

Elucidation of strain-specific interaction of a GII-4 norovirus with HBGA receptors by site-directed mutagenesis study

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ABSTRACT

Noroviruses interact with histo-blood group antigen (HBGA) receptors in a strain-specific manner probably detecting subtle structural differences in the carbohydrate receptors. The specific recognition of types A and B antigens by various norovirus strains is a typical example. The only difference between the types A and B antigens is the acetamide linked to the terminal galactose of the A but not to the B antigen. The crystal structure of the P dimer of a GII-4 norovirus (VA387) bound to types A and B trisaccharides has elucidated the A/B binding site on the capsid but did not explain the binding specificity of the two antigens. In this study, using site-directed mutagenesis, we have identified three residues on the VA387 capsid that are sterically close to the acetamide and are required for binding to A but not B antigens, indicating that the acetamide determines the binding specificity between the A and B antigens. Further mutational analysis showed that a nearby open cavity may also be involved in binding specificity to HBGAs. In addition, a systematic mutational analysis of residues in and around the binding interface has identified a group of amino acids that are required for binding but do not have direct contact with the carbohydrate antigens, implying that these residues may be involved in the structural integrity of the receptor binding interface. Taken together, our study provides new insights into the carbohydrate/capsid interactions which are a valuable complement to the atomic structures in understanding the virus/host interaction and in the future design of antiviral agents.

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Introduction

Noroviruses, a group of single-stranded, positive sense RNA viruses within the family *Caliciviridae*, are the major viral pathogens of epidemics of acute gastroenteritis worldwide. The norovirus capsid is composed of 180 monomers of the capsid protein that organizes into 90 dimers (Prasad et al., 1999). The capsid protein can be divided into two principle domains, the S domain constitutes the interior icosahedral shell, while the P domain forms an arch-shaped protrusion emanating from the shell. The P domain can be further divided into P1 and P2 subdomains, with the P2 subdomain located at the outer surface of the viral capsid. The primary sequence of the P2 subdomain is highly variable among norovirus strains, suggesting its involvement in host interaction. Expression of the P domain *in vitro*

results in dimerization (P dimer) and the formation of P particles that retain HBGA-binding function (Tan, Hegde, and Jiang, 2004; Tan, Meller, and Jiang, 2006; Tan and Jiang, 2005b). The involvement of the P2 subdomain in receptor binding has been confirmed by mutagenesis and crystallographic studies (Bu et al., 2008; Cao et al., 2007; Tan et al., 2003).

Noroviruses recognize human histo-blood group antigens (HBGAs) as receptors, in a strain-specific manner [Reviewed in (Tan and Jiang, 2005a; Tan and Jiang, 2007)]. Eight distinct HBGA-binding patterns of noroviruses have been described (Huang et al., 2003; Huang et al., 2005). Strain VA387 belongs to GII-4 genotype that is predominant in many countries (Ramirez et al., 2008; Siebenga et al., 2008; Tu et al., 2008; Verhoef et al., 2008). VA387 represents one of the eight receptor binding patterns and binds to A, B and H antigens. Other HBGA-binding patterns include the H and A binders of Norwalk virus and C59, the A binder of BUDS, the B binder of Snow Mountain virus, the A and B binder of MOH and etc (Huang et al., 2005). Human HBGAs are complex carbohydrates that are composed of 3–8 monosaccharides linked to proteins or lipids on the surface of red blood cells and mucosal epithelia of the respiratory, genitourinary, and digestive

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tracts, or as free oligosaccharide in biological fluids such as saliva. HBGAs are highly polymorphic and three major HBGA families, namely the ABO, Lewis, and secretor families, are involved in norovirus recognition (Tan and Jiang, 2005a; Tan and Jiang, 2007). Direct evidence linking HBGAs to norovirus infections and illness has been obtained in volunteer studies by challenging with Norwalk virus (Hutson et al., 2005; Hutson et al., 2002; Lindesmith et al., 2003). Outbreak studies of GII-4 viruses also support this linkage (Tan et al., 2008; Thorven et al., 2005). However, such evidence for other strains representing other receptor binding patterns remains lacking.

Recently, the 2.0 Å crystal structures of the P dimer of VA387 complexed with the type A- $\{\alpha\text{-L-fucose-(1,2)-}[\alpha\text{-D-acetyl-galactosamine-(1,3)]\text{-D-galactose}\}$ and type B- $\{\alpha\text{-L-fucose-(1,2)-}[\alpha\text{-D-galactose-(1,3)]\text{-D-galactose}\}$ trisaccharides, respectively, have been determined (Cao et al., 2007) (Fig. 1). This has led to the identification of the HBGA-binding interface on the outermost P2 surface and the recognition of the importance of dimerization of the P domain in the receptor binding. The crystal structure revealed an extensive hydrogen-bonding network between the trisaccharides and the nearby amino acid residues of the receptor-binding interface. Three groups of amino acids, each interacting with different sugars of the trisaccharide, have been described (Cao et al., 2007). Although both the A- and the B-trisaccharides were observed to bind to the same open pocket, the crystal structures did not provide detailed information on the binding specificity of noroviruses to HBGAs. In fact, no direct interaction was observed between P domain and the acetamide of the A-trisaccharide, the only structural difference between the A and the B antigens (Cao et al., 2007).

In this study, we performed systematic mutagenesis to explore the binding specificity of the HBGA-binding interface of VA387 and identified a site defined by three amino acids (Q₃₃₁, K₃₄₈, and I₃₈₉) that are sterically close to the acetamide of the A antigen and that is required for binding to A- but not to the B-antigens, suggesting the involvement of the site generated by these residues in interactions with the acetamide of the A antigen. Similar effects were also observed in a nearby open cavity. In addition, a group of other residues that do not have direct contact with HBGAs but are required for binding has been identified. These new data provide important insights into the carbohydrate/capsid interactions and would be useful for future antiviral design against noroviruses.

Results

Evidence for the role of the acetamide of the A antigen in norovirus-HBGA binding specificity

Our study began with the characterization of the site on the capsid interacting with the $\alpha\text{-1,3-galactose}$ of the B-trisaccharide as suggested by the crystal structure (Cao et al., 2007). The A- and B-antigens are structurally identical except for the presence of an acetamide group at the terminal sugar $\alpha\text{-1,3-N-acetylgalactosamine}$ of the A-antigen, but not the $\alpha\text{-1,3-galactose}$ of the B-antigen (Fig. 1). Three amino acids (A₃₄₆, S₄₄₁, and K₃₄₈) were predicted to interact via solvent molecules with the $\alpha\text{-1,3-galactose}$ of the B-antigen based on the crystal structure (Cao et al., 2007) (Fig. 1). Mutations A₃₄₆S and S₄₄₁A completely prevented binding of the mutant P particles to all A-, B-, and H-antigens (Figs. 2B and D, Table 1), indicating that these two residues are critical for binding to both A and B antigens. However, the third mutation (K₃₄₈A) resulted in complete loss of binding to the A- but did not affect binding to the B-antigen in both saliva- and synthetic oligosaccharide-based binding assays (Figs. 2C and I, Table 1), suggesting that this residue is involved in the binding specificity to the A antigen. The crystal structure of the HBGA-binding interface (Fig. 1) indicates that residue K₃₄₈ is positioned away from the center of the binding interface, close to the acetamide of the A-antigen, although no direct interaction between the acetamide and K₃₄₈ is seen (Cao et al.,

