Original Research

MOLECULAR CHARACTERISTICS OF NOROVIRUS IN ASIATIC HARD CLAM IN HANOI IN 2016

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ABSTRACT

Aims: Norovirus (NoV) is a common foodborne virus in bivalve molluscs. This study aimed to identify genotypes of NoV genogroups I and II (GI and GII) in Asiatic hard clam (*Meretrix meretrix*) marketed in Hanoi, Viet Nam, in 2016.

Methods: A total of 39 Asiatic hard clam samples positive for NoV GI and/or NoV GII was genotyped using nested RT-PCR targeting fragment spanning the ORF1/ORF2 overlap region. PCR products were sequenced using Sanger sequencing and phylogenetic analysis was carried out using MEGA 11 software.

Results: NoV genotyping has been successfully performed for 23 samples. For NoV GI, four genotypes were detected: GI.2 (13.3%), GI.5 (46.7%), GI.6 (33.3%) and GI.8 (6.7%). We found five distinct genotypes of NoV GII, including GII.3 (61.1%), GII.4 (11.1%), GII.6 (11.1%), GII.13 (11.1%) and GII.17 (5.6%). Phylogenetic analysis revealed these NoV genotypes displayed high similarity with clinical strains circulating worldwide, especially strains from Asian countries.

Conclusions: The contamination of NoV GI and NoV GII in Asiatic hard clams in Hanoi was complex with multiple genotypes. Two worldwide epidemic genotypes GII.4 and GII.17 were identified in the present study, however, the dominant NoV genotypes were GI.5 and GII.3.

Keywords: *Norovirus, foodborne virus, genotyping, Asiatic hard clam, phylogenetic tree*

I. INTRODUCTION

Norovirus (NoV) is one of the most common causes of acute gastroenteritis, which in some cases can cause persistent diarrhea, leading to dehydration and possibly death if not treated promptly [1]. According to recent epidemiological statistics, NoV is considered one of the main causes of foodborne illness worldwide. In the United State of America, NoV is responsible for two-thirds of all foodborne illnesses caused by micro-organisms [2]. In Europe, more than 5.7 million children under the age of 5 suffer from diarrhea caused by NoV every year, of whom more than 50,000 require hospitalization [3]. In Japan, 40.2% of diarrhea cases are attributable to NoV [4].

The principal route of NoV infection is through the consumption of foods, mainly bivalve molluscs, which accumulate NoV via filter feeding.

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In Vietnam, Asiatic hard clam (*Meretrix meretrix*) is one of the most commonly cultivated bivalve molluscs with high export volume and revenue.

However, data on the situation of foodborne virus contamination in Asiatic hard clam remains scarce. In addition, the diversity of NoV as well as the emergence of new variants pose various difficulties to trace the disease back to contaminated food. Therefore, it is of importance to study the molecular characteristics of NoV in Asiatic hard clam in Vietnam, thus contributing to the risk assessment process of NoV in bivalve molluscs.

II. METHODS

2.1. Materials

Asiatic hard clams

Asiatic hard clam samples were collected from retail markets and supermarkets in Hanoi every fortnight throughout the year of 2016. Samples were analyzed for NoV contamination by ISO 15216-2:2013 method. In total, 39 samples were positive for NoV GI and/or NoV GII. RNAs extracted from these samples were used for genotyping.

Oligonucleotides

Table 1. *Primers and probes used for Real-time RT-PCR*

Target	Primer/probe name	Sequence (5’ – 3’)
Mengovirus [5]	Mengo 110	GCGGGTCCTGCCGAAAGT
	Mengo 209	GAAGTAACATATAGACAGACGCACAC
	Mengo 147	ATCACATTACTGGCCGAAGC
NoV GI [5]	QNIF4	CGCTGGATGCGNTTCCAT
	NV1LCR	CCTTAGACGCCATCATCATTTAC
	NVGG1p	TGGACAGGAGAYCGCRATCT
NoV GII [5]	QNIF2	ATGTTCAGRTGGATGAGRTTCTCWGA
	COG2R	TCGACGCCATCTTCATTCACA
	QNIFs	AGCACGTGGGAGGGCGATCG

Table 2. *Primers used for Nested RT-PCR*

Target	Primer name	Sequence (5’ – 3’)	Function	Amplicon size (bp)
NoV GI [6]	COG1F	CGYTGGATGCGNTTYCATGA	Outer primer	381
	G1SKR	CCAACC CARCCA TTRTACA		
	G1SKF	CTGCCCGAATTYGTAAATGA	Inner primer	318
	G1SKR	CCAACC CARCCA TTRTACA		
NoV GII [6]	COG2F	CARGARBCNATGTTYAGRTGGATGAG	Outer primer	378
	G2SKR	CCRCCNGCATRHCCRTTRTACAT		
	G2SKF	CNTGGG AGGGCG ATCGCAA	Inner primer	344
	G2SKR	CCRCCN GCATRH CCRTTR TACAT		

2.2. Experimental procedures

All experimental procedures were performed at the School of Biotechnology and Food Technology, Hanoi University of Science and Technology.

