Molecular research and epidemiology of gastroenteric viruses

Fang-Tzy Wu, Che-Yuan Chang, Ching-Yi Wu, Ho-Sheng Wu
Centers for Disease Control, Taiwan

Summary:

Norovirus (NoV) is the majority cause of acute gastroenteritis in winter season in Taiwan. In 2012-2013, a new norovirus strain named GII.4 Sydney caused 45.8% (253/552) of reported food-borne-associated and diarrhea-associated outbreaks in Taiwan. Norovirus GII.4 Sydney replaced former GII.4 New Orland became to be the predominant cause agent of gastroenteritis outbreaks since 2012. Besides, norovirus GII.4 Sydney affected all ages, but most of the GII.4 2012 reported outbreaks were from schools. This is different from other GII.4 strains which were reported caused most of the outbreaks in long-term care facility or nursing care center. In this study, we analyze the transmission association of this novel NoV strain among different age groups.

Purpose:

Norovirus (NoV) is the major etiological agent of viral acute gastroenteritis in humans and second greatest burden of all infectious diseases worldwide. A systematic review and meta-analysis from Embase, Medline and Global Health database of 175 articles in 2008-2014, showed that the pooled prevalence of norovirus with acute gastroenteritis was 18%. The prevalence trend is higher in AGE cases in community and outpatients setting than in inpatients settings (1). It's estimated about 800 deaths and 70,000 hospitalizations in United States every year (2). 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 (2,3), or by touching the object surface which is contaminated from patient's vomitus droplet and then be infected through oral-fecal route (4).

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, six genogroups (GI-GVI) 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) (5,6). 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 (7-11). Recently, GII.4 new variants, including GII.4 2008, GII.4 2009 and GII.4 2010 strains were recognized in many countries (10,12). 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 persistently circulating in human population (13,14). In this study, we found novel GII.4 Sydney which results in most of acute gastroenteritis outbreaks and replaced former GII.4 strains in a short period then became a predominant cause agent during 2012-2014. Aslo, most of the outbreaks were reported from schools, but not from long-term care facilities. Through the molecular surveillance, we identified that there were several NoV strains caused outbreaks in Taiwan in the past years. We hypothesis that most of the adults have had NoVs infected before, and this novel NoV infection initiated from young children then transmitted to other elderly aged groups, which may be with low exiting antibody to this novel viral strain.

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 (15). The definition of NoV outbreak is at least one specimen which was molecular identified as NoV positive in an outbreak.

Specimen collection and RNA extraction

A total of 2,471 stool specimens were collected from outbreak reporting patients in 2012-2013. Fecal samples were diluted to 10% suspension with phosphate-buffered saline then clarified by centrifuge at 3,000 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 (16). 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 (17) software with 1,000 replications.

Nucleotide sequence accession numbers

GII.4 reference variant strains included GII.4 Den Haag 2006, GII.4 New Orleans 2009, and GII.4 Sydney 2012as well as GII.4 strains detected in Taiwan from 2004-2012: 04-R-2 (Hunter 2004), 06-AM-11(2006a), 07-B-1(2006b), 08-F-2(2008a), 08-W-1 (Apeldoorn 2007), 09-L-4 (2009a), 09-BI-2-1(New Orleans 2009), 12-AY-1(New Orleans 2009), 12-BA-1(Sydney 2012), 12-BQ-1(Sydney 2012), 12-CD-2-4(Sydney 2012), 12-CG-2-4(Sydney 2012), 13-AS-1(Sydney

2012),13-AV-1(Sydney 2012), 13-BE-4 (Sydney 2012) and 13-Z-2 (Sydney 2012). Nucleotide sequence data determined in this study were deposited in GenBank under the following 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(KJ533132), 12-BQ-1(KC792281), 12-CD-2-4(KC792282),12-CG-2-4(KJ533133), 13-AS-1(KJ433968), 13-AV-1 (KJ33970), 13-BE-4(KJ51059) and 13-Z-2 (KJ533134) . These data also included the following reference GII.4 variants: Sydney 2012 strains (JX459908 and JX629458), New Orleans 2009 strains (JN595867 and GU445325), Apeldoorn 2007 strains (GQ246794, AB541274, AB491291, AB445395, HQ009513 and GU270580), Yerseke 2006a strains (EF126963, GQ849126, AB447458 and EF126964), DenHaag 2006b strains (EF126965 and EF684915), Hunter 2004 strains (DQ078814 and AY883096), Camberwell strain (AF145896, AY030098) and Lordsdale (X76716, X86557).