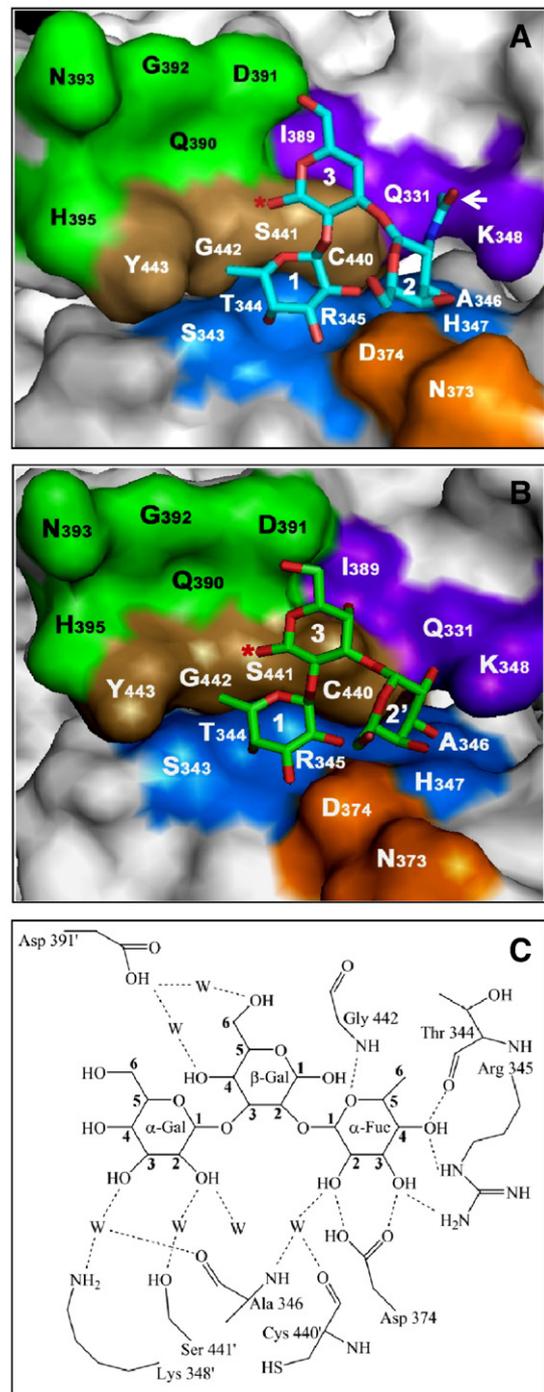


Fig. 1. The HBGA-binding interface of the norovirus capsid of VA387 (GII-4) interacting with the type A- and B-trisaccharides. The binding interfaces in (A) and (B) are in surface representation. The A- and B-trisaccharides are shown in stick representation, with nitrogen, oxygen, and carbon atoms colored blue, red, and cyan (A-antigen) or green (B-antigen), respectively. The saccharides are indicated as 1 = $\alpha\text{-1,2-fucose}$, 2 = $\alpha\text{-1,3-N-acetylgalactosamine}$, 2' = $\alpha\text{-1,3-galactose}$, 3 = $\beta\text{-1,3-galactose}$. The star symbol indicates the junction linking to the remaining portion of the antigen. The white arrow shows acetamide group in (A). The nearby open pocket is shown in green at the top-left corner. (C) schematically shows hydrogen bonds (dotted lines) between the individual saccharides of the B-trisaccharide and the nearby amino acids of the capsid. The water-mediated hydrogen bonds are indicated by W. (A) and (B) were prepared by software PyMOL version 1.0 (Delano Scientific), while (C) by software ChemDraw Pro version 11.0 (Adept Scientific).

2007). Interestingly, replacement of two other residues (Q₃₃₁ and I₃₈₉) in the vicinity of K₃₄₈ (Fig. 1) with alanines also resulted in the loss of binding to the A- but not B- antigen (Figs. 2E–F, J–K; Table 1). These

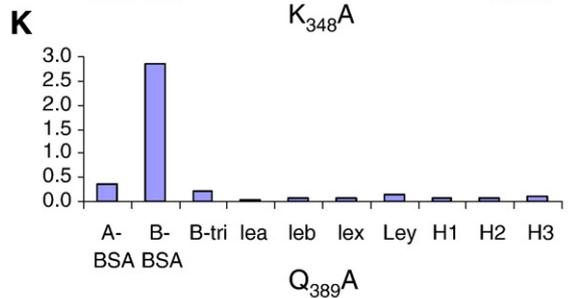
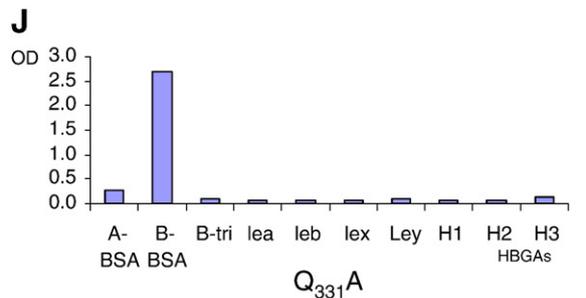
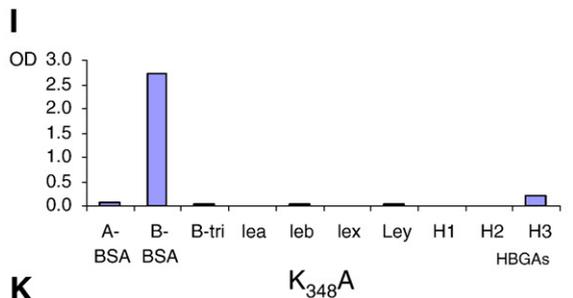
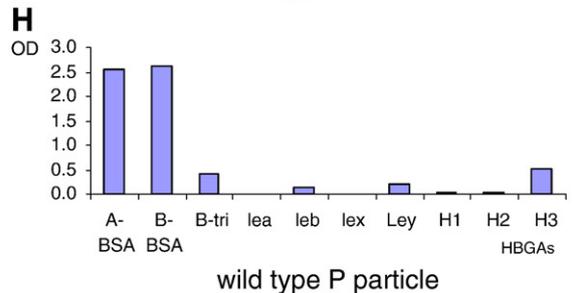
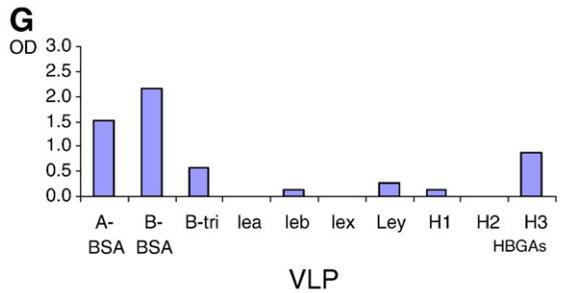
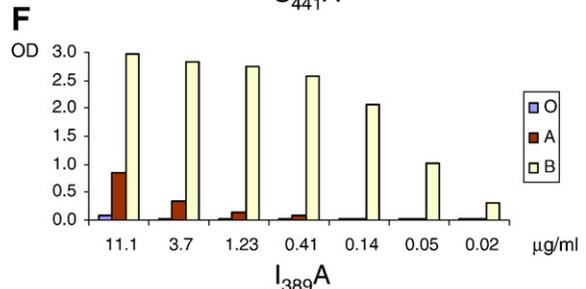
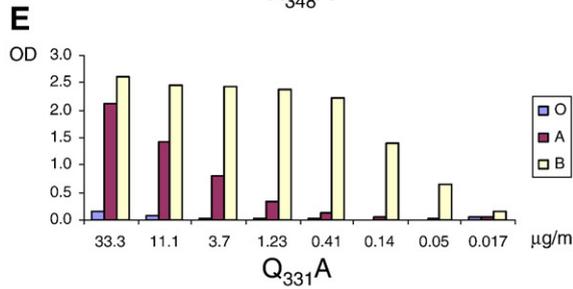
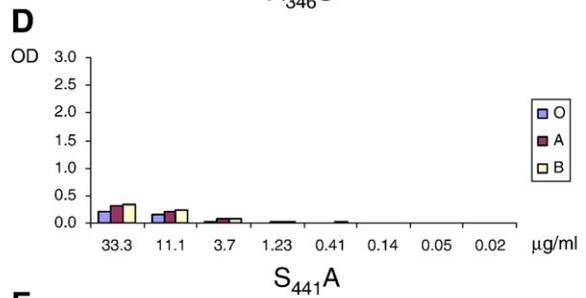
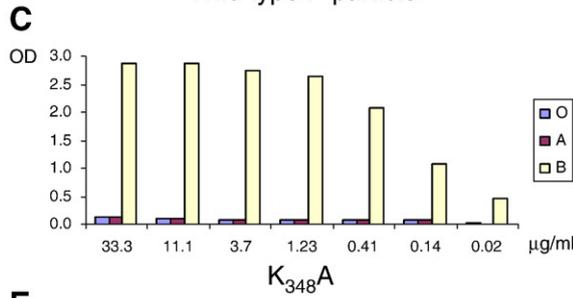
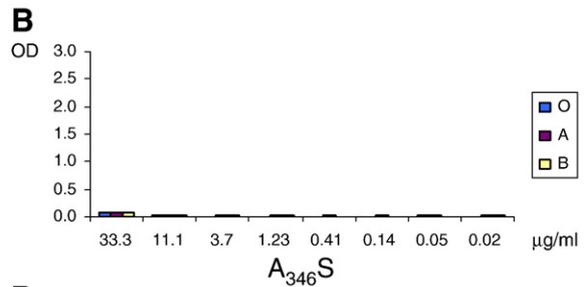
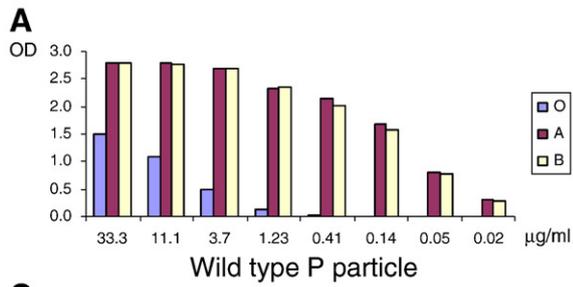


Table 1

Summary of the systematic mutagenesis analysis on residues in and around the HBGA-binding interface of VA387 norovirus

