Develop the methods to investigate the mechanism of Norovirus evolution

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Summary:

In 2012, a new norovirus strain named GII.4 2012 caused 37**%** (112/303) of reported food-borne-associated and diarrhea-associated outbreaks in Taiwan. It was first identified in a bacteria co-infection diarrhea-associated outbreak in February. Then norovirus GII.4 2012 became to be the predominant cause agent of gastroenteritis outbreaks, during September to December. Besides, norovirus GII.4 2012 affected all ages, especially in age group older than 60 years old. The suspected transmission mode was through person-to-person contact than by the mode of contaminated food. Most of the GII.4 2012 reported outbreaks were happened in school. Analysis of the complete capsid and P2 region sequences observed that the amino acid substitutions in P2 subdomain among GII.4 2012 strains.

Purpose:

Norovirus (NoV) is the major etiological agent of viral acute gastroenteritis in humans worldwide. It's estimated about 800 deaths and 70,000 hospitalizations in United States every year (1). In the developing countries, NoV causes 20,000 people deaths each year. NoV gastroenteritis outbreaks occur most common in closed settings such as hospitals, long-term care facilities, prison and military. Outbreaks also occur in the settings where people stay together for a long time, including schools, cruise ships and restaurants. All ages can be affected by NoV, especially young children, elders and immunocompromised patients. Norovirus can either infect humans via fecal-oral transmission or by person-to-person contact (1, 2), or by touching the object surface which is contaminated from patient's vomitus droplet and then be infected through oral-fecal route (3).

NoVs belong to *Caliciviridae* family, *Norovirus* genus and they are small round (27-38 nm in diameter), nonenveloped and with a 7.5 kb positive-sense RNA genome. Currently, five genogroups (GI-GV) and at least 40 genotypes are recognized by capsid gene nucleic acid similarity. Strains of GI and GII affect humans mainly, and the most commonly identified in NoV gastroenteritis outbreaks is genogroup II genotype 4 (GII.4) (4, 5). In the past two decades, GII.4 strain lead to severe gastroenteritis outbreaks worldwide and can be segregated into multiple major clusters using phylogenetic analysis. At least five major GII.4 strains caused NoV

epidemic outbreaks globally, for example, the Houston strain caused over half of outbreaks in the United States and the Netherlands from 1995 to 2002; the Farmington Hill strain lead to major outbreaks from 2002 to 2004; the Hunter strain was reported worldwide in 2004 and 2006; and the Laurens (2006a)/Minerva (2006b) strains replaced the Hunter strain in 2006 (6-10). Recently, GII.4 new variants, including GII.4 2008, GII.4 2009 and GII.4 2010 strains were recognized in many countries (9, 11). Because the continuously evolutionary changes in epitopes of NoV GII.4 which leads to an escape of existing herd immunity; and human protective antibodies are not last long and effective, therefore, NoV GII.4 can persistently circulating in human population (12, 13). In this study, we found a novel GII.4 variant in Taiwan which results in most of acute gastroenteritis outbreaks and replaces former GII.4 strain in a short period.

Method:

Reporting system in Taiwan and epidemiological data

Suspected cluster of NoV infection was monitored via food-borne associated outbreak reporting system and diarrhea syndrome outbreak reporting system. The reporting items include patients and workers, age, gender, setting, and date of onset. Local public health agency involves in most of outbreak control and investigating of possible transmission route. Outbreak is defined as at least two patients suffer diarrhea or vomiting in the same setting and time (14). And the definition of NoV outbreak is at least one specimen is NoV positive by laboratory in an outbreak.

Specimen collection and RNA extraction

A total of 1,418 stool specimens were collected from outbreak reporting patients in 2012. Fecal samples were diluted to 10% suspension with phosphate-buffered saline then clarified by centrifuge at 3000 rpm for 15 min at 4℃. Viral RNA was extracted from stool suspension using MagNA Pure Compact system (Roche Diagnostics, Indianapolis, IN) according to the manufacture's instruction.