Result:

Norovirus identified in food-borne associated and diarrhea syndrome outbreaks

In 2012-2013, a total of 2,471 stool specimens were collected from 552 outbreaks, from food-borne associated outbreak reporting system and diarrhea syndrome outbreak reporting system in Taiwan. A total of 731 cases were identified as NoV-positive from 253 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 (16) using neighbor-joining phylogenetic analysis. A new variant strain GII.4 Sydney was identified in February 2012, which was the major NoV strain and led to 65.2% (165/552) of gastroenteritis outbreaks in the 2012-2013 NoV-season (Fig 1). In the beginning of 2012, most of the NoV outbreaks were identified by several non-GII.4 strains. The new variant GII.4 Sydney replaced almost non-GII.4 strains since mid-2012. Interestingly, non-GII.4 Sydney strains dramatically decreased from March and were almost replaced by GII.4 Sydney strain thereafter (Fig1).

Epidemiology of NoVs and GII.4 Sydney outbreaks

Data analysis of age-groups of all reporting cases, almost half of the reporting cases (45.1%, 1,114/2,471) was under 24 years old. In this age-group, 29.8% (332/1,114) was lab-identified as NoV positive including 14.2% (158/1,114) identifies as GII.4 Sydney. Besides, there were 12.8% (316/2,471) cases reporting in age-group of more than 60 years old, but with 55.3% (175/316) cases molecular identified as NoV positive and 46.2% (146/316) were confirmed as GII.4 Sydney infection (Table

1).

Data analysis of settings of all 552 reporting outbreaks, 253 NoV associated outbreaks including 165 sequenced-confirmed GII.4 Sydney outbreaks. The most frequently reported settings of GII.4 Sydney outbreaks were schools (59/165, 35.8%) and long-term care facilities (40/165, 24.2%), followed by restaurants (28/165, 17%), hospitals (21/165, 12.7%), and prisons or military bases (3/165, 1.8%). Furthermore, the number of reporting outbreaks in school was much higher in 2012 than in 2013. Also, the number of reporting outbreaks in long-term care facilities and restaurants were higher in 2013 than in 2012 (Table 2).

It seems that NoV infection risk in school was higher than in long-term care facilities in 2012. In order to know the epidemic pattern, we re-analyze NoV GII.4 Sydney infection in different age groups by week during 2012-2013 (Fig 2). Only a few numbers of cases were found in age group 37-48 and 49-60 at week 8 and 10, then 7-12, and 13-24 at week 12. Increased cases were found mostly in age group 0-6 in the following weeks since week 13 and peak was in week 37(Fig 2). After week 37, cases of GII.4 Sydney infection increased sharply and infected cases separated in all age groups (Fig1, 2). Moreover, data showed the weekly epidemic trends extended longer and more cases were identified in 2013 in age group of > 60 than in other age groups.

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. (Fig 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) (18). 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 (Fig 4). 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 (19). 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 (20). 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 Sydney caused a widespread of gastroenteritis outbreaks in Europe, Australia, United State, Hong Kong and Canada (21-26). The first GII.4 Sydney strain was isolated almost the equal time period from Taiwan and Australia in March. Outbreaks of GII.4 Sydney increased sharply in late 2012 and the similar trend was also observed in Taiwan. Based on epidemiological data in Taiwan, GII.4 Sydney strain affected all ages but elders were more vulnerable. School was the most common setting where GII.4 Sydney outbreaks occurred. Phylogenetic analysis revealed that GII.4 Sydney strain is closer to GII.4 2009b, GII.4 2008b and GII.4 2010 clusters. Nucleotides similarity calculation showed that GII.4 Sydney 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 or GII.4 2009b was possible the ancestor of GII.4 Sydney. In early 2012, outbreaks only caused by few GII.4 2009b and none by GII.4 2008b were observed; we speculate that GII.4 Sydney 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 (27). 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 (28).