Mutants ^c	Relative binding affinity to HBGAs ^a					
	P particles ^b			P dimers		
	O	A	B	O	A	B
Wild type	+	++++	++++	+	++	++
The α -fucose-binding site:						
T ₃₄₄ A*	+	++++	++++	+	++	++
R ₃₄₅ A*	-	-	-	-	-	-
A ₃₄₆ S*	-	-	-	/	/	/
N ₃₇₃ A	-	-	-	/	/	/
D ₃₇₄ A*	-	-	-	-	-	-
C ₄₄₀ A*	+	++++	++++	+	++	++
G ₄₄₂ A*	-	-	-	-	-	-
Y ₄₄₃ A	-	-	-	-	-	-
P ₄₄₄ A	-	-	-	/	/	/
Residues that support the conformation of the α -fucose-binding site:						
Q ₃₃₆ A	-	-	-	/	/	/
T ₃₃₈ A	-	-	-	-	-	-
S ₃₄₃ A	+/-	++	++	+/-	+	+
H ₃₄₇ A	-	-	-	/	/	/
The α -glucose/ α -N-acetylglactosamine-binding site:						
Q ₃₃₁ A	-	+	++++	/	/	/
A ₃₄₆ S*	-	-	-	/	/	/
A ₃₄₆ G*	+++	++++	++++	/	/	/
K ₃₄₈ A*	-	-	++++	/	/	/
I ₃₈₉ A	-	-	++++	/	/	/
I ₃₈₉ V	+	+++	++++	/	/	/
S ₄₄₁ A*	-	-	-	/	/	/
β -Galactose-binding site:						
D ₃₉₁ A*	+	+++	+++	/	/	/
2nd open cavity:						
Q ₃₉₀ A	+/-	+++	+++	/	/	/
G ₃₉₂ A	-	+	+++	/	/	/
N ₃₉₃ A	+/-	+++	+++	/	/	/
H ₃₉₅ A	+/-	++	+++	/	/	/
Negative control:						
T ₃₃₅ S	+	++++	++++	/	/	/
M ₃₃₃ A	+	++++	++++	/	/	/
R ₃₃₉ A	+	++++	++++	+	++	++
D ₅₁₇ A	+	++++	++++	+	++	++
S ₅₁₈ A	+	++++	++++	+	++	++

^a Number of "+" indicated the relative binding affinity of the mutant P particles and P dimers to HBGAs. "-" indicated a complete loss of binding. "/" = not determined.

^b The P particle formation of wild type and all mutants were confirmed by gel filtration.

^c Amino acids with a star symbol are predicted to interact with type A- and/or B-trisaccharides by crystallographic study.

data suggested that the three clustered residues Q₃₃₁, K₃₄₈, and I₃₈₉ might form an interaction interface specifically needed by the A antigen.

Systematic analysis of residues in and around the binding interface

The findings of the two residues (Q₃₃₁ and I₃₈₉) that have no direct contact with the trisaccharides but affect the receptor binding suggested that a systematic analysis of individual residues in and surrounding the HBGA-binding interface might be informative. The crystal structure of the VA387 P dimer bound to the A- and B-trisaccharides revealed eleven amino acids in the receptor-binding interface that interact with the trisaccharide via direct or water-mediated hydrogen bonds (Cao et al., 2007) (Fig. 1). We replaced all of them with either alanines or serines, including the three residues interacting with the α -1,3-galactose described above, and found that

the mutations affected HBGA-binding as predicted by the crystal structures (see below).

In addition, 12 amino acids that have no direct contact with the trisaccharides but surround the binding interface as shown by the crystal structures (Fig. 1) were also subjected to mutagenesis. Many of them also affected HBGA-binding (see below), indicating that these residues are also required for the structural integrity of the receptor binding interface. Finally, five other mutations (T₃₃₅S, M₃₃₃A, R₃₃₉A, D₅₁₇A, and S₅₁₈A) that are far away from the binding interface were introduced into the P particles and none of them affected the HBGA-binding function. None of the single amino-acid mutations described in this study affected P particle or P dimer formation as shown by gel filtration analysis (Tan and Jiang, 2005b; Tan, Hegde, and Jiang, 2004; Tan, Meller, and Jiang, 2006).

The α -fucose plays a central role in norovirus/receptor interactions

The crystal structures suggested six amino acids (T₃₄₄, R₃₄₅, A₃₄₆, D₃₇₄, C₄₄₀, and G₄₄₂) interacting with the α -1,2-fucose of the B-trisaccharide (Cao et al., 2007). These residues were replaced by alanine or serine and 4 of the mutants lost their binding to all A-, B-, and H- antigens completely or nearly completely (G₄₄₂A) (Fig. 3 and Table 2). The binding to HBGAs of the remaining 2 mutants (T₃₄₄A and C₄₄₀A) did not change, although the crystal structures showed hydrogen bonds between the two amino acids and the fucose (Fig. 1). This discrepancy could be explained by the fact that both residues interact with the α -1,2-fucose through their backbone oxygen atoms (Cao et al., 2007) (Fig. 1), which would remain the same in the substituted alanines. Thus all six fucose-interacting amino acids predicted by the crystal structures are most likely required for binding to the carbohydrate receptors.

In addition, seven other amino acids, none of which have contact with the A- and B-trisaccharides, were also examined. Four (S₃₄₃, Q₃₃₆, T₃₃₈, and H₃₄₇) of them were suggested to support the conformation of the α -fucose-binding site (Cao et al., 2007), while the other three (N₃₇₃, Y₄₄₃, and P₄₄₄) were nearby. Replacement of each of these seven amino acids with alanines decreased binding to all A-, B- and H-antigens significantly (Fig. 4 and Table 2). Taken together, except the six amino acids that have direct contact with the α -fucose, at least seven additional residues are likely to be required for the structural integrity of the binding site, further highlighting the importance of this sugar in the norovirus/HBGA interaction as suggested by previous studies (Cao et al., 2007; Huang et al., 2005; Tan and Jiang, 2005a; Tan and Jiang, 2007).

The β -galactose may not be critical for HBGA-binding

The crystal structures indicated that the β -1,3-galactose of both type A- and B-trisaccharide is relatively distant from the P dimer surface (Cao et al., 2007) (Fig. 1). Only D₃₉₁ was seen to interact with the β -galactose via two water-bridged hydrogen bonds (Fig. 1C). However, replacement of D₃₉₁ with an alanine only slightly reduced binding to HBGAs (Fig. 5C and Table 1), suggesting that the interaction between D₃₉₁ and the β -1,3-galactose may not be critical for binding to HBGAs.

A nearby pocket affects binding to the A-antigen

Another open pocket near the binding interface that is made up of residues Q₃₉₀ and H₃₉₅ in the bottom and D₃₉₁, G₃₉₂, N₃₉₃ and Y₄₄₃ as

Fig. 2. Binding of mutant P particles with amino acid substitutions in or around the α -galactose-binding site to the saliva samples (A–F) and synthetic oligosaccharide representing different HBGAs (G–K). The X-axes of (A) to (F) show the protein concentrations of the P particles and the Y-axes indicate the optical densities at 450 nm (OD₄₅₀) that were the average value of triplicate experiments. "O", "A", and "B" represent the type O (containing H antigen), A, and B saliva, respectively. The X-axes of (G) to (K) shows a panel of synthetic oligosaccharides representing 9 different HBGAs (A, B, Le^a, Le^b, Le^x, Le^y, H1, H2, and H3). BSA indicates that those oligosaccharides were linked to bovine serum albumin as backbones (A-BSA and B-BSA), while the remaining oligosaccharides were conjugated to polyacrylamide (PAA) as backbones. The protein concentrations of VLP (G) and P particles (H–K) were 1 μ g/ml and 2 μ g/ml, respectively.