Reverse transcription and polymerase chain reaction (RT-PCR)

To amply the partial capsid gene, the cDNA synthesis was carried out first with One-step RT-PCR kit (Invitrogen, Carlsbad, CA, USA). With 6.8 μl of the RNA in 20 μl of the reaction mixture containing 0.15 μg/μl random primer , 1× Superscript III RT buffer, 10 mM DTT, 0.4 mM of each dNTP, 1.5 U RNase inhibitor, and 10 U Superscript RT III. RT was performed at 37°C for 15 min, followed by 1 hr at 50°C. For NoV genogroup I (GI) PCR, G1SKF and G1SKR primer pairs were used and for NoV GII PCR, G2SKF and G2SKR primer pairs were used (15). Viral capsid gene

RT-PCR was performed by primer sets of G2SKF-Clone (5'-CACCCNTGGGAGGGCGATCGCAA-3') and TX30SNX (5'-GACTAGTTCTAGATCGCGAGCGGCCGCCC$(T)_{30}$-3'). Specifically, 8μl RNA extraction incubate with 1μL TX30SXN (10μM) primer at 66℃ for 5min, then on ice for 2 min. RT reaction mix is added to each vial and react at 50℃ for 90 min, then inactivate at 85℃ for 10 min. PCR reaction was conducted using KOD-Plus Neo system (Toyobo, Tokyo, Japan). PCR mix (50μl) contains 1X Blend-Taq Plus PCR buffer, 200nM dNTP, 200nM G2SKF-Clone and TX30SXN primers, and 1.25U Blend-Taq enzyme. PCR reaction starts after denaturation at 94℃ for 3 min, and with program of 40 cycles of 94℃ for 30 sec, 60℃ for 30 sec, 72℃ for 3 min and final extension at 72 ℃ for 10 min.

Sequencing and phylogenetic analysis

RT-PCR products were purified by the T-pro gel extraction kit (T-pro Biotechnology, Taipei, Taiwan). Nucleotide sequence reactions were prepared with the terminator cycle sequence kit (version 3.1) and determined with the ABI 3100 Avant sequencer (PE Biosystems, USA). Nucleotide sequences were aligned with ClustalW and phylogenetic dendrograms were generated using neighbor-joining method by MEGA 4.0 (16) software with 1,000 replications.

Nucleotide sequence accession numbers

The nucleotide sequence data determined in this study have been deposited in GenBank under accession numbers: 04R-2(HQ456320), 06-AM-11(KC792278), 07-B-1(HQ456329), 08-F-2(HQ456335), 08-W-1(HQ456341), 09-L-4(HQ456343), 09-BI-2-1(HQ456346), 12-AY-1(KC792279), 12-BA-1(KC792280), 12-BQ-1(KC792281), 12-CD-2-4(KC792282) and 12-CG-2-4(KC792283), and also included reference GII.4 strains as: Sydney 2012 strains (JX459908 and JX629458), 2010 strain(JN595867), 2009b strain(GU445325), 2008b strains (GQ246794, AB541274, AB491291, AB445395, HQ009513 and GU270580), 2006a strains (EF126965, GQ849126, AB447458 and EF126964), 2006b strains (EF126965 and EF684915), Hunter strains (DQ078814 and AY883096), AB220921, DQ369797, AY485642, AY502023, DQ364459, EU310927, AB434770, AF080558, AJ004864, AF145896, AY030098, AB294779, AB303929, X76716, X86557 and VA387 strain (AY038600)

Result:

Characteristic of pathogen distribution in gastroenteritis outbreaks

During 2012, a total of 1,418 stool specimens were collected from 303

gastroenteritis outbreaks in Taiwan, 511 cases were identified as NoV-positive from 176 NoV associated outbreaks. We confirmed the NoV-associated outbreaks using RT-PCR diagnosis. The determination of NoV genotype was based on N/S domain sequence (15) using neighbor-joining phylogenetic analysis. In 2012, the gastroenteritis outbreaks due to NoV GII were 58.1%, 0.3% were NoV GI, the non-NoV virus was accounted for 14.9% (2.9% rotavirus, 2.9% sapovirus, 5.6% astrovirus, 2.6% adenovirus, 0.6% aichi virus and 0.3% echovirus), and the others are bacteria (9.9%) and unknown (16.8%). The major pathogen of gastroenteritis outbreaks was NoV GII, with GII.4 strains the most prevalent. A new variant strain GII.4 2012 was identified which lead to 37% (112/303) of gastroenteritis outbreaks (Table). The first GII.4 2012 outbreak was detected in February 2012 with many other GII.4 subgenotypes in the same month; since then the number of gastroenteritis caused by GII.4 2012 strain has increased dramatically over time. Now the GII.4 2012 NoV accounts for the majority of gastroenteritis outbreaks (Figure 1).