Tan et al. first predicted the HBGA binding pocket on norovirus VA387 model (18). 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 (13). 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 (19). 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 (29). 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(30) and Taiwan's strains have the same motif except for GII.4 2008b and GII.4 Sydney. In Taiwan GII.4 2008b and GII.4 Sydney 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 (14). Our study showed GII.4 Sydney 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 (13). Our study indicates the norovirus GII.4 strain emerging and pandemic outbreaks occur in 2012-2013 in Taiwan. GII.4 Sydney strain almost replaced other norovirus genotypes 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. Furthermore, epidemiological information indicated that NoV infection risk in school was higher than in long-term care facilities in 2012. Moreover, data showed the weekly epidemic trends extended longer and more cases were identified in 2013 in age group of > 60 than in other age groups. In this study, we speculate that novel GII.4 Sydney first incubation in sporadic cases then initiated propagation in young children group which may not have immunity against such virus or epitopes. Then, viral pooled were selected and spread

to all age group populations. Moreover, data showed the weekly epidemic trends extended longer and more cases were identified in 2013 in age group of > 60 than in other age groups. It may indicate that elder people, especially in nursing center, who may with less antibodies against NoVs and amplify the epidemic cases formed foundation of epidemic cases. This possible mechanism should be further identified and confirmed by either retrospective study or viral strains blockade assay.

Table 1. Number of case-patient reporting and NoV identified by age groups in 2012-2013.

Age group (year)	No. (%) case-patients		
	Case No. (n=2471)	NoV (+) (n=731)	2012 Sydney (n=423)
0~6	187 (7.6%)	70 (9.6%)	31 (7.3%)
7~12	408 (16.5%)	132 (18.1%)	62 (14.7%)
13~24	519 (21.0%)	129 (17.6%)	65 (15.4%)
25~36	327 (13.2%)	73 (10.0%)	38 (9.0%)
37~48	364 (14.7%)	72 (9.8%)	41 (9.7%)
49~60	350 (14.2%)	80 (10.9%)	40 (9.5%)
61~	316 (12.8%)	175 (23.9%)	146 (34.5%)

Table 2. Number of GII.4 Sydney outbreaks by settings in 2012 and 2013.

	GII.4 Sydney (n=165)		
Year	2012 (n=110)	2013 (n=55)	Total (%)
Setting			
School	42 (38.1)	17 (30.9)	59 (35.8)
Long-term care facility	26 (23.6)	14 (25.5)	40 (24.2)
Restaurant	17 (15.5)	11 (20.0)	28 (17.0)
Hospital	16 (14.5)	5 (9.1)	21 (12.7)
Prison/military	1 (0.9)	2 (3.6)	3 (1.8)
Other/unknown	8 (7.3)	6 (10.9)	14 (8.4)

Fig 1. Weekly distribution of reporting case-patient, norovirus positive and GII.4 Sydney from reporting food-borne associated and diarrhea syndrome outbreaks in Taiwan, 2012-2013.