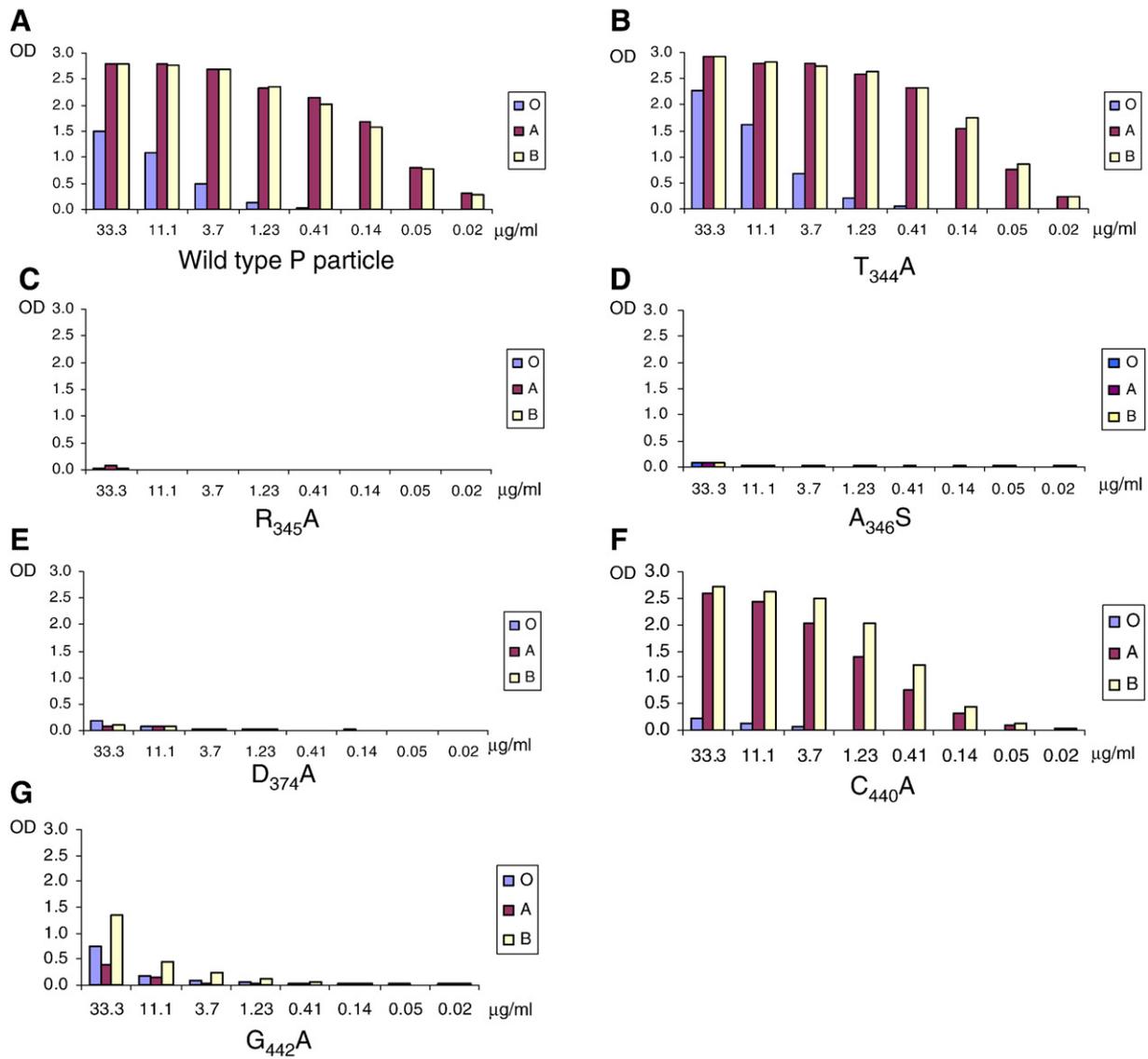


Fig. 3. Binding of mutant P particles with amino acid changes in or around the α -fucose-binding site to the saliva samples. The X-axes show the protein concentrations of the P particles, while the Y-axes indicate the optical densities at 450 nm (OD_{450}) that were the average value of triplicate experiments. "O", "A", and "B" represent the type O (containing H antigen), A, and B saliva samples, respectively.

the walls of the pocket was suggested to have additional interaction with the HBGAs (Cao et al., 2007) (Fig. 1). When these residues were replaced by alanines, except Y_{443} that has been shown to be important for binding to A-, B-, and H-antigens probably by interacting with the α -1,2-fucose (Fig. 3H), only G_{392} and H_{395} showed reduction in binding to A- but not B-antigens (Figs. 5A to F). Interestingly, the reduction in binding to A-antigen by $G_{392}A$ and $H_{395}A$ mutations occurred only in the saliva- but not the synthetic oligosaccharide-based binding assays (Figs. 5H to I). These data suggest that some unknown features of the native HBGAs (saliva) that are missing on the synthetic oligosaccharides may be responsible for the interaction with this nearby pocket.

The receptor binding interface is highly conserved among known GII-4 viruses

Following the identification of the receptor binding interface and the functional involvement of the amino acids described above, we performed a sequence alignment (Fig. 6A) of the major GII-4 variants, including several strains with known binding patterns to all A-, B-

and H-antigens and two strains that lost binding to all A-, B- and H-antigens (Huang et al., 2003; Huang et al., 2005; Lindesmith et al., 2008), in an attempt to elucidate the mechanism and residues that are responsible for the receptor binding changes. Among the 19 amino acids (Fig. 6A and Table 1) important for HBGA-binding identified in this study, only two amino acids have changed since 1987: A_{346} to a glycine (G) in 2000 and I_{389} to a valine (V) in 2001 (Fig. 6A). These are very similar amino acids with only a methyl group (CH₃) difference in their side chains between A and G or between I and V. These changes appear not to correlate with the phenotypic changes of the binding patterns (Huang et al., 2003; Huang et al., 2005; Lindesmith et al., 2008). Mutant $A_{346}G$ developed for VA387 did not affect the binding to types A and B saliva but increased binding to type O saliva (Fig. 6C), mutant $I_{389}V$ showed a small decrease in binding to type A but not B saliva (Fig. 6D), while mutant with double mutations of $A_{346}G$ and $I_{389}V$ resulted in stronger reduction in binding to both A- and B-antigens but the bindings to all A-, B- and H-antigens clearly remained (Fig. 6E). These data suggested that subtle changes of the binding interface can vary receptor binding, but neither A_{346} nor I_{389} is responsible for the

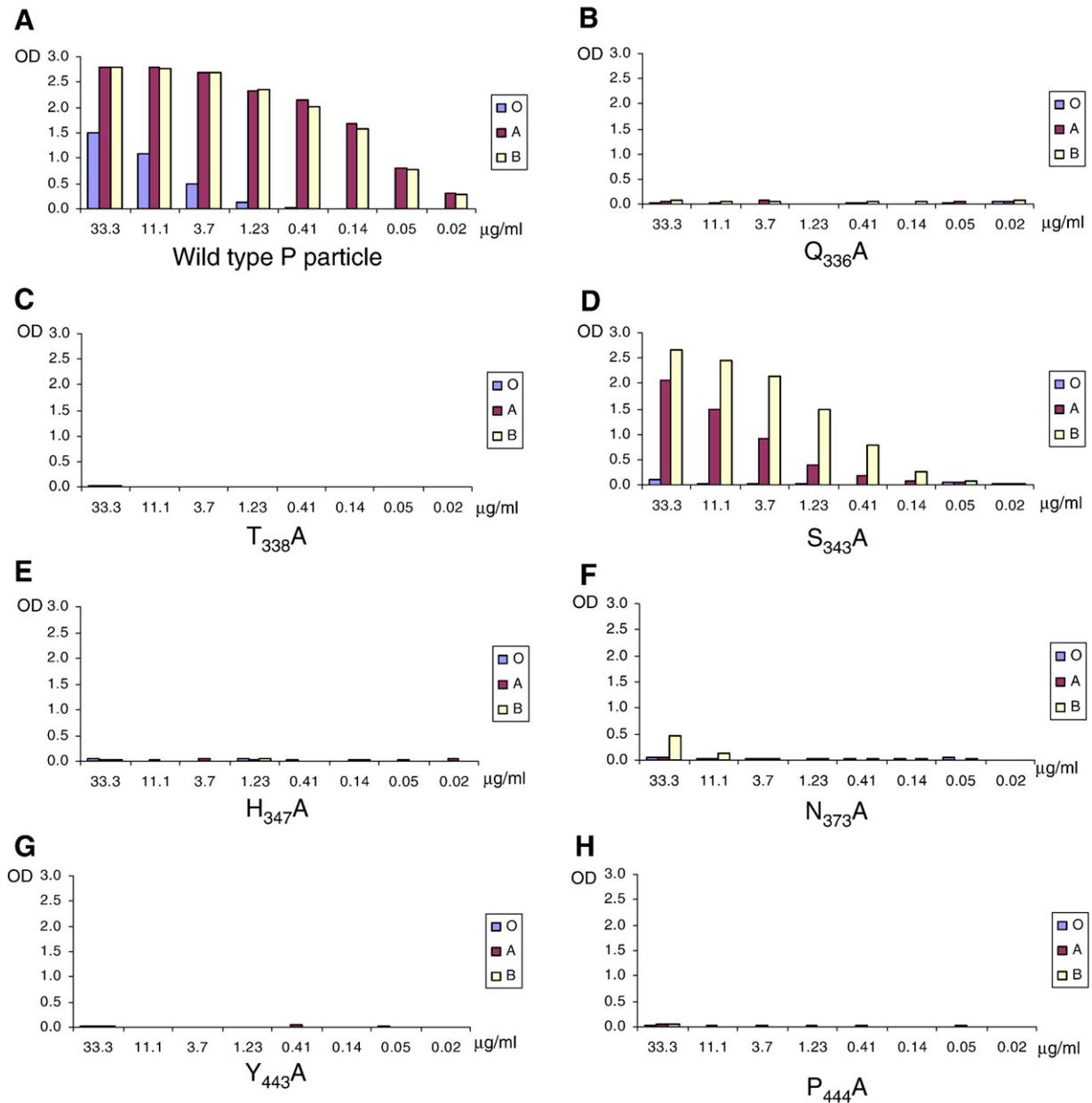


Fig. 4. Binding of mutant P particles with single mutations at the 4 amino acids that were predicted to support the structural integrity of the α -fucose-binding site as well as 3 other amino acids nearby. The X-axes show the protein concentrations of the P particles, while the Y-axes indicate the optical densities at 450 nm (OD_{450}) that were the average value of triplicate experiments. "O", "A", and "B" represent the type O (containing H antigen), A, and B saliva samples, respectively.

complete loss of HBGA-binding of the two variants, Hunter and Sakai viruses (Lindesmith et al., 2008).