Epidemiology and transmission mode of NoVs GII.4 2012

Epidemiological data revealed in all reporting cases that GII.4 2012 was the majority infection etiology in age group 71-80 years old at 49% and 55% in age group 81-90 years old. Furthermore, GII.4 2012 strain accounted for 91% of NoV cases in 61-70 age group, with 78% in 71-80 age group, 78% in 81-90 age group and 65% in 91-100 age group (Figure 2a). GII.4 2012 outbreaks most occurred in schools (38.2%), 23.6% in long-term care facilities, 14.5% in hospitals, 15.5% in restaurants, 0.9% in prison and 7.2% in other places (Figure 2b). Since transmission of NoV can be either through food-borne or person-to-person contact (1, 2). In our study we defined the food-borne mode as patients develop symptoms after consuming the same food; and person-to-person contact mode as patients stay in closed settings for a long time and no direct evidence of food-borne is associated; whereas unknown is defined as the occurrence of an outbreak which is not in a closed setting or uncertain if food is correlated. We observed the mode of person-to-person contact accounted for 71.8% (79/110) with GII.4 2012, and 55.4% (36/65) for non-GII.4 2012 NoVs. In food-borne mode, GII.4 2012 accounted for 26.4% (29/110), and 21.5% (14/65) for non-GII.4 2012 NoVs. Evidently, the transmission route was higher in person-to-person contact mode than food-borne mode, no matter in GII.4 2012 or non-GII.4 2012 outbreaks (Figures 2c, 2d). Interestingly, non-GII.4 2012 strains dramatically decreased from March and were almost replaced by GII.4 2012 strain thereafter (Figures 2c, 2d).

Phylogenetic analysis of NoV GII.4

Routinely, we used G2SKF/R primer pairs in RT-PCR for NoV detection. The

products of G2SKF/R RT-PCR contain partial ORF1/2 were sequenced and analyzed for genotyping. In order to differentiate the changing pattern and the possible evolutionary, different clusters of GII.4 in previous years were selected according to the result of genotyping for further full-length capsid region analysis by G2SKF/TX30SXN primers set. G2SKF/TX30SXN primers yield 2.5kb product which cover the capsid region, VP1 and VP2, of norovirus. Phylogenetic tree were generated in major capsid domain (VP1) nucleotide sequences region by the neighbor joining method. The reference GII.4 strain included GII.4 2012 Sydney which was isolated in March from Australia. NoV strains isolated from Taiwan CDC in the phylogenetic tree from year in 2004, 2006, 2008, 2009, 2010 and 2012, which named as 04R-2 (Hunter), 06-AM-11(2006a), 07-B-1(2006b), 08-F-2(2008a), 08-W-1(2008b), 09-L-4 (2009a), 09-BI-2-1(2009b), 12-AY-1(2010), 12-BA-1(2012), 12-BQ-1(2012), 12-CD-2-4(2012) and 12-CG-2-4(2012) were included to analyze the evolutionary of GII.4 in Taiwan. Phylogenetic tree showed that GII.4 2012 is a new cluster and most likely evolution from the ancestor of strains GII.4 2006b, 2009b and 2010. (Figure 3).

Norovirus GII.4 2012 variation

GII.4 P2 subdomain is considered the most variation domain and the entry binding site with human cellular HBGA (Histo-blood group antigen) (17). Therefore, analysis of the complete capsid and P2 region sequences observed that the amino acid substitutions in P2 subdomain among GII.4 strains from 2004 to 2012 in Taiwan. Data showed that amino acid sequences of P2 subdomain was observed frequently changed in Taiwan's local GII.4 strains and focus on the P2 subdomain three important immunoloops (Table 2). According to Tan et al. previous studies, first predicted that NoV P2 subdomain loop1 is located on 294-297, loop2 is located on 371-374 and loop3 is located on 390-393 (18). Also, previous studies showed the human cellular HBGA binding sites, including site 1 (position 343-345, 374) and site 2 (position 393-395) are also located on P2 subdomain (19). From sequences of GII.4 2012 strains isolated in Taiwan, indicated there were at least 6 positions different from other strains within P2 subdomain and HBGA binding site at position 294, 340, 341, 372, 373 and 393. Interestingly, we found highly variation at the position 294, 340, 393 and 413.