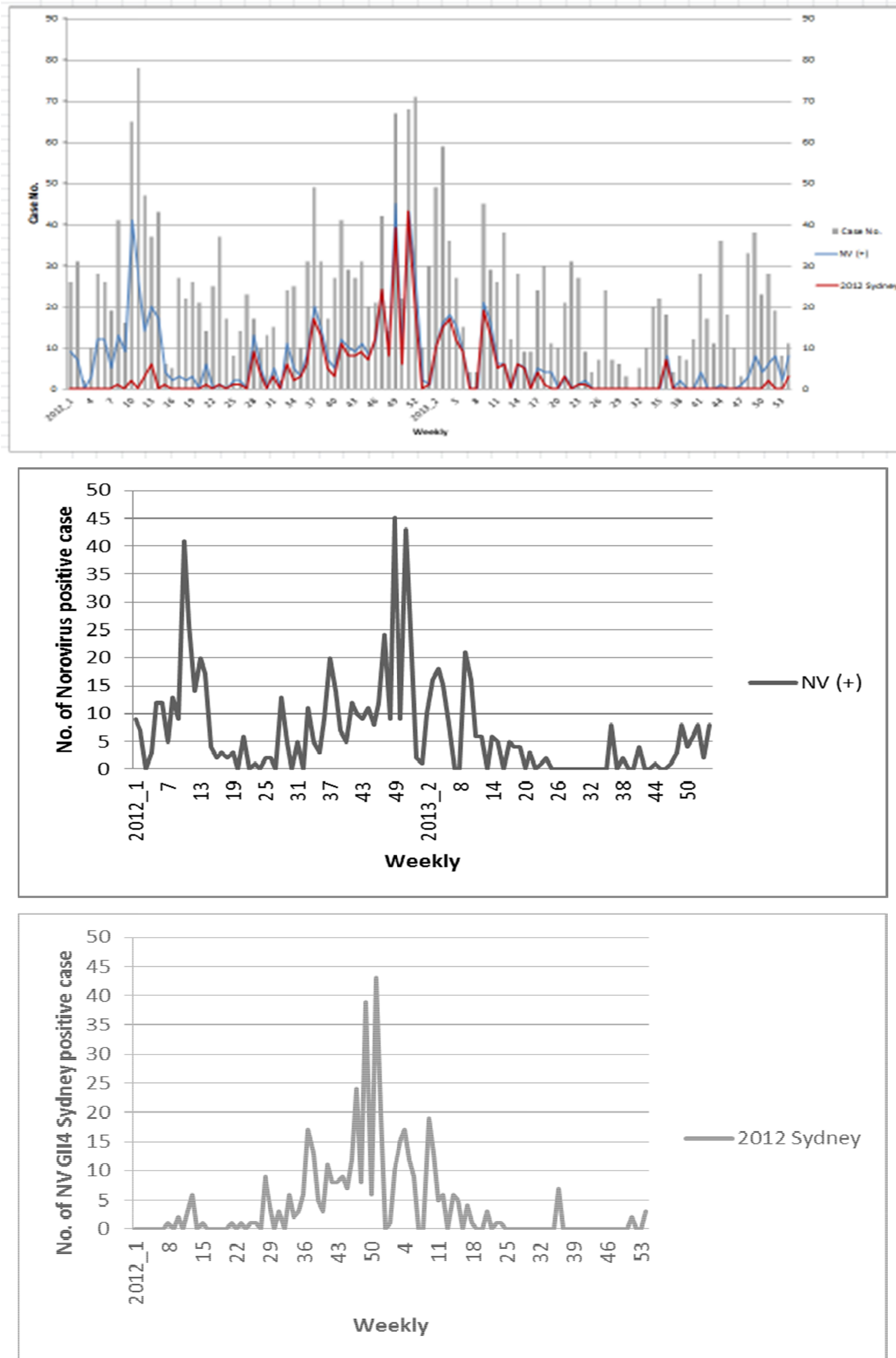

Fig 2. Weekly distribution of NoV GII.4 Sydney infection cases, by age group, 2012-2013.