Discussion

In this study we performed systematic mutagenesis on 27 amino acids in and around the HBGA-binding interface of a GII-4 virus (VA387) to examine their roles in norovirus-receptor binding affinity and specificity. Among 11 amino acid residues that have direct contact with the carbohydrate receptors based on the crystal structures (Cao et al., 2007), 7 residues are absolutely required for receptor-binding as they knock out binding to both A- and B-antigens when they are replaced by alanines. One residue (K_{348}) is required for binding to A- but not to B-antigens and the remaining three residues (T_{344} , C_{440} and D_{391}) are also likely involved in the binding although the possibility exists that we might not have selected proper replacement of amino

acids in our mutagenesis. In conclusion, except T_{344} , C_{440} , and D_{391} , whose roles for HBGA-binding remain to be confirmed, all remaining 8 amino acids constituting the binding interface observed in the crystal structure (Cao et al., 2007) participate in norovirus receptor binding.

In addition, we have identified another 11 amino acids that are important for HBGA-binding, although they do not make direct contact with the carbohydrate receptors according to the crystal structures. These residues are likely responsible for the conformational integrity of the receptor binding interface. Finally, we have demonstrated that the HBGA-binding interface is highly conserved among the known GII-4 viruses, which is consistent with our observation that the majority of GII-4 viruses bind to all A, B and H antigens (unpublished data) and appear to explain their predominance in the epidemiology of noroviruses. However, we still cannot exclude the possibility that additional residues other than those described in this study are also involved in norovirus-HBGA binding

Table 2
Primers used for site-directed mutagenesis to generate single amino acid mutation

#	Name	Primer sequence (5' to 3')	Sense	Mutation
1	P981	CGTGGGAAAGATCGCAGCATGCTACCCAAACC	+	Q331A
2	P982	GGTTTGGGTGAGCATGCGCTGCGATCTTCCCCAG	-	Q331A
3	P983	GGGAAAGATCAAGCGCGCTACCCAAACCAC	+	M333A
4	P984	GTGGTTTGGGTGAGCGCGCTTGGATCTTCC	-	M333A
5	P1102	CAGTGAGCACTGGGACTGTCCACTCACTCC	+	S335A
6	P1103	GGAGTGAAGTGGACAGTCCCAGTGTCTACTG	-	S335A
7	P985	CCAAGGCATGCTCACCGCAACCACAAGAGAGGATGGC	+	Q336A
8	P986	GCCATCTCTTGTGGTGTGGGTGAGCATGCTCTGG	-	Q336A
9	P929	GCATGCTCACCCAAACCACAAGAGGATGGCTCG	+	T338A
10	P930	CGAGCCATCTCTTGGCGTGGTGGTGGAGCATGC	-	T338A
11	P931	GCTCACCCAAACCACAAGAGGATGGCTCGAC	+	R339A
12	P932	GGTCGAGCCATCTCTGCTGTGGTGGTGGTGAGC	-	R339A
13	P943	CACAAGAGAGGATGGCGGACCCGCGCCAC	+	S343A
14	P944	GTGGGCGCGGTGCGCCATCTCTTGTG	-	S343A
15	P925	GAGAGGATGGCTCGCCCGCCCAAAAGC	+	T344A
16	P926	GCTTGTGGGCGCGCCGAGGATGCTCTCTC	-	T344A
17	P927	GAGGATGGCTCGACCGCCGCCACAAAGTTAC	+	R345A
18	P928	GTAGCTTGTGGGCGCGCTCGAGCCATCTCTC	-	R345A
19	P991	GGATGGCTCGACCGCTCCCAAGAGTACAGTG	+	A346S
20	P992	CACTGTAGCTTGTGGGAGCGGGTTCGAGCCATCC	-	A346S
21	P1285	GGTCGACCCGCGCCACAAGCTACAG	+	A346G
22	P1286	CTGTAGCTTGTGGCCGCGGTGAGCC	-	A346G
23	P1100	GCTCGACCCGCGCCCAAGCTACAGTGGC	+	H347A
24	P1101	GCTCACTGTAGCTTGGCGGCGCGGTGAGC	-	H347A
25	P1001	CGACCCGCGCCACGACTACAGTGGAGC	+	K348A
26	P1002	GTGCTCACTGTAGCTGGTGGGCGCGGTGCG	-	K348A
27	P993	CCACTGACACAACGCTGATTTCAAAGTGGC	+	N373A
28	P994	GCCAGTTTAAAATCAGCGTTTGTGTCACTGG	-	N373A
29	P933	CACTGACACAACAATGCTTTCAAAGTGGC	+	D374A
30	P934	GGCCAGTTTAAAAGCTTGTGTGTCACTGG	-	D374A
31	P995	GACACAACAATGATGCTCAAAGTGGCCAAACACC	+	F375A
32	P996	CGTGTGGTGGCAGTTTGGAGCATATTGTGTGTC	-	F375A
33	P1259	CACCCAGTCGCGCGCTCGCCAGGATGGTAATAACC	+	I389A
34	P1260	GGTATTACCATCTGGGCGACCGGACTGGGGTG	-	I389A
35	P1287	CCAGTCGCGCTGTCAGGATGGTAATAACC	+	I389V
36	P1288	GGTATTACCATCTGGACGACCGGACTGGG	-	I389V
37	P1261	CCAGTCGCGCTCGCCGAGTGGTAATAACCACC	+	Q390A
38	P1262	GGTGGTATTACCATCCGCGATGACCGGACTGG	-	Q390A
39	P1106	CGGCGTCATCCAGGCTGGTAATAACCACC	+	D391A
40	P1107	GGTGGTATTACCAGCCTGGATGACGCCG	-	D391A
41	P1263	GCGTCATCCAGGATGCTAATAACCACCAAAATG	+	G392A
42	P1264	CATTTTGGTGTATTAGCATCTCGGATGACGC	-	G392A
43	P1265	CGTCATCCAGGATGGTGTAAACCACCAAAATGAAC	+	N393A
44	P1266	GGTTCATTTTGGGTGAGCACCATCTGGATGACG	-	N393A
45	P1267	CCAGGATGGTAATAACGCCAAAATGAACCCAGC	+	H395A
46	P1268	GCTGGGTTTCAATTTGGCGTATTACCATCTGG	-	H395A
47	P1108	CCAATATGCGCGGCTAGCGGTTATCC	+	C440A
48	P1109	GGGATACCCGCTAGCCCGGCGATAGTCC	-	C440A
49	P1110	CTATGCCCGGTGTGCCGGTATCCCAAC	+	S441A
50	P1111	GTTGGGATACCCGACACCCGCGGATAG	-	S441A
51	P997	GCCCGGTTGAGCGGCTATCCCAACATG	+	G442A
52	P998	CATGTTGGGATACGCGCTACACCCGGGC	-	G442A
53	P935	CCCGGTTGAGCGGGCTCCCAACATGAATCTGG	+	Y443A
54	P936	CCAGATTCATGTTGGAGCCCGCTACCCCGGG	-	Y443A
55	P999	GGGTGAGCGGATGCCAACARGAATCTGG	+	P444A
56	P1000	CCAGATTCATGTTGCATACCCGCTACACCC	-	P444A
57	P937	CCAATGTTAATTTAGATTTGCTTCTGGGTCAACCAG	+	D517A
58	P938	CTGGTTGACCCAGGAAGCAATCTAAAATAACCATTGG	-	D517A
59	P939	GGTATTTTATGATTTGATGCTGGTCAACCAAGTTCTAC	+	S518A
60	P940	GTAGAAGTGGTGGACCCAGGCATCAAACTAAAATAACC	-	S518A

because some GII-4 viruses lose complete or partial receptor-binding but retain the conserved binding interface. Further investigations of this issue are necessary.

Here we have provided direct evidence suggesting that the acetamide group of the A antigen is responsible for the binding specificity differences between the A and the B antigens. The identification of three amino acids (H₃₄₈, Q₃₃₁, and I₃₈₉) responsible for the binding specificity may help future genetic analysis and classification of noroviruses. For example, noroviruses differ in recognizing the A and B antigens. VA387 (GII-4) described in this study and a few other strains (MOH, MxV, and Paris Island virus)

recognize both A and B antigens; the prototype Norwalk virus and BUDS recognize A but not B; while the Snow Mountain virus recognizes the B but not A antigen (Huang et al., 2005; Tan and Jiang, 2005; Tan and Jiang, 2007; Tan et al., 2004). The identification of the site responsible for acetamide recognition on the capsid would allow correlation between sequence and phenotype and consequently allow a more detailed classification of norovirus strains.

