG antibody (diluted 1:3000). Subsequently, tetramethylbenzidine (TMB) was introduced as the chromogenic substrate for peroxidase and allowed to undergo a 10 min reaction at 37 °C. Following termination of the substrate reaction using 2 M H_2SO_4, the optical density (OD) values were quantified at 450 nm. A sample was deemed positive if its net OD value exceeded predetermined cut-off and reached a minimum of 0.2 OD [25], while the highest reciprocal serum dilution that yielded absorbance >2-fold over the background value was considered as the final titer. Counting final titer of individual mice to determine the geometric mean titers (GMTs) with 95% confidence intervals (CIs) for each immunization group.

2.6. HBGAs binding assay

The HBGAs binding assay was conducted using VLPs and various sources of HBGAs, including human saliva (types A, B, O, and non-secretor) as well as PGM type III (Sigma-Aldrich). 96-well microtiter plates were coated with diluted saliva samples (1:1000) and PGM (1 mg/ml) diluted saliva samples, respectively. After being treated with 5% skimmed milk to block, the saliva samples and PGM were exposed to VLPs at concentrations of 1 μg/ml for 1 h at a temperature of 37 °C. Following this, the plates underwent incubation with antisera obtained from mice that had been immunized with VLPs for an hour at 37 °C. Subsequently, they were subjected to incubation with a secondary HRP-conjugated goat anti-mouse IgG antibody (diluted at a ratio of 1:3000) for half an hour at 37 °C. The TMB Liquid Substrate System was utilized to detect the signal. The samples were analyzed in triplicate, and a sample was defined positive as the net OD value exceeding the predetermined set cut-off and at least 0.2 OD.

2.7. HBGAs blockade assay

The blockade assay was employed to assess the ability of antibodies blocking the interaction between VLPs and ligands. In brief, microtiter plates were coated with PGM at 4 °C overnight. Subsequently, a blocking step was performed using 5% skimmed milk. Mouse sera were diluted two-fold (starting dilution 1:100) and incubated with VLPs (1 μg/ml) for 1 h at 37 °C. The pre-incubated mixtures were then added to plates and incubated for another hour at 37 °C. Following this, the plates were exposed to sera for an additional hour at 37 °C, after which a secondary HRP-conjugated goat anti-mouse IgG antibody (diluted to 1:3000) was applied and left to incubate for half an hour at 37 °C. The TMB Liquid Substrate System was utilized to detect the signal. Samples were analyzed in triplicate. The blocking index (%) was determined by calculating (OD wells without serum - OD wells with serum)/OD wells without serum × 100%.

2.8. Statistical analysis

The serological data were represented by the geometric mean titers (GMT). Statistical analysis was conducted using GraphPad prism8.0 software. The binding properties were evaluated through Schapiro-Wilk test and Mann-Whitney *U* test. One-way ANOVA was employed to determine significant differences among various groups, and $P < 0.05$ was statistically significant.

3. Results

3.1. Evolutionary and spatio-temporal analysis

Over the past decades, a collection of 3504 sequences that contain VP1 genes of norovirus GII.4 were gathered. Norovirus GII.4 has been reported in 43 countries (Fig. 1a), and China (21.69%) accounted for the largest number, followed by Japan (16.38%) and the United States (14.67%). The Bayesian Information Criterion (GTR + G) was utilized for the construction of the phylogenetic tree, depicting the evolutionary relationships and diversification patterns among different variant of GII.4 (Fig. 1b). In the past few decades, a series of variants have emerged in a chronological order and remained prevalent for periods until being replaced by subsequent new variants (Fig. 1c). To date, a pandemic scale has witnessed the emergence of six significant variants – US95_96, Farmington Hills, Hunter, Den Haag 2006b, New Orleans, and Sydney. Hence, a comprehensive analysis was conducted on seven GII.4 variants, encompassing the six prominent epidemic variants and the ancestral variants, to investigate the evolution among chronologically emerging GII.4 noroviruses.

3.2. Production and morphological observation of the VLPs

The baculovirus expression system was utilized to produce the VLPs of seven GII.4 variants, which have emerged and prevailed during different periods over the past decades. The VP1 proteins expressed were purified through ultrafast centrifugation. To validate

the formation of VLPs through self-assembly of VP1 proteins, the purified samples were observed using transmission electron microscopy (TEM). Results showed that all these GII.4 VP1 proteins were successfully self-assembly into VLPs (Fig. 2 a-g).

3.3. Preparation and titer determination of antiserum

After four immunizations, antiserums of GII.4 norovirus variants were successfully prepared, and the titers of the sera all reached 10^5 or above, as shown in Fig. 3. Ancestral GMT values are: ancestral: 256000 (95% CI = 256000-256000). US 95/96:168897 (95% CI = 24670–549470); Farmington Hills: 111430 (95% CI = 79661–150738); Hunter: 776046 (95% CI = 119876–182572); Den Haag 2006b: 194011 (95% CI = 29969–456430); New Orleans: 97005 (95% CI = 11588–218811); Sydney: 128000 (95% CI = 128000-128000).