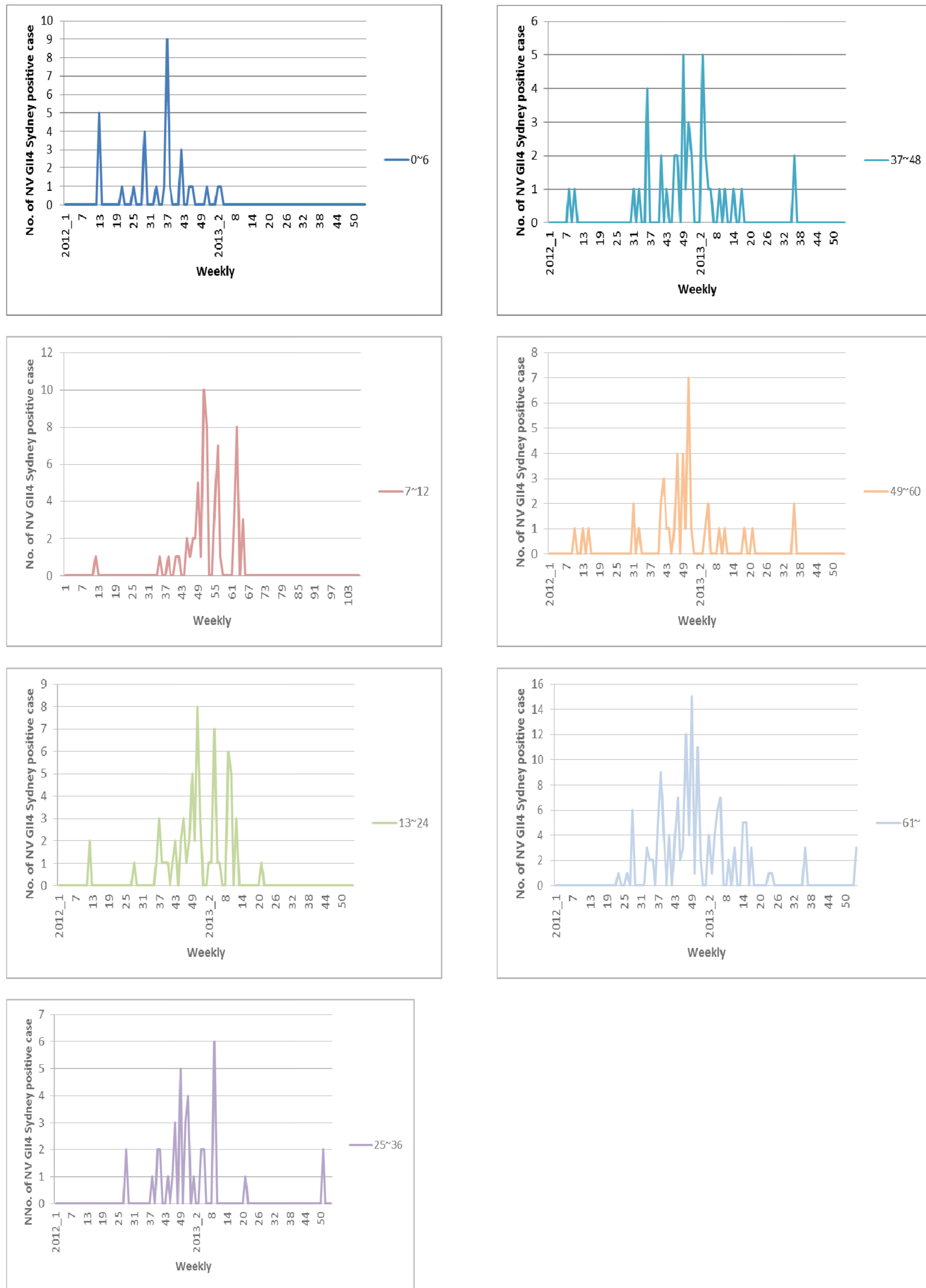

Fig 3. Phylogenetic analysis of norovirus GII.4 ORF2 gene with Taiwan strains and compared with references from GenBank. Full length ORF2, VP1 gene (1623bp) of all GII.4 strains were aligned and the tree was generated via Kimura-2 parameter mode of neighbor-joining method in using MEGA 4.0 software. Bootstrap values of 1,000 replication were shown on the branches.