Real-time RT-PCR

Virus detection was carried out using real-time reverse-transcription PCR assays following ISO 15216–2:2013 procedure [5]. All reactions were prepared using the Ultrasens quantitative RT-PCR kit (Life Technologies) on the MasterCycler RealPlex4 system (Eppendorf). Five microliters of nucleic acid extract were added to 20 µl of reaction mixture containing 12,5 µl of 2X reaction mix; 0,5 µl of SuperScript™ III RT/Platinum™ *Taq* Mix; 0,5 µM of primer and 0,4 µM of probe (Table 1). Reverse transcription was carried out at 55ºC for 60 min, followed by denaturation at 95ºC for 5 min. cDNA was amplified immediately with 45 PCR cycles of 15 s at 95ºC, 1 min at 60ºC, and 1 min at 65ºC.

Nested RT-PCR

Samples identified as positive for NoV GI and NoV GII underwent molecular typing by nested RT-PCR. Primers used in nested RT-PCR are provided in Table 2. Firstly, RT-PCR reactions were all assembled in 25 µl, containing 5 µl of RNA; 1X of Reaction Mix (SuperScript™ III One-Step RT-PCR System); 1 µl of SuperScrip III RT/Platinum *Taq* Mix and 0.2 µM of each primer. Reverse transcription was carried out at 50ºC for 30 min, followed by denaturation at 94ºC for 2 min. cDNA was amplified immediately with 40 PCR cycles of 15 s at 94ºC, 30s at 50ºC, and 30s min at 72ºC and a final extension at 72°C for 2 min. The secondary PCR was prepared in a total volume of 50 µl containing 1 µl of RT-PCR product, 1 X Q5 Hot Start High-Fidelity 2X Master Mix and 0.5 µM of each primer. The second PCR reaction was subjected to the following thermal cycling parameters: 30s at 98°C followed by 25 cycles of 5s at 98°C, 30s at 52°C, and 30s at 72°C. The reactions were completed with a final extension for 2 min at 72°C. The Nested RT-PCR products were visualized by gel electrophoresis (1.5% agarose gel) with Ethidium Bromide staining.

Phylogenetic analysis

PCR products were purified by QIAquick PCR purification kit and were sequenced by 1st Base Company (Malaysia). Sequence quality was checked with BioEdit software v7.2. Phylogenetic trees were contructed with the sequences from this study and reference sequences obtained from GenBank. Phylogenetic relationship was inferred by the ClustalW and Maximum Likelihood method based on the Kimura 2-parameter model with Gamma distribution using MEGA 11 software.

III. RESULTS

3.1. Molecular characteristics of Norovirus GI

Phylogenetic analysis of 26 samples positive with NoV GI by ISO 15216-2:2013 method was performed based on sequences spanning the ORF1/ORF2 overlap region. Nested RT-PCR generated a total of 15 amplicons, which were sequenced by the primer G1SKF. BLASTN analysis showed these 15 sequences displayed high homology (> 99%) with clinical NoV GI sequences in GenBank. The phylogenetic tree (Figure 2) was constructed using 15 sequences from this study and 20 reference sequences covering 9 genotypes of human NoV GI (GI.1 - GI.9).

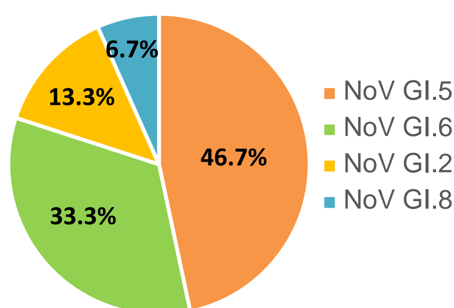

Figure 1. *Genotype distribution of Norovirus GI in Asiatic hard clam marketed in Hanoi*

Figure 2. *Phylogenetic tree of NoV GI strains.*

The tree was constructed using the Maximum likelihood method based on the Kimura 2-parameter model with Gamma distribution. Bootstrap values of >50% are shown. The bar at the bottom of the tree indicates distance.