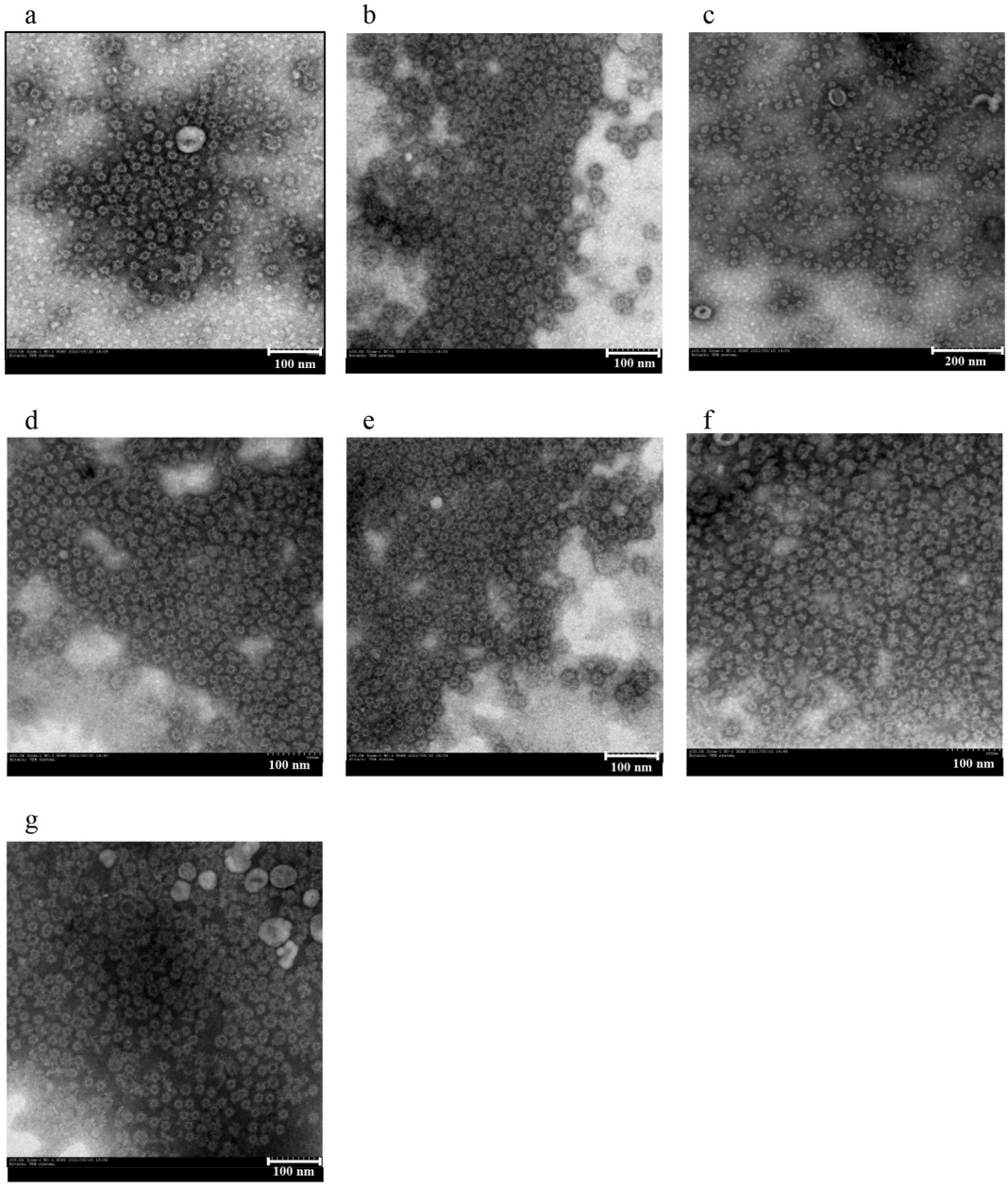


Fig. 2. The morphological observation of the VLPs of the GII.4 norovirus variants. (a)–(g) displayed the morphology observed by the TEM for the Ancestral, US 95/96, Farmington Hills 2002, Hunter, Den Haag 2006b, New Orleans 2009, and Sydney 2012, respectively.

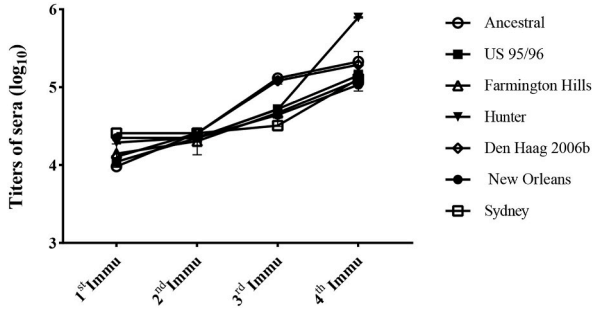


Fig. 3. Titers of GII.4 sera antibodies. The vertical axis represents serum titers, expressed as geometric mean titers (GMT), and the horizontal axis represents the serum titers of the first to fourth immunizations. The different annotations represent different strains of variation.

3.4. HBGA binding profile of GII.4 VLPs

96-well plates with different types of salivary receptors and PGM were assembled to measure the VLP HBGAs binding ability of a series of GII.4 norovirus variants. The results indicated that all 7 variants could bind to the salivary receptors of type A, type B, type O and PGM, but hardly bind to the non-secretory type (Fig. 4a). Nevertheless, diverse variants exhibited distinct advantages in their binding affinity towards various HBGAs subtypes. For type A, Ancestral and US 95/96, which were epidemic in the earlier years, displayed a similar binding ability with Farmington Hills and Hunter (OD450 = 0.3–0.6). The newly epidemic strains Den Haag 2006b, New Orleans and Sydney showed stronger binding ability to type A (OD450 = 0.4–0.8) than the above four variants. For type B, Hunter, Den Haag 2006b, New Orleans and Sydney variants (OD450 = 1.0–1.2) had stronger binding ability than those Ancestral, US 95/96 and Farmington Hills variants (OD450 = 0.5–0.6). Among type O, Hunter exhibited the highest binding ability while US 95/96 showed the lowest binding affinity. In addition, none of these variants had the ability to bind to non-secretory HBGAs, except for the New Orleans and Sydney variants, which had a slight ability to bind to non-secretory HBGAs. (Fig. 4b).

3.5. Cross-reactivity of GII.4 antiserum

To investigate the cross-reactivity of GII.4 norovirus variant VLPs in binding with antiserum, the ELISA method was used for detection. The results, as shown in Fig. 5, indicated that the antiserum of all seven variants can react with their own VLPs, and can also generate varying degrees of cross-reactivity with VLPs from other variants. The antiserum obtained from the VLPs (Ancestral, US 95/96) which was epidemic in earlier years had a high degree of cross-reactivity with its homologous variants (OD450 = 1.2–1.25). However, its response to the Farmington Hills, which gained popularity in the early 21st century, was comparatively subdued. (OD450 = 0.8–0.95). In addition, its cross-reactivity to the subsequent epidemic variants like Hunter, Den Haag 2006b, New Orleans, and Sydney was significantly reduced (OD450 = 0.6–0.8). The antiserum derived from the VLPs of Farmington Hills and Hunter exhibited a high degree of cross-reactivity with their corresponding VLPs, while displaying reduced reactivity toward VLPs of other variants. The antiserum obtained from the VLPs of the Den Haag 2006b and New Orleans exhibited strong homologous reactivity, as well as cross-reactivity with VLPs of those epidemic in similar years (e.g. the New Orleans antiserum with the Sydney variant VLP). It is noteworthy that the antiserum derived from the Sydney variant exhibited a significantly reduced reactivity towards VLPs of earlier variants that were epidemic in previous years, as compared to its own VLPs ($P < 0.001$).