Discussion

In 2012, NoV GII.4 2012 caused a widespread of gastroenteritis outbreaks in Europe, Australia, United State, Hong Kong and Canada (20-25). The first GII.4 2012 strain was isolated almost the equal time period from Taiwan and Australia in March. Outbreaks of GII.4 2012 increased sharply in late 2012 and the similar trend was also

observed in Taiwan. Based on epidemiological data in Taiwan, GII.4 2012 strain affected all ages but elders were more vulnerable. School was the most common setting where GII.4 2012 outbreaks occurred. Phylogenetic analysis revealed that GII.4 2012 strain is closer to GII.4 2009b, GII.4 2008b and GII.4 2010 clusters. Nucleotides similarity calculation showed that GII.4 2012 is 93.3% and 92.5% identical to strains GII.4 2008b and GII.4 2009b in P2 subdomain and with 95.3% and 94.3% identical to strains GII.4 2008b and GII.4 2009b in ORF1(data not shown), suggested that strain GII.4 2008b was possible the ancestor of GII.4 2012. In early 2012, outbreaks only caused by few GII.4 2009b and none by GII.4 2008b were observed; we speculate that GII.4 2012 may be evolved from GII.4 2008b or GII.4 2009b.

Because of norovirus belongs to RNA virus, RNA dependent RNA polymerase doesn't have the proofreading function; hence, the mutation rate of RNA virus is higher than DNA virus. Additionally, norovirus recombination can occur naturally, so new variant evolve rapidly (26). Furthermore, norovirus have low infectious dose (< 18 viral particles) and high resistance to chemicals, pressure and temperature. Hall et al. indicated noroviruses might be the perfect human pathogens (27).

Tan et al. first predicted the HBGA binding pocket on norovirus VA387 model (17). HBGAs could bind to receptor binding (RBD) sites of norovirus. RBDs were defined to site 1 (position 343-345, 374) and site 2 (position 393-395). Once RBD positions altered, the HBGA binding activity would be change and elevate the immunity escape probability (12). Our study indicated one amino acid of site 2 was substituted compared with GII.4 2009b strain. Tan et al. found that norovirus P domain can be expressed by E. coli system and form a 24 copies P particle naturally. This P particle is not only highly antigenic but also a good vaccine platform. Three potential immunoloops on the surface determined the epitopes of norovirus P particle. Loop 1, 2 and 3 are position 294-297, 371-374 and 390-393, respectively (18). We also found amino acids in loop 1, loop 2 and loop3 were replaced. Allen et al. demonstrated the putative epitopes site A (position 296-298) and site B (position 393-395) on P2 subdomain are important in antibody recognition (28). They predicted site A is the major epitope while site B is minor epitope but contribute antigentic diversity. The frequencies of "STT" motif on site B increased from 2004-2011(29) and Taiwan's strains have the same motif except for GII.4 2008b and GII.4 2012. In Taiwan GII.4 2008b and GII.4 2012 strains, amino acid substitution at site 393 were from S to D and G, which may contributed variation of antigenicity and help virus escape from existing host immunity. Lindesmith et al. elucidated the early serum from norovirus-infected patients couldn't recognize recently norovirus VLPs. Contrarily, recent serum couldn't recognize early norovirus VLPs. They also predicted 5 major

epitopes, A to E, in P2 region and demonstrated the A and D are the most important epitopes (13). Our study showed GII.4 2012 has multiple variations and some changed positions are coincidence with important epitope sites published in previous studies.

Norovirus infect human persistently and the epochal evolution of epitopes plays a crucial role in herd immunity escape (12). Our study indicates the norovirus GII.4 strain emerging and pandemic outbreaks occur every 2-3 year in Taiwan. GII.4 2012 strain almost replaced other norovirus genotype to be a predominant strain since mid-2012. Amino acid substitutions were observed in potential epitopes and it could be a possible reason which caused pandemic outbreaks.