In total, four genotypes were detected (Figure 1); of which GI.5 was the most dominant, accounting for 46.7%; followed by GI.6 at 33.3%. Overall, NoV GI strains detected in this study had high similarities with those circulating in Asia. Specifically, all of the GI.5, GI.6 and GI.8 sequences had high homology to clinical strains from China, Korea, and Taiwan. Meanwhile, two GI.2 sequences had high similarity to a clinical strain from Russia.

3.2. Molecular characteristics of Norovirus GII

For NoV GII, phylogenetic analysis of 34 samples positive with real-time RT-PCR has produced 18 sequences. BLASTN analysis showed these 18 sequences had high similarity with NoV GII sequences in GenBank. The phylogenetic tree was constructed using 18 sequences from this study and 29 reference sequences covering 18 genotypes of human NoV GII (GII.1 – GII.10, GII.12 – GII.18, GII.20).

As shown in Figure 3, five genotypes were identified within GII, including GII.3, GII.4, GII.13, GII.17 and GII.6. GII.3 was the dominant genotype, accounting for 61.1%; followed by GII.4, GII.13 and GII.17 at 11.1% each. Of note, GII.3 strains detected in this study encompasses the most diverse sequences having high homology with clinical strains from South Africa, China, Vietnam, the Netherlands, Japan, South Korea and the United States of America. Interestingly, only two GII.4 and two GII.17 strains were identified in the present study while these GII.4 and GII.17 are the main epidemic strains worldwide. However, it is worthy to note that the strain N2016.08ST02 was almost identical to the pandemic GII.4 Sydney strain, suggesting that variants of this pandemic strain were still circulating in Vietnam in 2016 at low prevalence. Similarly, the strain N2016.08C01 had high homology to the novel predominant strain GII.17 Kawasaki 2014, which emerged as a major cause of NoV outbreaks in several Asian countries during the period 2014-2015.

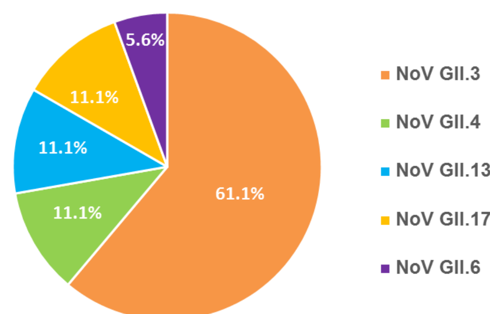

Figure 3. *Genotype distribution of Norovirus GII in Asiatic hard clam marketed in Hanoi*