The demonstration of the involvement of the open pocket near the binding interface in the HBGA interaction is also significant since it indicates that additional features of the HBGA molecules may also participate in norovirus recognition. The involvement of this site in binding to HBGAs was also observed previously through a mutant VLP (D₃₉₃G) of another GII-4 strain (Lindesmith et al., 2008). In addition, the binding difference of the two mutants (G₃₉₃A and H₃₉₅A) to the native HBGAs in saliva and synthetic carbohydrate receptors (Fig. 5) further supports our hypothesis. The native HBGAs are much more complicated containing 3 to 8 saccharides either free or linked to proteins or lipids as backbones, while the synthetic carbohydrates used in the crystallographic and the mutagenesis studies contain only 2 to 4 sugars either free or linked to bovine serum albumin (BSA) or polyacrylamide (PAA) as backbones. Thus either additional sugars or the backbones of the native receptors could be involved in the interaction with the nearby pocket. The impact of the backbones in norovirus-receptor interaction has also been previously suggested (Huang et al., 2005) but the site on the viral capsid for this interaction remains unknown. The potential interacting site of additional sugars could be investigated by co-crystallization of the viruses or P dimers with more complicated carbohydrate molecules.

Our study also provides new evidence supporting the central role of the α -1,2-fucose- (H-epitope) in norovirus-host interaction which was first suggested by our previous characterization of receptor binding variations of variable noroviruses (Huang et al., 2003; Huang et al., 2005) and then by the crystal structure of a GII-4 virus (Cao et al., 2007). Structurally the α -1,2-fucose is the terminal saccharide of the H-related antigens, including A, B, Le^b, Le^y, and H types 1, 2, 3 antigens, and thus is the key determinant for binding to noroviruses. We now show functional evidence for the requirement of the 6 residues interacting with the α -1,2-fucose in GII-4 norovirus binding to all A-, B-, and H-antigens. In addition, we demonstrated the involvement of a few other residues interacting with the α -1,3-N-acetylglactosamine- (A-epitope), which may further strengthen the binding affinity and determine the binding specificity. The complete loss of binding activity to all A-, B- and H-antigens by single mutations in these binding sites imply that they may be important targets for broadly effective antivirals against different noroviruses with different binding patterns.

The observed involvement of the acetamide of the A antigen in the binding specificity of VA387 provides an example of how a minor structural modification of a HBGA can affect the receptor binding pattern and potentially change the host range of a norovirus. Human HBGA system is highly polymorphic under the control of multiple gene families. In addition, modifications by other chemical groups such as the sialic epitopes and subtypes of the ABO, secretor and Lewis families of HBGAs make the human HBGA system and its recognition with noroviruses even more complicated. The demonstration of the relationship between the three amino acids (Q₃₃₁, K₃₄₈, and I₃₈₉) and the acetamide helps to explain the mechanism of the strain-specific norovirus-HBGA interaction. Another example are the residues in the nearby open cavity that could interact with additional structures of HBGAs and reduce binding to the A- more than to the B-antigen [Fig. 5, (Lindesmith et al., 2008)], although the detailed mechanism still needs to be confirmed.

In an attempt to explore the mechanism of the HBGA-binding variations, evolution, and epidemiology of different GII-4 viruses

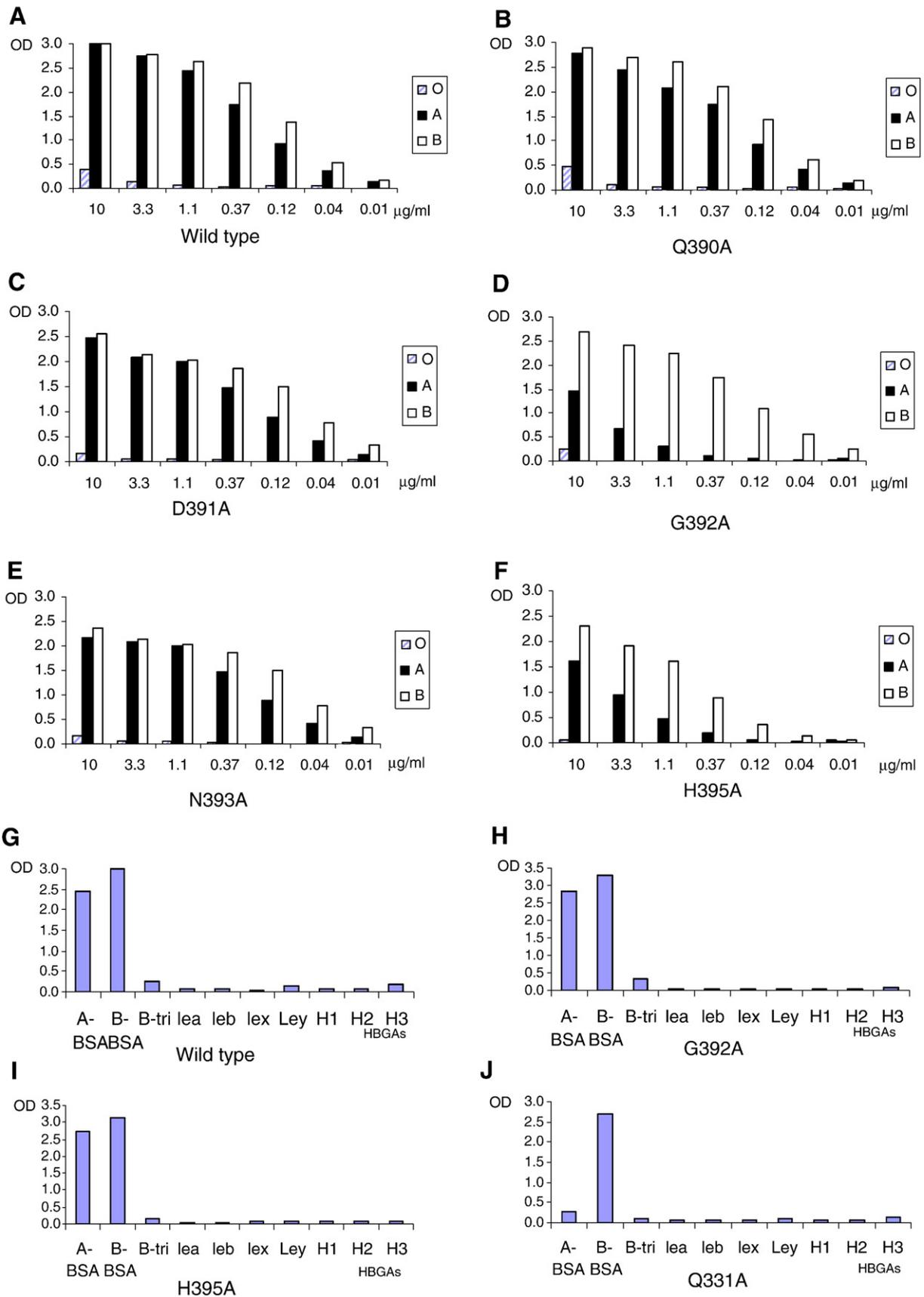


Fig. 5. Binding of mutant P particles with single amino acid change at the second open pocket to the saliva samples (A–F) and synthetic oligosaccharides representing different HBGA (G–J). The X-axes of (A) to (F) show the protein concentrations of the P particles and the Y-axes indicate the optical densities at 450 nm (OD₄₅₀) that were the average value of triplicate experiments. “O”, “A”, and “B” represent the type O (containing H antigen), A, and B saliva, respectively. The X-axes of (G) to (J) shows a panel of synthetic oligosaccharides representing 9 different HBGA (A, B, Le^a, Le^b, Le^x, Le^y, H1, H2, and H3). BSA indicates that those oligosaccharides were linked to bovine serum albumin as backbones (A-BSA and B-BSA), while the remaining oligosaccharides were conjugated to polyacrylamide (PAA) as backbones. The protein concentrations of the P particles in G to J were 2 µg/ml. Mutant Q331A (J) served as control of selective inhibition of binding to A-antigen.