Table 1. Etiology of gastroenteritis outbreaks in 2012

Pathogen	Outbreaks	%
Norovirus (genotype, subgenotype)		
GII.4 2006	1	0.3%
GII.4 2009a	4	1.3%
GII.4 2009b	1	0.3%
GII.4 2010	2	0.7%
GII.4 2012	93	30.7%
GII.4 2012 co-infection[a]	19	6.3%
GII (non-GII.4)[b]	30	9.9%
GI[c]	1	0.3%
Mixed-infection[d]	26	8.6%
Non-NoV viruses[e]	45	14.9%
Bacteria[f]	30	9.9%
Unknown[g]	51	16.8%
Total	303	100%

a. Norovirus GII.4 2012 co-infection either with GI/GII norovirus, other viruses or bacteria

b. Norovirus non-GII.4 outbreaks includes 1 GII.2, 10 GII.5, 3 GII.6 and 16 GII unknown genotype

c. Norovirus GI unknown genotype

d. Mixed-infection includes 18 with GI/non-GII.4 2012 norovirus, 3 with other virus and 3 with bacteria

e. Non-norovirus includes 7 rotavirus, 8 sapovirus, 17 astrovirus, 8 adenovirus, 2 aichi virus and 1 echovirus

f. Bacteria causing diarrhea includes 6 *Vibrio*, 16 *Staphylococcus aureus*, 1 *Shigella* and 5 *Salmonella* outbreaks

g. No pathogen isolated.

Figure 1. Epidemic curve of norovirus outbreaks by different GII.4 strains in Taiwan, 2012, by month

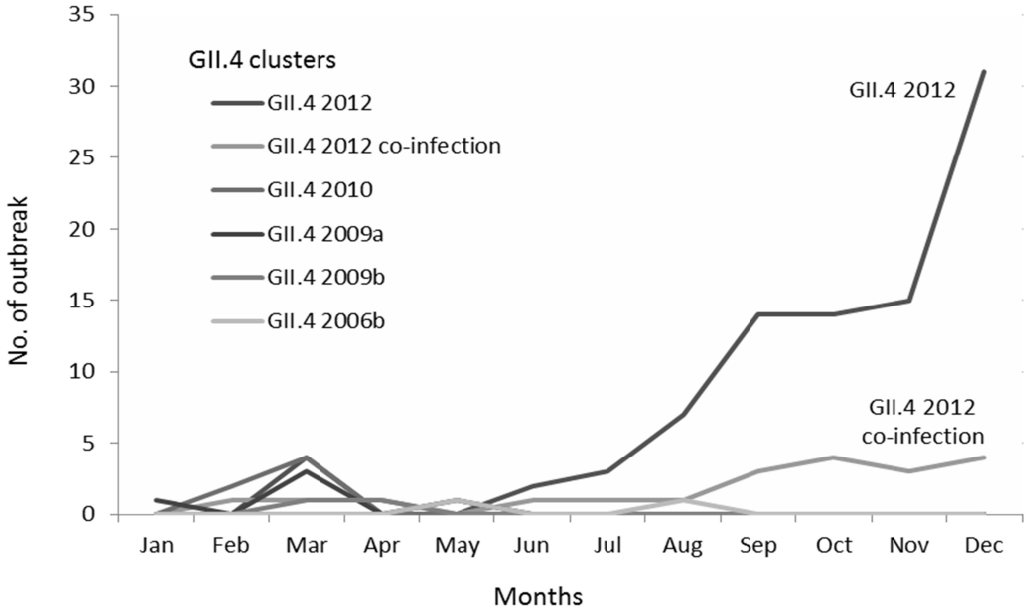


Figure 2. Epidemiological features of outbreaks caused by GII.4 2012 strain

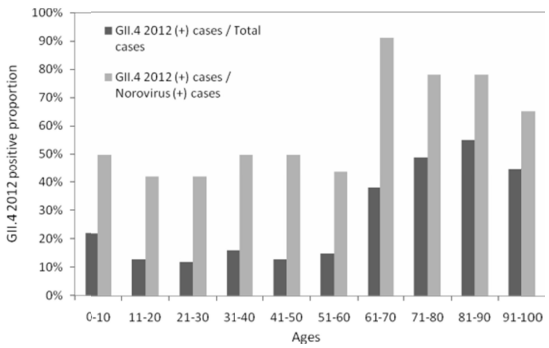
a. Proportion of norovirus GII.4 2012 positive cases by age group, compare to all reported cases and identified NoV positive cases.

b. Proportion of norovirus GII.4 2012 outbreaks by settings.