3.6. Cross-blocking activity among different GII.4 antisera

By coating with PGM on a 96-well plate, we explored the HBGAs binding blocking ability of GII.4 norovirus antiserum, and the results are shown in Fig. 6a. The high blocking titers were exhibited when tested against the corresponding VLP and antiserum, with EC50 values exceeding 2000. (e.g., the EC50 of Sydney variant antiserum blocking Sydney variant VLP HBGAs binding is 4932). Different blocking abilities were exhibited among heterologous antiserum, which can be divided into moderate blocking ability and no blocking ability. Interestingly, the antiserum of the ancestral variant only demonstrated HBGAs binding blocking ability against its own VLP and US 95/96, but exhibited no such activity against the epidemic variants that emerged after US 95/96. The antiserum of US 95/96 variant showed similar results. The antiserum of Farmington Hills and Hunter variants exhibited strong HBGAs binding blocking ability against their own VLP, as well as moderate cross-blocking ability against adjacent variants (such as Hunter variant antiserum can cross-block the HBGAs binding of Farmington Hills and Den Haag 2006b). However, they lacked the ability to block other variants with a longer time interval. In contrast, the antiserum of the subsequent epidemic variants Den Haag 2006b and New Orleans exhibited a more extensive cross-blocking ability, especially the antiserum of Den Haag 2006b variant. The antiserum of Sydney variant had an extremely strong HBGAs binding blocking ability against its own VLP, with an EC50 value of up to 4932. Overall, the antiserum of GII.4 norovirus variants exhibit strong HBGAs binding blocking ability against their own VLP, moderate blocking ability against variants with a similar time interval, and no blocking ability against variants with a greater time interval (Fig. 6b–c).

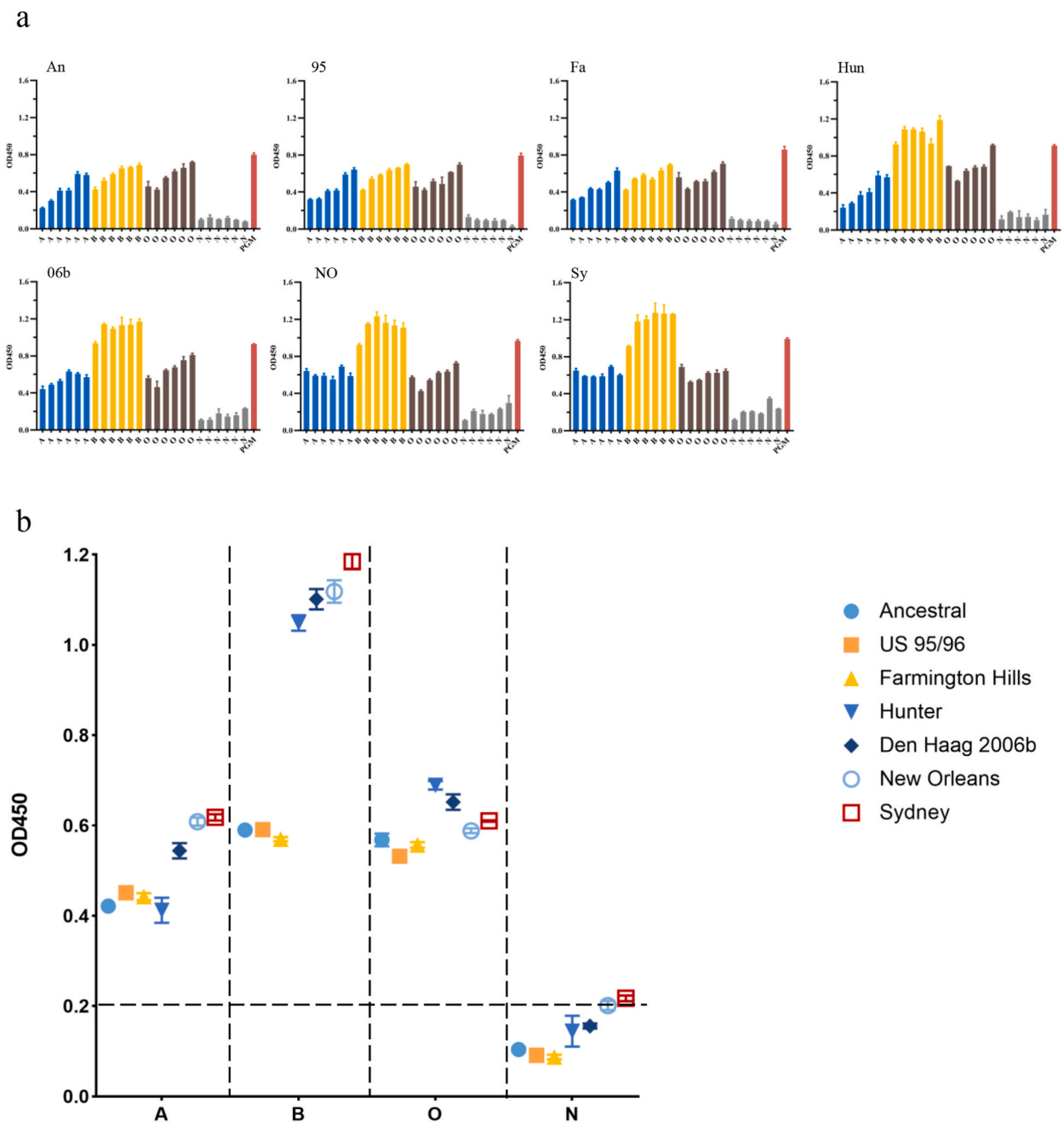


Fig. 4. Receptor Binding Abilities of GII.4 Norovirus. (a) Analysis of the binding ability of different variants to receptors of different types. The vertical axis represents the OD450 value, the horizontal axis represents different types of saliva receptors, and each saliva receptor is tested with three replicates, which are represented by different colors in the graph. (b) The vertical axis represents the average OD450 value of binding ability to different types of saliva receptors, and the horizontal axis represents different types of saliva receptors. Different strains are indicated by the legend on the right side of the graph. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.7. Amino acid alignment of antigenic and HBGAs binding sites