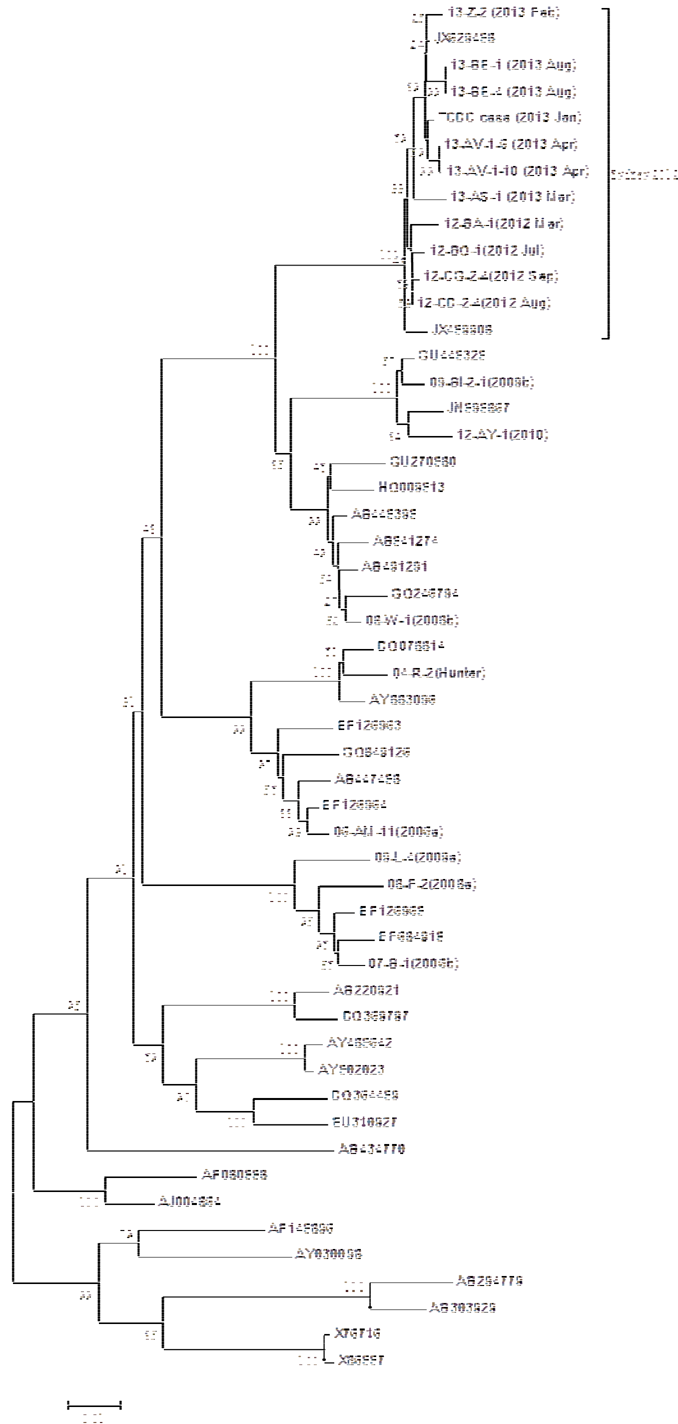

Fig 4. Comparison the amino acids changes of GII.4 strains in predicted potential immunoloops within P2 subdomain and HBGA binding pocket. Three immunoloops contain loop1 (position 294-297), loop2 (position 371-374) and loop3 (position 390-393). HBGA binding sites include site 1 (position 343-345, 374) and site 2 (position 393-395). Gray color means the variation between GII.4 Sydney strain and other GII.4 strains. Dark color means the variation between GII.4 Sydney strains in 2012 and 2013.

Immunoloops	Loop 1														Loop 2			
HBGA binding sites										1								1
Epitope sites	A		A					C						A		A		
	294	295	296	297	298	305	310	340	341	343	344	345	359	368	371	372	373	374
08-W-1 (Apeldoorn 2007)	T	S	S	R	N	T	S	A	D	S	T	R	A	A	T	D	N	D
12-AY-1 (New Orleans 2009)	P					S	S	T	N				S	A			N	
12-BA-1 (Sydney 2012)(2012-Mar)	T					S	N	T	D				A	E			H	
12-BQ-1 (Sydney 2012)(2012-Jul)	T					S	N	T	D				A	E			H	
12-CD-2-4 (Sydney 2012)(2012-Aug)	T					S	N	T	D				A	E			H	
12-CG-2-4 (Sydney 2012)(2012-Sep)	T					S	N	T	D				A	E			H	
13-Z-2 (Sydney 2012)(2013 Feb)	T					S	N	T	D				A	E			R	
13-AS-1 (Sydney 2012)(2013 Mar)	T					S	N	T	D				A	E			R	
13-AV-1 (Sydney 2012)(2013 Apr)	T					S	N	T	D				A	E			H	
13-BE-4 (Sydney 2012)(2013 Aug)	T					S	N	T	D				A	E			R	