A

Year accession #	330	340	350	370	390	400	P2<>P1	440
2006 (AB291542):	IQGVLTQTTK	GDGSTR	GHKATV	YVYTGSA	FF	FSDT	TENDEF	ETHQNT
2005 Sakai (AB220922):	IQGVLTQTTK	GDGSTR	GHKATV	STGSDV	FF	FATD	TNDDEF	FETGQNT
2004 Hunter (DQ078794):	IQGVLTQTTTR	RDGSTR	GHKATV	STGSDV	VHF	FSDT	SNDDEF	FETGQNT
2004 (DQ078794):	IQGVLTQTTTR	RDGSTR	GHKATV	STGSDV	VHF	FSDT	SNDDEF	FETGQNT
2003 (AY581254):	IQGVLTQTTTR	RDGSTR	GHKATV	STGSDV	VHF	FNTD	TNDEF	FETGQNT
2002 (AY588018):	IQGVLTQTTTR	RDGSTR	GHKATV	STGSDV	VHF	FNTD	TNDEF	FETGQNT
2002 (AY502023):	IQGVLTQTTTR	RDGSTR	GHKATV	STGSDV	VHF	FNTD	TNDEF	FETGQNT
2002 (DQ364459):	IQGVLTQTTTR	RDGSTR	GHKATV	STGSDV	VHF	FNTD	TNDEF	FETGQNT
2001 (AJ583672):	IQGVLSQTTR	RDGSTR	GHKATV	STGSDV	VHF	YTTD	TNDEF	FETGQNT
2000 (AF427117):	IQGVLTQTTTR	RDGSTR	GHKATV	STGSDV	VHF	FNTD	TNDEF	FETGQNT
1999 (AF427116):	IQGVLTQTTTR	RDGSTR	GHKATV	STGSDV	VHF	YTTD	TNDEF	FETGQNT
1998 VA387 (AY038600):	IQGVLTQTTTR	RDGSTR	GHKATV	STGSDV	VHF	YTTD	TNDEF	FETGQNT
1998 (AY532123):	IQGVLTQTTTR	RDGSTR	GHKATV	STGSDV	VHF	YTTD	TNDEF	FETGQNT
1997 (DQ078829):	IQGVLTQTTTR	RDGSTR	GHKATV	STGSDV	VHF	YTTD	TNDEF	FETGQNT
1996 (AF080558):	IQGVLTQTTTR	RDGSTR	GHKATV	STGSDV	VHF	FNTD	TNDEF	FETGQNT
1995 (AF080551):	IQGVLTQTTTR	RDGSTR	GHKATV	STGSDV	VHF	YTTD	TNDEF	FETGQNT
1995 (AJ277619):	IQGVLSQTTR	RDGSTR	GHKATV	STGSDV	VHF	YTTD	TNDEF	FETGQNT
1994 (AF145896):	IQGVLTQTTTR	RDGSTR	GHKATV	STGSDV	VHF	FNTD	TNDEF	FETGQNT
1993 (X76716):	IQGVLTQTTTR	RDGSTR	GHKATV	STGSDV	VHF	FNTD	TNDEF	FETGQNT
1992 (X86557):	IQGVLTQTTTR	RDGSTR	GHKATV	STGSDV	VHF	FNTD	TNDEF	FETGQNT
1987 (AY032605):	IQGVLTQTTTR	RDGSTR	GHKATV	STGSDV	VHF	FNTD	TNDEF	FETGQNT

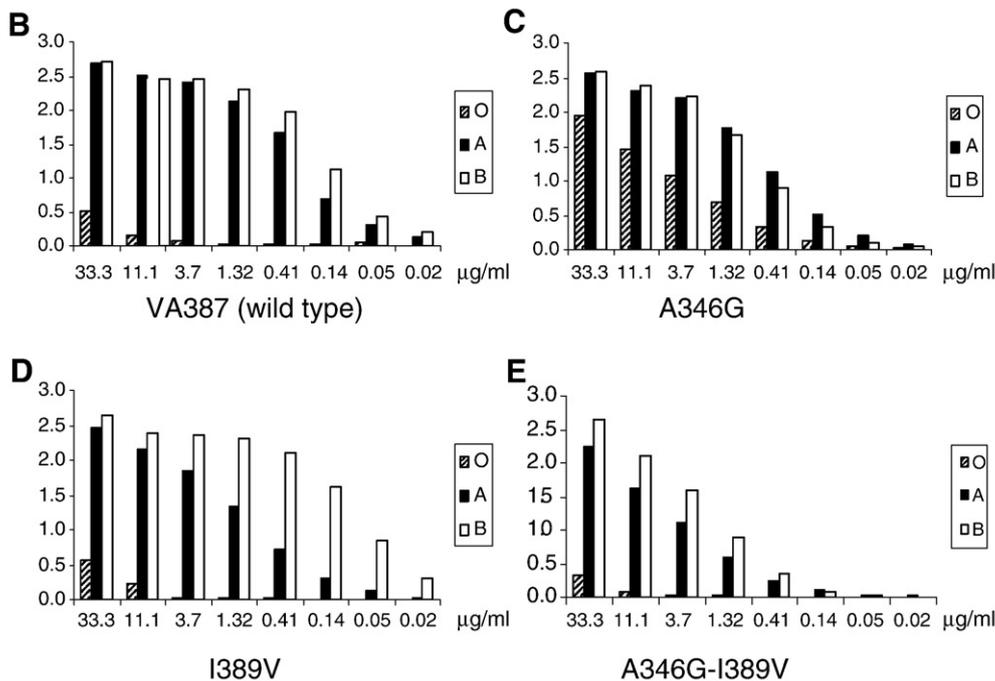


Fig. 6. Sequence alignment (A) of the HBGA-binding interfaces and the surrounding regions in the P domain of GII-4 viruses selected from different years since 1987. Each virus is indicated by the year of isolation and its accession number in the GenBank. Dark background indicates highly conserved residues and gray background represents less conserved amino acids. Dots in the dark background indicate omission of conserved residues. The numbers on the top indicate the positions of the residues according to VA387 (underlined). The Hunter virus (2004, DQ078794) and Sakai virus (2005, AB220922) that loss their binding activities (Lindsmith et al., 2008) are highlighted by bold letters. “#” indicates amino acids that are required for HBGA-binding, “*” indicates residues that affect the binding specificity, “+” indicates amino acids that form the nearby open pocket and “<>” indicates the boundary of the P1 and P2 subdomains. B to E, binding of mutant P particles of VA387 with single mutations [A₃₄₆G (C) or I₃₈₉V (D)], or double mutation [A₃₄₆G and I₃₈₉V (E)] to saliva samples. The X-axes show the protein concentrations of the P particles (µg/ml) and the Y-axes indicate the optical densities at 450 nm (OD₄₅₀) that were the average value of triplicate experiments. “O”, “A”, and “B” represent the type O (containing H antigen), A, and B saliva, respectively.

from different countries, we have shown that the majority of GII-4 strains isolated in the past 20 years retain a broad spectrum of host specificity to all A, B and H antigens, although binding to each of the antigen may be variable [(Huang et al., 2005), unpublished data]. In this study we performed a sequence alignment of different GII-4 strains and found that 17 of the 19 amino acids that are critical for HBGA-binding are highly conserved among all GII-4 viruses, while the remaining two residues are replaced by very similar amino acids. These data suggest that the human HBGAs might be a selection marker in the evolution of noroviruses and our observa-

tion that the majority of GII-4 viruses bind to all A, B and H antigens (unpublished data) appears to explain the predominance of GII-4 viruses over other types that recognize narrower spectrum of human HBGA receptors. Two mutations (A₃₄₆G and I₃₈₉V) affected the binding specificity in a different way suggesting that a subtle conformational change in the binding interface could result in changes of the binding pattern. Unfortunately, we were unable to identify any genetic markers responsible for the phenotypic change of the GII-4 variants and the reason for the complete loss of binding to the A, B and/or H antigens of Hunter and Sakai viruses

(Lindesmith et al., 2008) remains unknown. One possibility is that residues other than those described in this study are also involved in HBGA-binding of noroviruses. Those residues could directly interact with the carbohydrate receptors by as yet unidentified structures or be involved in defining the local or global conformation that is required for the receptor binding function. One example is the arginine cluster at the C-terminus of the P domain that is far from the HBGA-binding interface but is required for receptor binding (Tan, Meller, and Jiang, 2006). These issues will require further structural and solution studies.

Materials and methods

Construction of mutant P particles and P dimers of VA387

The wild type P particle and the P dimer of VA387 were constructed and expressed in bacteria as described previously (Tan and Jiang, 2005b; Tan, Hegde, and Jiang, 2004; Tan, Meller, and Jiang, 2006). The mutant P particles and P dimers of VA387 with single amino acid substitutions were constructed by site-directed mutagenesis using the expression constructs of P particle (P-RGD4C in pGEX-4T-1) (Tan and Jiang, 2005b; Tan, Meller, and Jiang, 2006) or P dimer (HP in pGEX-4T-1) (Tan and Jiang, 2005b; Tan, Hegde, and Jiang, 2004), respectively, as templates. Site-directed mutagenesis was performed using the QuickChange Site-Directed Mutagenesis Kit (Stratagen, La Jolla, CA) and the corresponding primer pairs (Table 2) as described elsewhere (Tan and Jiang, 2005b; Tan, Hegde, and Jiang, 2004; Tan, Meller, and Jiang, 2006). The wild type and mutant P particles, as well as P dimers, were expressed and purified as described previously (Tan and Jiang, 2005b; Tan, Hegde, and Jiang, 2004; Tan, Meller, and Jiang, 2006). Briefly, after sequence confirmation through DNA sequencing, the mutant constructs were expressed in *E. coli* strain BL21 with IPTG (0.5 mM) induction at room temperature (~25 °C) overnight. The P protein-GST fusion proteins were purified using the Glutathione Sepharose 4 flow (GE Health Care Bio-Sciences, Piscataway, NJ) according to the manufacturer's protocol. P proteins were released from GST by thrombin (GE Health Care Bio-Sciences, Piscataway, NJ) digestion. The formation of P particle and P dimer was determined by gel filtration using a size-exclusion column Superdex 200 (GE Health Care Bio-Sciences, Piscataway, NJ) powered by an AKTA-FPLC system (model 920, GE Health Care Bio-Sciences, Piscataway, NJ), in which the P particles form a peak at ~830 kDa and the P dimer at ~69 kDa, respectively (Tan and Jiang, 2005b; Tan, Hegde, and Jiang, 2004; Tan, Meller, and Jiang, 2006).