Antigenic sites were defined by Lindesmith et al., Tohma et al., and Tohma et al. [11,26,27]. Here, Alignment of antigenic and HBGAs binding sites on VP1 of seven GII.4 norovirus variants was conducted. Among the 48 sites analyzed, 15 were conserved (31.25%), and 33 were variable (68.75%). Notably, except for the Loop-3 binding domain, β-sheet, Loop-1, and Loop-2 all contained variable sites that overlapped with different antigenic domains. In the β-sheet, positions 340 and 341 were antigenic site C residues,

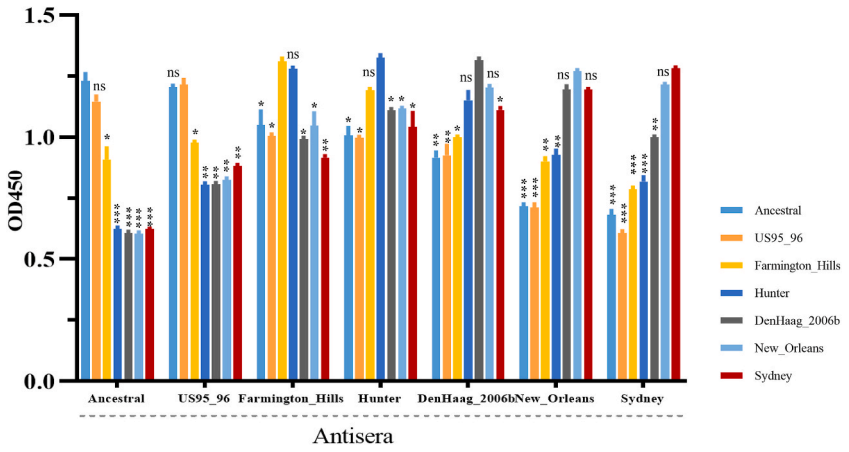


Fig. 5. Cross-reactivity of homologous and heterologous antiserum against GII.4 norovirus. The horizontal axis represents the antiserum against seven GII.4 norovirus variants, while the vertical axis represents the OD450 values. The legend shows different colors representing different GII.4 norovirus variant VLPs. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

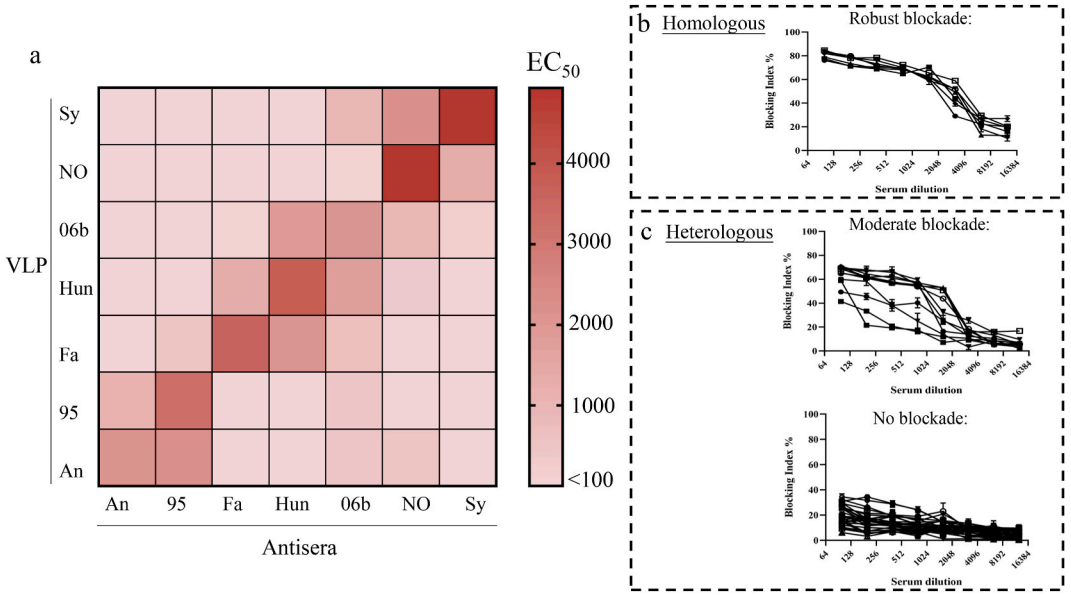


Fig. 6. Receptor-binding blocking ability of GII.4 norovirus variant antiserum. The left figure a is a heat map of EC50 blocking ability, with the horizontal axis representing variant antiserum and the vertical axis representing variant VLP, and the color gradient ranging from light to dark indicating the increasing EC50 values. The right figure b and c shows the detailed HBGAs binding blocking ability among homologous and heterologous, displaying strong, moderate, and no blocking ability. The corresponding data for each line in b and c can be found in the appendix Fig. 1. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

with 340 exhibiting high variability. Loop-1 contained antigenic sites A and C, with positions 372 and 373 undergoing variations Asn-Ser-Glu-Asp and Asn-Arg, respectively, and positions 376, 377, and 378 undergoing variations Gln-Glu, Thr-Ala, and Gly-His-Asn, respectively. Antigenic site D residues 393–396 in Loop-2 were also highly variable, with position 394 being blank in the Ancestral and US 95/96 strains but then gaining a new residue Gly during the evolution from US 95/96 to Farmington Hills and subsequently mutating to Thr. Positions 393, 395, and 396 undergoing variations Asp-Asn-Ser-Gly, His-Asn-Thr, and His-Pro, respectively. Antigenic site E exhibited similar variability as shown in Fig. 7. In conclusion, the evolution of critical residues within the antigenic and HBGAs binding sites has been a driving force behind the evolutionary trajectory of GII.4 noroviruses.