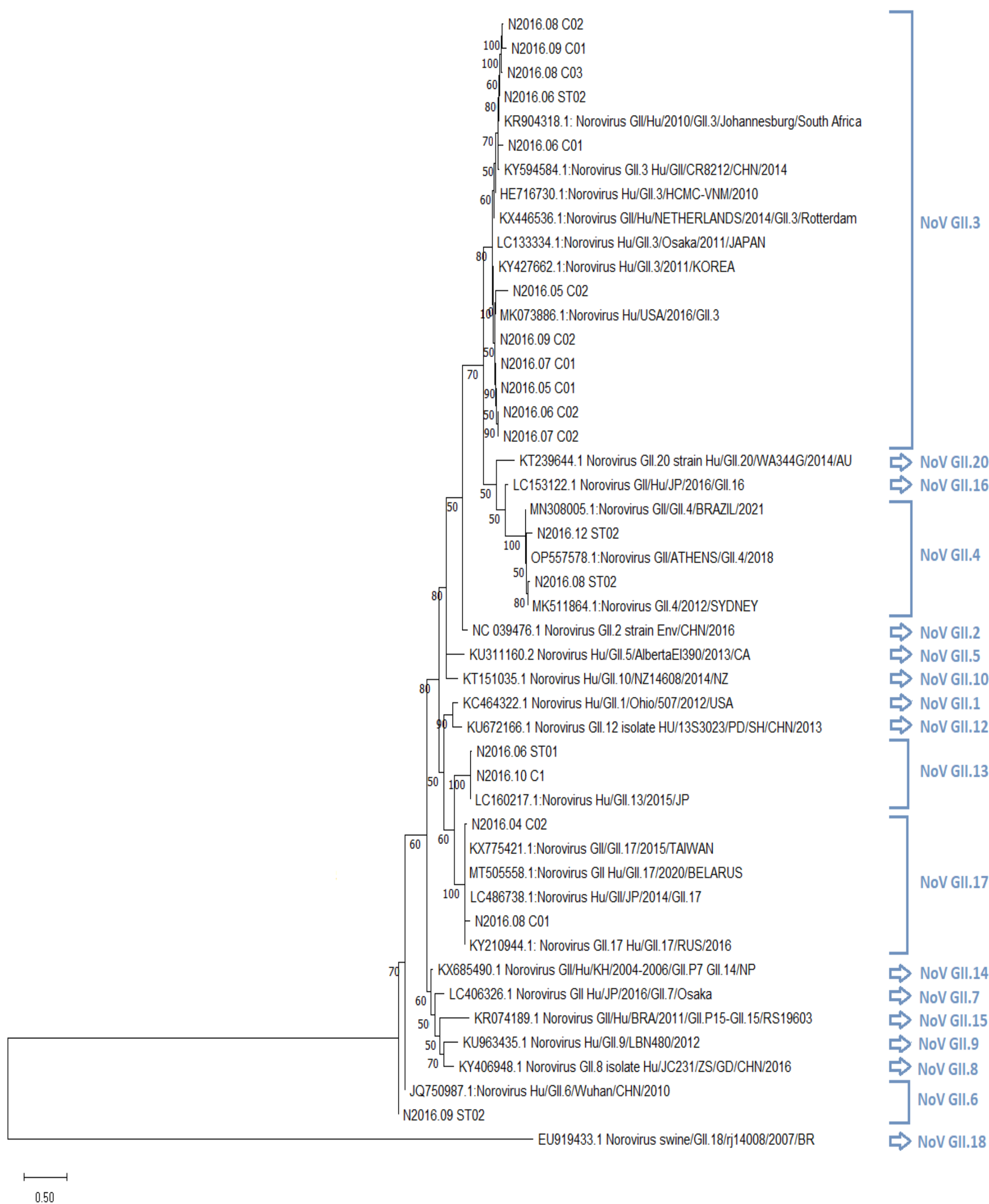

Figure 4. *Phylogenetic tree of NoV GII strains.*

The tree was constructed using the Maximum likelihood method based on the Kimura 2-parameter model with Gamma distribution. Bootstrap values of >50% are shown. The bar at the bottom of the tree indicates distance.

IV. DISCUSSION

NoV GI and GII are now recognized as the leading cause of acute gastroenteritis globally. The emergence and spread of novel epidemic NoV strains has been associated with (i) point mutations on two different genes: ORF1, encoding the non-structural proteins, and ORF2, encoding the major capsid protein (VP1); and (ii) recombination events that create chimeric viruses. NoV infections can happen several times in a lifetime as the result of limited duration of acquired immunity and high antigenic diversity of the virus. Since the mid-1990s, NoV outbreaks have been predominantly attributed to the rapidly evolving GII.4 genotype with periodic emergence of GII.4 variants every 2–3 years [7]. During the winter of 2014–2015, a novel GII.17 strain has emerged as a major cause of gastroenteritis outbreaks in Asia, and this variant replaced the previously dominant GII.4 Sydney 2012 variant [8, 9]. Contaminated bivalve molluscs, especially oysters and clams, are the most frequent source of infections.

In this study, genetic analysis of the ORF1/ORF2 overlap region has revealed the occurrence of multiple genotypes of NoV GI (GI.2, GI.5, GI.6 and GI.8) and NoV GII (GII.3, GII.4, GII.6, GII.13 and GII.17) in Asiatic hard clam marketed in Hanoi during 2016, indicating that multiple NoV genotypes co-circulate in Vietnam. Unexpectedly, the most common genotypes were GII.3 and GI.5. However, when comparing to surveillance data in the literature, we found that GII.3 were also the predominant genotypes in several study [10, 11]. Recently, GI.5 genotype was involved in several NoV outbreaks in China and Spain [12, 13].

To date, in Vietnam, NoV studies have mainly been conducted on clinical samples. A study by Nguyen Van Trang from 2007 to 2013 showed the dominance of the NoV GII.4 genotype among the circulating genotypes; followed by genotype GII.3. In our study, two strains N2016.08ST02 and N2016.08C01 had high similarity with the pandemic GII.4 Sydney strain and the novel predominant strain GII.17 Kawasaki 2014, indicating that these highly infectious strains were still circulating in Vietnam in 2016.

V. CONCLUSION

The study shows that the contamination of NoV in Asiatic hard clam in Vietnam was highly complex with the co-circulation of multiple genotypes. Variants of the pandemic GII.4 Sydney strain and the novel predominant strain GII.17 Kawasaki 2014 were also detected in our study. Our finding indicates the need for regular control measures in bivalve molluscs farming areas in order to minimize the risk of acute gastroenteritis due to the contamination of NoV.

Conflict of interest

The authors have no competing interests

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