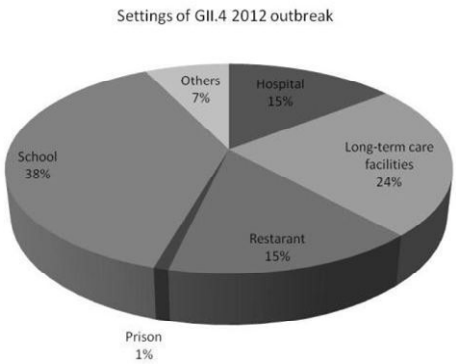
c. Possible transmission mode of outbreaks by GII.4 2012 strain, by month.

d. Possible transmission mode of outbreaks by non-GII.4 2012, by month.

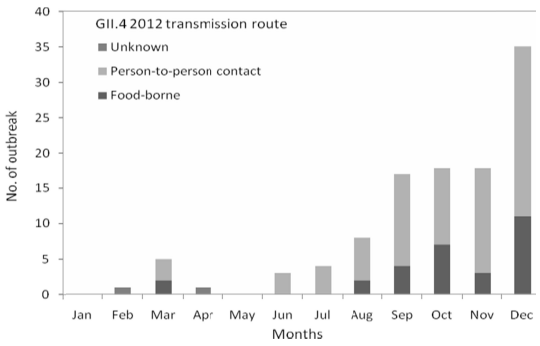
a.



b.



c.



d.

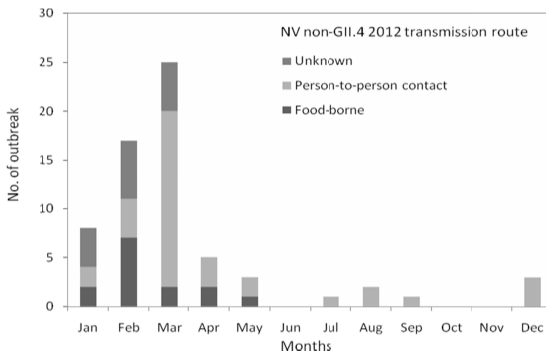


Figure 3. Phylogenetic analysis of full-length norovirus GII.4 VP1 gene.

Full length VP1 (1623bp) of all GII.4 strains aligned and the tree was generated via neighbor-joining method using MEGA 4.0 software. Bootstrap values of 1000 replication were shown on the branches.