4. Discussion

Norovirus is a prevalent foodborne virus that frequently colonizes the surface of various foods, including shellfish and vegetables [28,29]. Consuming food contaminated with norovirus often results in the development of acute gastroenteritis in humans. Norovirus

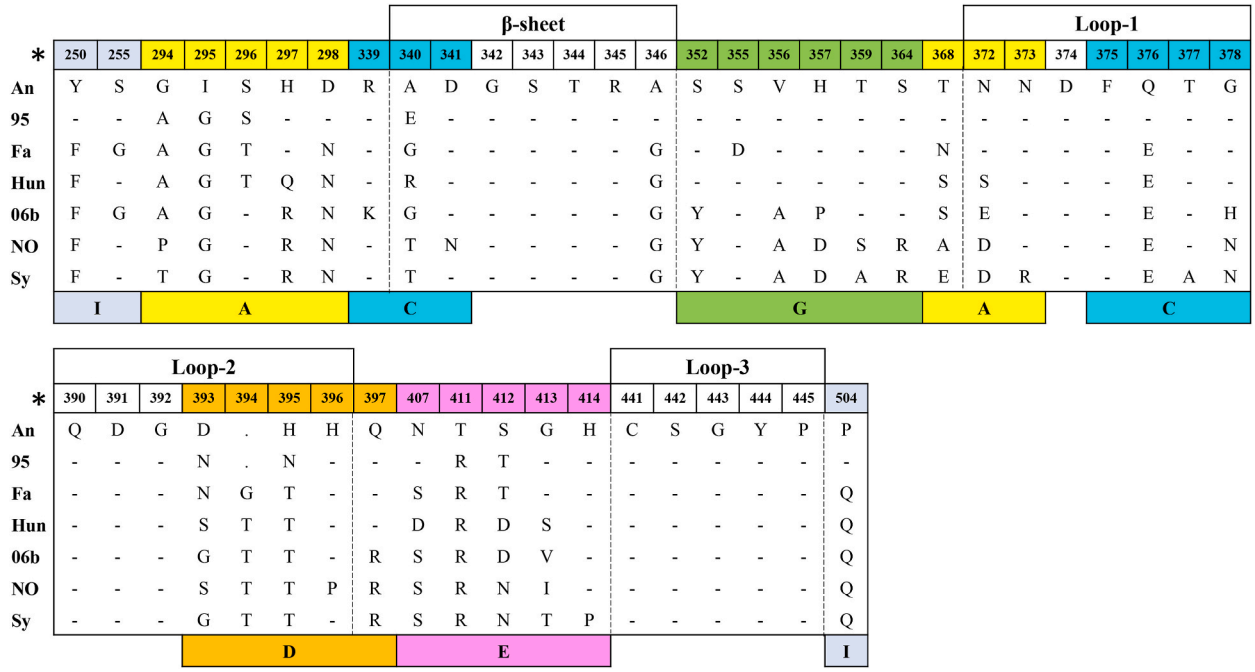


Fig. 7. Amino acid alignment of antigenic and receptor binding site of GII.4 norovirus. * in the figure denoting the specific site of antigen-receptor binding. The dashed box represents the receptor binding site, which includes the β-sheet, Loop-1, Loop-2, and Loop-3. The antigenic site is marked with different colors, with sites A, C, D, E, G, and I represented by yellow, cyan, orange, pink, green, and gray, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

outbreaks of varying degrees have been reported worldwide every year since its initial discovery, particularly in settings where individuals congregate such as schools, nursing homes, and hospitals [2,30,31]. This study aims to investigate the variances in HBGAs binding capacity and immunogenicity of GII.4 norovirus, which is the most common genotype responsible for foodborne norovirus infection. The findings will provide a foundation for comprehending the evolutionary mechanism of norovirus and contribute to enhancing prevention and control measures against foodborne norovirus.

Evolutionary analysis utilizing big data is a scientific methodology for comprehensively understanding viruses. Tohma et al. conducted genome-wide analyses of all human norovirus genotypes, and provided insights on evolutionary dynamics [32]. This study utilized big data and the norovirus strain resources accumulated by the research group to analyze the global distribution of GII.4 norovirus. The results indicate that norovirus has been documented across all six inhabited continents, with China reporting the highest incidence globally (Fig. 1). And this is associated with factors such as population size, local medical capacity, and the extent of virus surveillance efforts. In some underdeveloped regions, limited-scale transmission of foodborne norovirus may occur. However, due to inadequate healthcare infrastructure, detection rates remain low. In terms of global norovirus data, GII.4 has maintained its dominant epidemic position for numerous years. Therefore, this study initially constructed the GII.4 phylogenetic tree utilizing the Bayesian optimal model GTR + G and subsequently visualized its epidemic dynamics, revealing a linear pattern of intergenerational evolution among variant strains. This evolutionary model of GII.4 was also confirmed by Kendra et al. and Tohma et al. [11,33]. Hence, even representative variants, including the six major epidemic variants that have been widely reported, and the ancestral variants, were chosen as research subjects to investigate the evolution among chronologically emerging GII.4 noroviruses.

As an RNA virus, GII.4 displays extensive genetic diversity and exhibits rapid evolution. Therefore, investigating its evolutionary process is crucial for a comprehensive understanding of this virus. The VP1 protein, which constitutes the major capsid of norovirus, plays a crucial role in both antigenicity and receptor binding, thus driving the evolutionary process of this virus [34–36]. Currently, VP1 is widely utilized as a research target to investigate the molecular mechanisms of various norovirus genotypes [37,38]. However, there remains a dearth of research on the HBGAs binding capacity and cross-immunoreactivity of GII.4 in relation to its temporal prevalence. Therefore, this research investigates the evolutionary pattern of GII.4 norovirus based on the HBGAs binding ability and immunogenicity of its major capsid protein. The baculovirus expression system is highly efficient in expressing exogenous genes and processing them for transcription, rendering it an optimal choice for protein expression. This platform facilitates the expression of virus-like particles (VLPs) that closely imitate the natural particles of norovirus in both structure and immunogenicity. The inadequacy of appropriate cell culture systems and animal models has rendered VLPs a viable alternative for investigating the mechanisms of the virus [39,40]. In the present investigation, a series of GII.4 norovirus mutant strains were expressed as VLPs using the baculovirus expression system and subsequently purified through high-speed centrifugation to prepare a range of antisera in mice, thereby establishing the groundwork for further analysis into the virus's epidemic and evolutionary mechanisms.