HBGA binding assay

The saliva- and synthetic oligosaccharide-based binding assays were performed basically as described elsewhere (Huang et al., 2003; Huang et al., 2005). The affinity-column purified P particles and P dimers were first diluted to 1 mg/ml as starting solutions, then they were diluted further in a 3-fold-series to 33.3 µg/ml (30×), 11.1 µg/ml (90×), 3.70 µg/ml (270×), 1.23 µg/ml (810×), 0.41 µg/ml (2430×), 0.14 µg/ml (7290×), 0.05 µg/ml (21,870×), 0.02 µg/ml (65,610×) directly on the Elisa plates, on which saliva- or synthetic oligosaccharide have been coated. Alternatively, the proteins were diluted to 10 µg/ml (100×), 3.3 µg/ml (300×), 1.1 µg/ml (900×), 0.37 µg/ml (2700×), 0.12 µg/ml (8100×), 0.04 µg/ml (24,300×), 0.01 µg/ml (72,900×). The three saliva samples used in this study were well-defined in our laboratory (Huang et al., 2003; Huang et al., 2005), each containing high level of A-, B-, and H-antigen as determined by monoclonal antibodies against A, B, H1, H2, H3 antigens (Signet Laboratories Inc, Dedham/MA). The synthetic oligosaccharide-based binding assays were performed using a panel of oligosaccharides representing 9 different HBGA (A, B, H1, H2, H3, Le^a, Le^b, Le^x, and Le^y) as reported previously (Huang et al., 2003; Huang et al., 2005).

Crystal structure visualization and analysis

The crystal structures of the P dimer of VA387 complexed with type A- and B-trisaccharides were analyzed using the PyMOL software (DeLano Scientific LLC, Palo Alto, CA) and the Polyview-3D server (<http://polyview.cchmc.org>). The PDB files of the P protein in complex with A-trisaccharide (2OBS) and with B-trisaccharide (2OBT) were downloaded from the Protein Data Bank at the Rutgers University, New Brunswick, NJ (<http://www.rcsb.org>).

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References

- Bu, W., Mamedova, A., Tan, M., Xia, M., Jiang, X., Hegde, R.S., 2008. Structural basis for the receptor binding specificity of Norwalk virus. *J. Virol.* 82 (11), 5340–5347.
- Cao, S., Lou, Z., Tan, M., Chen, Y., Liu, Y., Zhang, Z., Zhang, X.C., Jiang, X., Li, X., Rao, Z., 2007. Structural basis for the recognition of blood group trisaccharides by norovirus. *J. Virol.* 81 (11), 5949–5957.
- Huang, P., Farkas, T., Marionneau, S., Zhong, W., Ruvoen-Clouet, N., Morrow, A.L., Altaye, M., Pickering, L.K., Newburg, D.S., LePendu, J., Jiang, X., 2003. Noroviruses bind to human ABO, Lewis, and secretor histo-blood group antigens: identification of 4 distinct strain-specific patterns. *J. Infect. Dis.* 188 (1), 19–31.
- Huang, P., Farkas, T., Zhong, W., Tan, M., Thornton, S., Morrow, A.L., Jiang, X., 2005. Norovirus and histo-blood group antigens: demonstration of a wide spectrum of strain specificities and classification of two major binding groups among multiple binding patterns. *J. Virol.* 79 (11), 6714–6722.
- Hutson, A.M., Airaud, F., LePendu, J., Estes, M.K., Atmar, R.L., 2005. Norwalk virus infection associates with secretor status genotyped from sera. *J. Med. Virol.* 77 (1), 116–120.
- Hutson, A.M., Atmar, R.L., Graham, D.Y., Estes, M.K., 2002. Norwalk virus infection and disease is associated with ABO histo-blood group type. *J. Infect. Dis.* 185 (9), 1335–1337.
- Lindesmith, L., Moe, C., Marionneau, S., Ruvoen, N., Jiang, X., Lindblad, L., Stewart, P., LePendu, J., Baric, R., 2003. Human susceptibility and resistance to Norwalk virus infection. *Nat. Med.* 9, 548–553.
- Lindesmith, L.C., Donaldson, E.F., Lobue, A.D., Cannon, J.L., Zheng, D.P., Vinje, J., Baric, R.S., 2008. Mechanisms of GII.4 norovirus persistence in human populations. *PLoS. Med.* 5 (2), e31.
- Prasad, B.V., Hardy, M.E., Dokland, T., Bella, J., Rossmann, M.G., Estes, M.K., 1999. X-ray crystallographic structure of the Norwalk virus capsid. *Science* 286 (5438), 287–290.
- Ramirez, S., Giammanco, G.M., De Grazia, S., Colomba, C., Martella, V., Arista, S., 2008. Genotyping of GII.4 and GIIB norovirus RT-PCR amplicons by RFLP analysis. *J. Virol. Methods* 147 (2), 250–256.
- Siebenga, J., Kroneman, A., Vennema, H., Duizer, E., Koopmans, M., 2008. Food-borne viruses in Europe network report: the norovirus GII.4 2006b (for US named Minerva-like, for Japan Kobe034-like, for UK V6) variant now dominant in early seasonal surveillance. *Euro. Surveill.* 13 (2).
- Tan, M., Hegde, R.S., Jiang, X., 2004. The P domain of norovirus capsid protein forms dimer and binds to histo-blood group antigen receptors. *J. Virol.* 78 (12), 6233–6242.
- Tan, M., Huang, P., Meller, J., Zhong, W., Farkas, T., Jiang, X., 2003. Mutations within the P2 domain of norovirus capsid affect binding to human histo-blood group antigens: evidence for a binding pocket. *J. Virol.* 77 (23), 12562–12571.
- Tan, M., Jiang, X., 2005a. Norovirus and its histo-blood group antigen receptors: an answer to a historical puzzle. *Trends Microbiol.* 13 (6), 285–293.
- Tan, M., Jiang, X., 2005b. The p domain of norovirus capsid protein forms a subviral particle that binds to histo-blood group antigen receptors. *J. Virol.* 79 (22), 14017–14030.
- Tan, M., Jiang, X., 2007. Norovirus-host interaction: implications for disease control and prevention. *Expert. Rev. Mol. Med.* 9 (19), 1–22.
- Tan, M., Jin, M., Xie, H., Duan, Z., Jiang, X., Fang, Z., 2008. 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), 1296–1301.

- Tan, M., Meller, J., Jiang, X., 2006. C-terminal arginine cluster is essential for receptor binding of norovirus capsid protein. *J. Virol.* 80 (15), 7322–7331.
- Tan, M., Zhong, W., Song, D., Thornton, S., Jiang, X., 2004. *E. coli*-expressed recombinant norovirus capsid proteins maintain authentic antigenicity and receptor binding capability. *J. Med. Virol.* 74 (4), 641–649.
- Thorven, M., Grahn, A., Hedlund, K.O., Johansson, H., Wahlfriid, C., Larson, G., Svensson, L., 2005. A homozygous nonsense mutation (428G→A) in the human secretor (FUT2) gene provides resistance to symptomatic norovirus (GII) infections. *J. Virol.* 79 (24), 15351–15355.
- Tu, E.T., Bull, R.A., Greening, G.E., Hewitt, J., Lyon, M.J., Marshall, J.A., McIver, C.J., Rawlinson, W.D., White, P.A., 2008. Epidemics of gastroenteritis during 2006 were associated with the spread of norovirus GI.4 variants 2006a and 2006b. *Clin. Infect. Dis.* 46 (3), 413–420.
- Verhoef, L., Depoortere, E., Boxman, I., Duizer, E., van Duynhoven, Y., Harris, J., Johnsen, C., Kroneman, A., Le Guyader, S., Lim, W., Maunula, L., Meldal, H., Ratcliff, R., Reuter, G., Schreier, E., Siebenga, J., Vainio, K., Varela, C., Vennema, H., Koopmans, M., 2008. Emergence of new norovirus variants on spring cruise ships and prediction of winter epidemics. *Emerg. Infect. Dis.* 14 (2), 238–243.