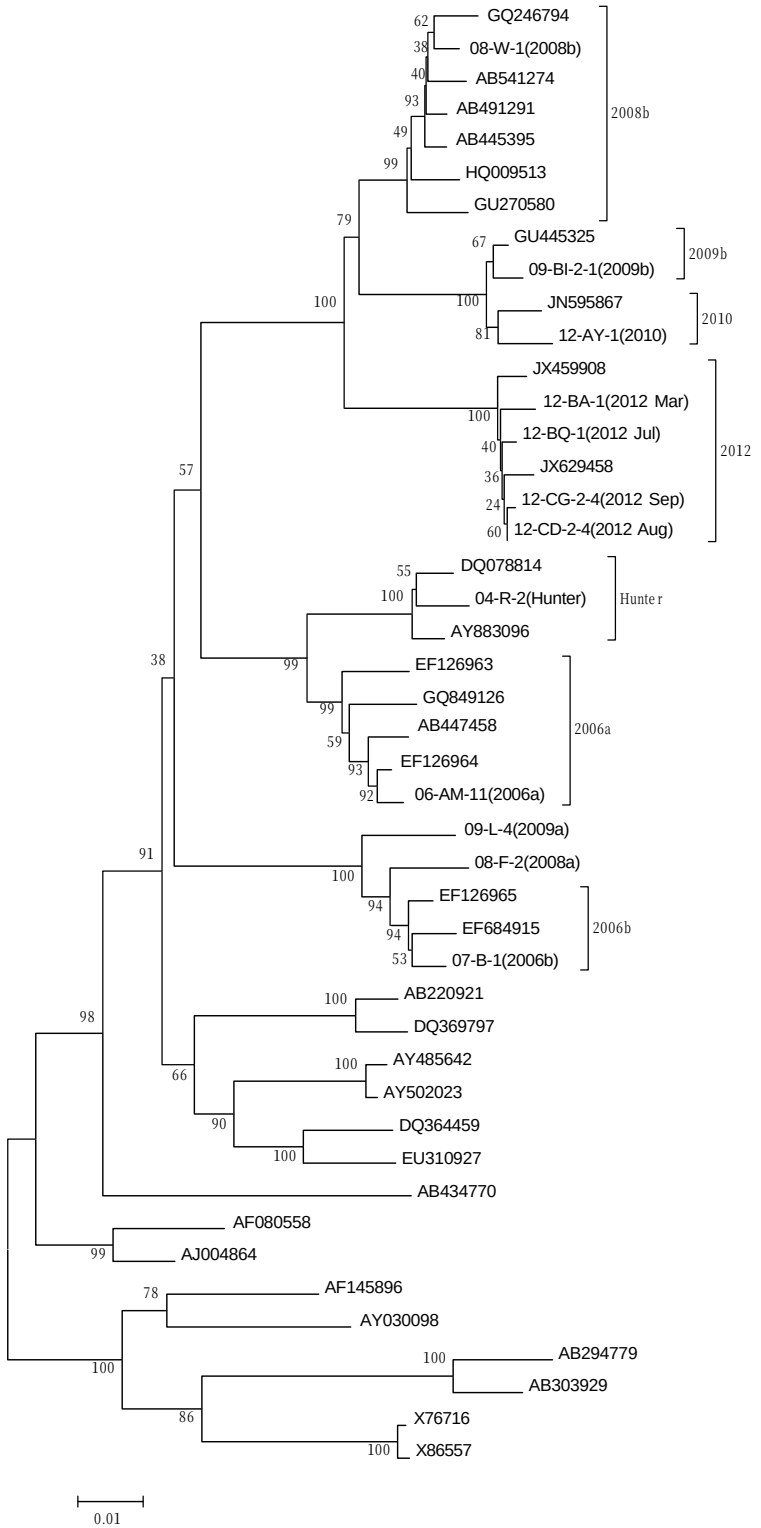


Table 2. Comparison of the Taiwan's GII.4 strains amino acids changes in predicted potential immunoloops within P2 subdomain and HBGA binding pocket. Three immunoloops contain loop1 (position 294-297), loop2 (position 371-374) and loop3 (position 390-393). HBGA binding sites include site 1 (position 343-345, 374) and site 2 (position 393-395). Gray color means the same variation between GII.4 2012 strain and GII.4 2008b strain. Diagonal stands for the variation different to past strains.

	294	295	296	297	305	310	340	341	343	344	345	357	359	364
07-B-1(2006b)	A	S	S	R	S	N	G	D	S	T	R	P	T	S
08-W-1(2008b)	T				T	S	A					D	A	R
09-BI-2-1(2009b)	P					S	T	N				D	S	R
12-AY-1(2010)	P					S	T	N				D	S	R
12-BA-1(2012-Mar)	T						T					D	A	R
12-BQ-1(2012-Jul)	T						T					D	A	R
12-CD-2-4(2012-Aug)	T						T					D	A	R
12-CG-2-4(2012-Sep)	T						T					D	A	R

	368	371	372	373	374	376	377	378	393	394	395	396	413	414
07-B-1(2006b)	S	T	E	N	D	E	T	H	S	T	T	H	V	H
08-W-1(2008b)	A		D			D	A	N	D		A			
09-BI-2-1(2009b)	A		D					N				P	I	
12-AY-1(2010)	A		D					N				P	I	
12-BA-1(2012-Mar)	E		D	H			A	N	G				T	
12-BQ-1(2012-Jul)	E		D	H			A	N	G				T	
12-CD-2-4(2012-Aug)	E		D	H			A	N	G				T	
12-CG-2-4(2012-Sep)	E		D	H			A	N	G				T	

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