Previous research has indicated that norovirus exhibits specific binding affinity to HBGAs, which are widely distributed in the

saliva, blood, and intestinal epithelial cells of infected individuals. It is known that norovirus can infect people of all ages. However, not all individuals exposed to the same contaminated food during a foodborne norovirus outbreak will necessarily become infected and subsequently develop symptoms of acute gastroenteritis. This could potentially be associated with the receptor type of HBGAs. Considering the high prevalence and persistence of GII.4 norovirus in foods such as shellfish, what changes occur in its HBGAs binding capacity during evolution? HBGAs binding ability of GII.4 variants, ranging from ancestral to current variants, was analyzed in this study. The findings revealed that all seven variants exhibited binding affinity towards A, B and O-type HBGAs; however, their binding profiles and potencies were distinct. In accordance with the chronological progression of epidemic evolution, the strength of HBGAs binding gradually intensified. Compared to ancestral variant, contemporary variants such as sydney exhibit significantly enhanced binding affinity to the aforementioned receptor types, particularly B-type, with a binding ability more than twice as strong as that of earlier variants. Lindesmith et al. have revealed human susceptibility to Norwalk virus infection in 2003 [41], and then Rougemont et al. had previously quantitatively analyzed the receptor binding ability of certain GII.4 variants, and this study's results on the HBGAs binding ability of early variant strains were consistent with theirs [42]. Furthermore, this study has updated the HBGAs binding capacity of GII.4, including recent contemporary variants such as New Orleans and Sydney. From the perspective of epidemic evolution and changes in binding ability, GII.4 has gradually increased its capacity to bind with various HBGAs types, particularly type B, while also exhibiting a tendency towards mild binding affinity to non-secretor. This implies that the infective dose required to contaminate food and cause infection in individuals with the same HBGAs type may be lower, thereby increasing the likelihood of infecting new populations such as non-secretor individuals, which could contribute significantly to the sustained prevalence of GII.4.

Antigenic changes are crucial inherent drivers for the emergence and evolution of novel pandemic variants [43]. A research paper that has been published in the Journal of Science demonstrated that H3N2 influenza viruses from phylogenetically distinct lineages exhibit numerous potential antigenic differences [44]. Kendra et al. have revealed the intricate genetic factors contributing to variations in antigenicity among GII.4 noroviruses through antigenic cartography [45]. The significance of antigenic variations in the evolutionary process of noroviruses cannot be disregarded. In antibody cross-reactivity assays, it was demonstrated that the antigenic response between variants and its own serum was significantly stronger than that between heterologous serum. The immune response elicited by variants from adjacent time periods was weaker than that between variants and its own serum, but still stronger than that between non-adjacent time-period variants. This suggests that the GII.4 variants exhibit varying levels of antigenicity, and novel variants may undergo mutations while retaining crucial ancestral antigenic epitopes or even developing new ones, leading to continuous accumulation and eventual antigenic drift. The alignment of amino acid sites also provides evidence supporting the statement mentioned above. In addition, the 394 site on antigenic epitope D inserts a new residue and further mutates during the evolution. The gradual accumulation of mutations in antigenic sites during evolution completes the process of antigenic drift, resulting in qualitative changes that enable variants to escape population protective immune responses.

HBGAs blocking assay is a crucial test for evaluating the neutralizing and protective capacity of serum. It also provides an effective solution to the challenge of lacking efficient cell systems for serum neutralization analysis and virus [22,46]. The results of HBGAs blocking assays indicate that the antisera exhibit potent binding activity against its own VLPs, while displaying moderate or negligible blocking activity against other VLPs. Considering the temporal aspect of incidence, it was determined that the GII.4 antiserum exhibits moderate HBGAs blocking activity against variants that emerged in close temporal proximity, but lacks such activity against those that arose at distant intervals. Studies indicate that the HBGAs binding domain of VP1, encompassing β-sheet, Loop-1, Loop-2 and Loop-3, plays a pivotal role in norovirus infection [47–50]. In this study, it was observed that the HBGAs binding domains, except for Loop-3, overlap with antigenic sites and undergo constant mutation due to strong immune selection pressure. This implies that the HBGAs binding domain of GII.4 norovirus is subjected to intense immune selection pressure and driven by both immunogenicity and surrounding residues, which collectively drive the evolution of GII.4, leading to the continuous emergence of novel variants that evade population immunity, thereby achieving sustained circulation. Each variant included in this study has a representative VLP; however, it should be noted that individual VLPs of each variant may exhibit variations within the analyzed potentially imposing limitations on the conclusions drawn. Nonetheless, this study still offers valuable data support for investigating the evolutionary mechanisms of norovirus.

5. Conclusion

This study employed the major capsid protein to comprehensively investigate the evolutionary mechanism of GII.4 strains, from ancestral to contemporary variants. The results indicate that strong immune selection pressure on the HBGAs binding region and gradual accumulation of mutations in immunogenicity collectively drive the evolution of GII.4, which lays a foundation for a comprehensive understanding of foodborne norovirus evolution.

Funding

This work was supported by the National Natural Science Foundation of China (32272436), the Natural Science Foundation of Guangdong Province (2021A1515011057), Plan on enhancing scientific research in GMU.