Immunoloops			Loop 3										
HBGA binding sites						2							
Epitope sites	C					D				E			
	376	377	390	391	392	393	394	395	396	407	412	413	414
08-W-1 (Apeldoorn 2007)	D	A				D	T	A	H	S	N	S	H
12-AY-1 (New Orleans 2009)	E	T				S		T	P			I	H
12-BA-1 (Sydney 2012)(2012-Mar)	E	A				G		T	H			T	H
12-BQ-1 (Sydney 2012)(2012-Jul)	E	A				G		T	H			T	H
12-CD-2-4 (Sydney 2012)(2012-Aug)	E	A				G		T	H			T	H
12-CG-2-4 (Sydney 2012)(2012-Sep)	E	A				G		T	H			T	H
13-Z-2 (Sydney 2012)(2013 Feb)	E	A				S		T	H			T	P
13-AS-1 (Sydney 2012)(2013 Mar)	E	A				G		T	H			T	P
13-AV-1 (Sydney 2012)(2013 Apr)	E	A				S		T	H			T	P
13-BE-4 (Sydney 2012)(2013 Aug)	E	A				S		T	H			T	P

References

1. Ahmed, S. M., Hall, A. J., Robinson, A. E., Verhoef, L., Premkumar, P., Parashar, U. D., Koopmans, M., and Lopman, B. A. (2014) Global prevalence of norovirus in cases of gastroenteritis: a systematic review and meta-analysis. *Lancet Infect. Dis.* 14, 725-730
2. Wikswo, M. E., Hall, A. J., Centers for Disease, C., and Prevention. (2012) Outbreaks of acute gastroenteritis transmitted by person-to-person contact--United States, 2009-2010. *MMWR Surveill. Summ.* 61, 1-12
3. Hall, A. J., Eisenbart, V. G., Etingue, A. L., Gould, L. H., Lopman, B. A., and Parashar, U. D. (2012) Epidemiology of foodborne norovirus outbreaks, United States, 2001-2008. *Emerg. Infect. Dis.* 18, 1566-1573
4. Ohe, M. (2013) A "blind spot" regarding the norovirus infection pathway. *Tohoku J Exp Med* 229, 125-128
5. Donaldson, E. F., Lindesmith, L. C., Lobue, A. D., and Baric, R. S. (2008) Norovirus pathogenesis: mechanisms of persistence and immune evasion in human populations. *Immunol Rev* 225, 190-211
6. Glass, R. I., Parashar, U. D., and Estes, M. K. (2009) Norovirus gastroenteritis. *N. Engl. J. Med.* 361, 1776-1785
7. Vinje, J., Altena, S. A., and Koopmans, M. P. (1997) The incidence and genetic variability of small round-structured viruses in outbreaks of gastroenteritis in The Netherlands. *J Infect Dis* 176, 1374-1378
8. Widdowson, M. A., Cramer, E. H., Hadley, L., Bresee, J. S., Beard, R. S., Bulens, S. N., Charles, M., Chege, W., Isakbaeva, E., Wright, J. G., Mintz, E., Forney, D., Massey, J., Glass, R. I., and Monroe, S. S. (2004) Outbreaks of acute gastroenteritis on cruise ships and on land: identification of a predominant circulating strain of norovirus--United States, 2002. *J Infect Dis* 190, 27-36
9. Siebenga, J., Kroneman, A., Vennema, H., Duizer, E., and 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
10. Greening, G. E., Hewitt, J., Rivera-Aban, M., and Croucher, D. (2012) Molecular epidemiology of norovirus gastroenteritis outbreaks in New Zealand from 2002-2009. *J Med Virol* 84, 1449-1458
11. Zheng, D. P., Widdowson, M. A., Glass, R. I., and Vinje, J. (2010) Molecular epidemiology of genogroup II-genotype 4 noroviruses in the United States between 1994 and 2006. *J Clin Microbiol* 48, 168-177