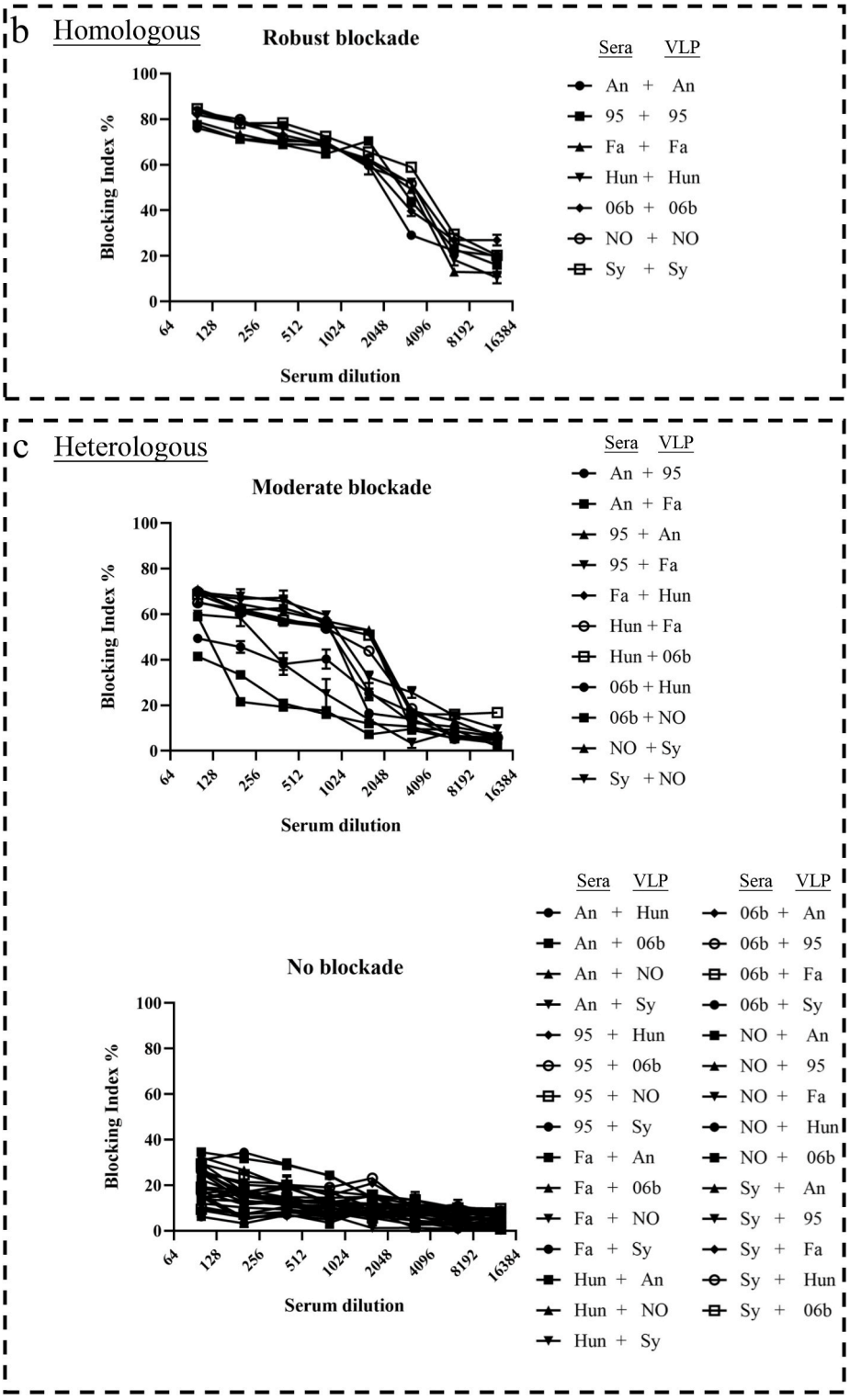
Ethics statement

The animal protocols conducted in this study received ethical approval from the Laboratory Animal Ethics Committee of Guangdong Institute of Microbiology(permission number GT-IACUC202207131). Human saliva used in this study was collected upon

approval.

Data availability statement

Data included in Supplementary File in article.



CRediT authorship contribution statement

Xiaojing Hong: Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Liang Xue:** Writing – review & editing, Resources, Funding acquisition, Conceptualization. **Yingwen Cao:** Methodology, Formal analysis, Data curation. **Ruiquan Xu:** Investigation, Data curation. **Jingmin Wang:** Formal analysis, Data curation. **Junshan Gao:** Methodology, Formal analysis. **Shuidi Miao:** Methodology, Data curation. **Yueting Jiang:** Resources, Formal analysis. **Xiaoxia Kou:** Writing – review & editing, Supervision, Resources, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e26567.