12. Vega, E., Barclay, L., Gregoricus, N., Williams, K., Lee, D., and Vinje, J. (2011) Novel surveillance network for norovirus gastroenteritis outbreaks, United States. *Emerg Infect Dis* 17, 1389-1395
13. Lindesmith, L. C., Donaldson, E. F., Lobue, A. D., Cannon, J. L., Zheng, D. P., Vinje, J., and Baric, R. S. (2008) Mechanisms of GII.4 norovirus persistence in human populations. *PLoS Med.* 5, e31
14. Lindesmith, L. C., Beltramello, M., Donaldson, E. F., Corti, D., Swanstrom, J., Debbink, K., Lanzavecchia, A., and Baric, R. S. (2012) Immunogenetic mechanisms driving norovirus GII.4 antigenic variation. *PLoS Pathog.* 8, e1002705
15. Iritani, N., Kaida, A., Kubo, H., Abe, N., Murakami, T., Vennema, H., Koopmans, M., Takeda, N., Ogura, H., and Seto, Y. (2008) Epidemic of genotype GII.2 noroviruses during spring 2004 in Osaka City, Japan. *J Clin Microbiol* 46, 2406-2409
16. Kojima, S., Kageyama, T., Fukushi, S., Hoshino, F. B., Shinohara, M., Uchida, K., Natori, K., Takeda, N., and Katayama, K. (2002) Genogroup-specific PCR primers for detection of Norwalk-like viruses. *J. Virol. Methods* 100, 107-114
17. Tamura, K., Dudley, J., Nei, M., and Kumar, S. (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* 24, 1596-1599
18. Tan, M., Huang, P., Meller, J., Zhong, W., Farkas, T., and 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, 12562-12571
19. Tan, M., Huang, P., Xia, M., Fang, P. A., Zhong, W., McNeal, M., Wei, C., Jiang, W., and Jiang, X. (2011) Norovirus P particle, a novel platform for vaccine development and antibody production. *J. Virol.* 85, 753-764
20. Shanker, S., Choi, J. M., Sankaran, B., Atmar, R. L., Estes, M. K., and Prasad, B. V. (2011) Structural analysis of histo-blood group antigen binding specificity in a norovirus GII.4 epidemic variant: implications for epochal evolution. *J Virol* 85, 8635-8645
21. van Beek, J., Ambert-Balay, K., Botteldoorn, N., Eden, J. S., Fonager, J., Hewitt, J., Iritani, N., Kroneman, A., Vennema, H., Vinje, J., White, P. A., Koopmans, M., and NoroNet. (2013) Indications for worldwide increased norovirus activity associated with emergence of a new variant of genotype II.4, late 2012. *Euro Surveill.* 18, 8-9
22. Bennett, S., MacLean, A., Miller, R. S., Aitken, C., and Gunson, R. N. (2013) Increased norovirus activity in Scotland in 2012 is associated with the

emergence of a new norovirus GII.4 variant. *Euro Surveill.* 18

23. (2013) Emergence of new norovirus strain GII.4 Sydney--United States, 2012. *MMWR Morb Mortal Wkly Rep* 62, 55
24. Chan, M. C., and Chan, P. K. (2013) Complete Genome Sequence of a Novel Recombinant Human Norovirus Genogroup II Genotype 4 Strain Associated with an Epidemic during Summer of 2012 in Hong Kong. *Genome Announc* 1
25. Fonager, J., Hindbaek, L. S., and Fischer, T. K. (2013) Rapid emergence and antigenic diversification of the norovirus 2012 Sydney variant in Denmark, October to December, 2012. *Euro Surveill.* 18
26. Hasing, M. E., Lee, B. E., Preiksaitis, J. K., Tellier, R., Honish, L., Senthilselvan, A., and Pang, X. L. (2013) Emergence of a New Norovirus GII.4 Variant and Changes in the Historical Biennial Pattern of Norovirus Outbreak Activity in Alberta, Canada (2008-2012). *J Clin Microbiol*
27. Siebenga, J. J., Vennema, H., Renckens, B., de Bruin, E., van der Veer, B., Siezen, R. J., and Koopmans, M. (2007) Epochal evolution of GGII.4 norovirus capsid proteins from 1995 to 2006. *J Virol* 81, 9932-9941
28. Hall, A. J. (2012) Noroviruses: the perfect human pathogens? *J Infect Dis* 205, 1622-1624
29. Allen, D. J., Noad, R., Samuel, D., Gray, J. J., Roy, P., and Iturriza-Gomara, M. (2009) Characterisation of a GII-4 norovirus variant-specific surface-exposed site involved in antibody binding. *Virol J* 6, 150
30. Zakikhany, K., Allen, D. J., Brown, D., and Iturriza-Gomara, M. (2012) Molecular evolution of GII-4 Norovirus strains. *PLoS One* 7, e41625