References

[1] B.A. Lopman, et al., The Vast and varied global burden of norovirus: prospects for prevention and control, PLoS Med. 13 (4) (2016) e1001999.
[2] H. Zhou, et al., The epidemiology of norovirus gastroenteritis in China: disease burden and distribution of genotypes, Front. Med. 14 (1) (2020) 1–7.
[3] R.L. Atmar, et al., Norwalk virus shedding after experimental human infection, Emerg. Infect. Dis. 14 (10) (2008) 1553–1557.
[4] D.S. Kondapi, et al., Norovirus in cancer patients: a review, Open Forum Infect. Dis. 8 (6) (2021) ofab126.
[5] C. Posovszky, et al., Acute infectious gastroenteritis in infancy and childhood, Dtsch Arztebl Int 117 (37) (2020) 615–624.
[6] C.P. Campillay-Véliz, et al., Human norovirus proteins: implications in the replicative cycle, pathogenesis, and the host immune response, Front. Immunol. 11 (2020) 961.
[7] P. Chhabra, et al., Updated classification of norovirus genogroups and genotypes, J. Gen. Virol. 100 (10) (2019) 1393–1406.
[8] P. Chhabra, et al., Corrigendum: updated classification of norovirus genogroups and genotypes, J. Gen. Virol. 101 (8) (2020) 893.
[9] L. Duan, et al., Prevalence of GII.4 sydney norovirus strains and associated factors of acute gastroenteritis in children: 2019/2020 season in guangzhou, China, Food Environ Virol 13 (3) (2021) 357–367.
[10] M. Farahmand, et al., Global prevalence and genotype distribution of norovirus infection in children with gastroenteritis: a meta-analysis on 6 years of research from 2015 to 2020, Rev. Med. Virol. 32 (1) (2022) e2237.
[11] K. Tohma, et al., Population genomics of GII.4 noroviruses reveal complex diversification and new antigenic sites involved in the emergence of pandemic strains, mBio 10 (5) (2019).
[12] G.I. Parra, et al., Static and evolving norovirus genotypes: implications for epidemiology and immunity, PLoS Pathog. 13 (1) (2017) e1006136.
[13] V.R. Tenge, et al., Glycan recognition in human norovirus infections, Viruses 13 (10) (2021).
[14] M. Tan, X. Jiang, The p domain of norovirus capsid protein forms a subviral particle that binds to histo-blood group antigen receptors, J. Virol. 79 (22) (2005) 14017–14030.
[15] M. Tan, X. Jiang, Norovirus-host interaction: multi-selections by human histo-blood group antigens, Trends Microbiol. 19 (8) (2011) 382–388.
[16] C.P. Cantelli, et al., Norovirus infection and HBGA host genetic susceptibility in a birth community-cohort, Rio de Janeiro, Brazil, Infect. Genet. Evol. 82 (2020) 104280.
[17] M. Tan, et al., Outbreak studies of a GII-3 and a GII-4 norovirus revealed an association between HBGA phenotypes and viral infection, J. Med. Virol. 80 (7) (2008) 1296–1301.
[18] X. Hong, et al., Association of fucosyltransferase 2 gene with norovirus infection: a systematic review and meta-analysis, Infect. Genet. Evol. (2021) 105091.
[19] Y. Yang, et al., Genetic and phenotypic characterization of GII-4 noroviruses that circulated during 1987 to 2008, J. Virol. 84 (18) (2010) 9595–9607.
[20] Y.T. Chen, et al., Crystallography of a lewis-binding norovirus, elucidation of strain-specificity to the polymorphic human histo-blood group antigens, PLoS Pathog. 7 (7) (2011).
[21] X. Jiang, et al., Expression, self-assembly, and antigenicity of the Norwalk virus capsid protein, J. Virol. 66 (11) (1992) 6527–6532.
[22] R.L. Atmar, et al., Comparison of microneutralization and histo-blood group antigen-blocking assays for functional norovirus antibody detection, J. Infect. Dis. 221 (5) (2020) 739–743.
[23] L. Fu, , et al.Cd-Hit, Accelerated for clustering the next-generation sequencing data, Bioinformatics 28 (23) (2012) 3150–3152.
[24] K. Katoh, D.M. Standley, MAFFT multiple sequence alignment software version 7: improvements in performance and usability, Mol. Biol. Evol. 30 (4) (2013) 772–780.
[25] H. Uusi-Kerttula, et al., Comparison of human saliva and synthetic histo-blood group antigens usage as ligands in norovirus-like particle binding and blocking assays, Microb. Infect. 16 (6) (2014) 472–480.
[26] L.C. Lindesmith, et al., Immunogenetic mechanisms driving norovirus GII.4 antigenic variation, PLoS Pathog. 8 (5) (2012) e1002705.
[27] K. Tohma, et al., Dynamic immunodominance hierarchy of neutralizing antibody responses to evolving GII.4 noroviruses, Cell Rep. 39 (2) (2022) 110689.
[28] M. Yang, et al., Contamination, bioaccumulation mechanism, detection, and control of human norovirus in bivalve shellfish: a review, Crit. Rev. Food Sci. Nutr. 62 (32) (2022) 8972–8985.
[29] A. Gobeil, B. Maherani, M. Lacroix, Norovirus elimination on the surface of fresh foods, Crit. Rev. Food Sci. Nutr. 62 (7) (2022) 1822–1837.
[30] J. Havumaki, et al., Immunologic and epidemiologic drivers of norovirus transmission in daycare and school outbreaks, Epidemiology 32 (3) (2021) 351–359.
[31] T.K. Trivedi, et al., Hospitalizations and mortality associated with norovirus outbreaks in nursing homes, 2009-2010, JAMA 308 (16) (2012) 1668–1675.
[32] K. Tohma, et al., Genome-wide analyses of human noroviruses provide insights on evolutionary dynamics and evidence of coexisting viral populations evolving under recombination constraints, PLoS Pathog. 17 (7) (2021) e1009744.
[33] J.A. Kendra, K. Tohma, G.I. Parra, Global and regional circulation trends of norovirus genotypes and recombinants, 1995-2019: a comprehensive review of sequences from public databases, Rev. Med. Virol. (2022) e2354.
[34] Y.L. Huang, et al., The role of conserved arginine and proline residues in enterovirus VP1 protein, J. Microbiol. Immunol. Infect. 55 (4) (2022) 590–597.
[35] T. Takano, et al., Immunodominant B-cell linear epitope on the VP1 P domain of a feline norovirus cat model, Pathogens 11 (7) (2022).

[36] M. Saito, et al., Molecular evolution of the capsid (VP1) region in human norovirus genogroup II genotype 3, Heliyon 6 (5) (2020) e03835.
[37] J.M. Devant, et al., Heterologous expression of human norovirus GII.4 VP1 leads to assembly of T=4 virus-like particles, Antivir. Res. 168 (2019) 175–182.
[38] R. Pogan, et al., Norovirus-like VP1 particles exhibit isolate dependent stability profiles, J. Phys. Condens. Matter 30 (6) (2018) 064006.
[39] M.E. Hardy, et al., Antigenic mapping of the recombinant Norwalk virus capsid protein using monoclonal antibodies, Virology 217 (1) (1996) 252–261.
[40] V. Lampinen, et al., Modular vaccine platform based on the norovirus-like particle, J. Nanobiotechnol. 19 (1) (2021) 25.
[41] L. Lindesmith, et al., Human susceptibility and resistance to Norwalk virus infection, Nat Med 9 (5) (2003) 548–553.
[42] A. de Rougemont, et al., Qualitative and quantitative analysis of the binding of GII.4 norovirus variants onto human blood group antigens, J. Virol. 85 (9) (2011) 4057–4070.
[43] J. Charon, S. Murray, E.C. Holmes, Revealing RNA virus diversity and evolution in unicellular algae transcriptomes, Virus Evol 7 (2) (2021) veab070.
[44] D.J. Smith, et al., Mapping the antigenic and genetic evolution of influenza virus, Science 305 (5682) (2004) 371–376.
[45] J.A. Kendra, et al., Antigenic cartography reveals complexities of genetic determinants that lead to antigenic differences among pandemic GII.4 noroviruses, Proc. Natl. Acad. Sci. U. S. A. 118 (11) (2021) e2015874118.
[46] S. Bhar, M.K. Jones, In vitro replication of human norovirus, Viruses 11 (6) (2019).
[47] B.K. Singh, M.M. Leuthold, G.S. Hansman, Human noroviruses' fondness for histo-blood group antigens, J. Virol. 89 (4) (2015) 2024–2040.
[48] S. Shanker, et al., Structural basis for norovirus neutralization by an HBGA blocking human IgA antibody, Proc. Natl. Acad. Sci. U. S. A. 113 (40) (2016) E5830–e5837.
[49] N. Hao, et al., Crystal structures of GI.8 Boxer virus P dimers in complex with HBGAs, a novel evolutionary path selected by the Lewis epitope, Protein Cell 6 (2) (2015) 101–116.
[50] J. Le Pendu, N. Ruvoën-Clouet, Fondness for sugars of enteric viruses confronts them with human glycans genetic diversity, Hum. Genet. 139 (6–7) (2020) 903–910.