pISSN 1229-1153 / eISSN 2465-9223 *J. Food Hyg. Saf.*
Vol. 32, No. 6, pp. 455~459 (2017)
https://doi.org/10.13103/JFHS.2017.32.6.455

Journal of Food Hygiene and Safety
Available online at http://www.foodhygiene.or.kr

Molecular Epidemiology of Norovirus in Asymptomatic Food Handlers in South Korea

Jeong Su Lee, Min Hee Jeong, Si Yeon Ju, Kyung Ah Kang, and In Sun Joo*

Food Microbiology Division, Food Safety Evaluation Department, National Institute of Food and Drug Safety Evaluation, Osong, Korea

(Received April 28, 2017/Revised May 20, 2017/Accepted October 11, 2017)

ABSTRACT - Norovirus (NoV) is the most common cause of acute gastroenteritis in all age groups worldwide. In this study, prevalence of asymptomatic norovirus infection was investigated in food handler being employed at food catering facilities in South Korea. A total of 2,729 fecal specimens from asymptomatic food handlers were analyzed, and 1.06% of food handlers (29/2,729) had asymptomatic NoV infection. Of these, 17.2% (5/29) were positive for NoV GI and 82.7% (24/29) were positive for NoV GII. Especially, sequencing and phylogenetic analysis showed that GII-4 was the most prevalent genotype and a large number of asymptomatic food handlers were infested with norovirus GII-4 strains. The results of this study show that asymptomatic food handlers may be potential transmission sources for NoV infection. These results emphasize the need for training of food catering employees about norovirus prevention. Asymptomatic norovirus infection should receive more attention.

Key words : Asymptomatic Food Handler, Genotype, Norovirus

The positive-sense polyadenylated single-stranded RNA virus family *Caliciviridae* contains four genera: Vesivirus, Sapovirus, Lagovirus, and Norovirus (NoV)[1]. The NoV is currently classified into 6 genogroups (GI to GVI)[2], and only NoV GI, GII, and GIV have been associated with human gastroenteritis[3]. The GI and GII genogroups of human NoV are further classified into 9 and 22 genotypes, respectively[4]. The virus is transmitted predominantly through ingestion of contaminated food as well as person-to-person by the fecal-oral route, airborne transmission and contact with contaminated surfaces[5]. In previous reports, infected food handlers have been implicated repeatedly as the source of infection in several outbreaks[6,7]. Asymptomatic NoV infections of food handlers may play a role in transmission[8]. NoV disease outbreaks are reported year-round in Korea. Furthermore, there have been several large outbreaks of NoV since 2003[9,10]. The NoV outbreak has exhibited a high prevalence during winter with cold temperate climates[10]. In other words, the environment with low temperature (average 2.1°C) and humidity (average 59.6%) may be a possible contributors to the transmission of enteric infections[12]. Many studies have reported the monitoring of NoV in facilities with outbreak[11,13,14]. However, little research is available about circulating viral strains in asymptomatic individuals in facilities without NoV outbreaks. Recently, another epidemiologic study of NoV outbreak in South Korea has reported that the excretion of NoV from asymptomatic food handlers may be an important portion of NoV outbreak[9,12,14,17]. The aims of this study were to investigate the molecular epidemiological characteristics of NoV detection from asymptomatic food handlers working at food catering facilities in South Korea from October 2012 to April 2013.

*Correspondence to: In Sun Joo, Food Microbiology Division, Food Safety Evaluation Department, National Institute of Food and Drug Safety Evaluation, Osong 28159, Korea
Tel: 82-43-719-4302, Fax: 82-43-719-4300
E-mail: jis901@korea.kr

Materials and Methods

Clinical samples

Rectal swab samples were collected from asymptomatic food handlers during regular physical examinations at five health centers (Tongyeong, Gyeonggi, Yeosu, Taean and Chenogju) in South Korea. Among 2,729 food handlers, the gender ratio of this study was 72% (1,965) female and 28% (764) male. Swab samples collected from randomly selected food handlers distributed in South Korea were suspended in 2 mL of phosphate buffered saline (pH 7.2), vortexed slightly, and then centrifugated at 3,000 rpm for 10 min to separate the supernatants. Supernatants were stored at −80°C until use.

Viral RNA extraction

The QIAamp Viral RNA Mini kit (Qiagen, Hilden, Germany) was used to perform the viral RNA extraction in

accordance with manufacturer's instructions. Viral RNA was eluted in 60 μL of AVE buffer and stored at −80°C until use.

Conventional nested RT-PCR

NoV was identified using conventional nested RT-PCR as previously described[17]. PCR amplification was performed to detect the ORF 1-2 junction region of the NoV. 5 μL of extracted RNA was used in the RT-PCR mixture with a total volume of 25 μL comprised of 10 μL one-step RT-PCR premix (with AMV reverse transcriptase), 6 μL of distilled water and 2 μL of each NoV GI and GII primer (20 pmol) (Table 1). The cycling conditions were: 30 min at 45°C for cDNA synthesis, 5 min at 94°C for predenaturation, then 35 cycles consisting of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 1 min 30 sec. The semi-nested PCR amplification procedure was followed using the first-round amplicon. 2 μL of amplicon was added to 48 μL of the PCR mixture containing 5 μL of 10× reaction buffer (Bioneer, Daejeon, Korea), 4 μL 10 mM dNTPs (Bioneer, Daejeon, Korea), 2.5 μL of each NoV GI and GII primer (20 pM), 1 μL of Top DNA polymerase (Bioneer, Daejeon, Korea) and 33 μL distilled water. The cycling conditions were: 5 min at 94°C for predenaturation, then 25 cycles consisting of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 1 min 30 sec. The amplification products were analyzed by 2% agarose gel electrophoresis and visualized with ultraviolet (UV) light after ethidium bromide staining. Samples that were NoV positive by conventional RT-PCR were further characterized (genotyped) by DNA sequencing. All PCR products were sequenced using an ABI Prism 3500×L genetic analyzer and BigDye Terminator cycle sequencing mix (Applied Biosystems, Foster City, CA, USA). For genotyping of sequenced products, the sequences were compared to those in the GenBank database using the NCBI BLAST search program. To confirm the genotype of NoV, phylogenetic analysis was performed and the phylogenetic trees were obtained using the CLUSTAL W method and MegAlign (Lasegene, DNAstar, Inc. Madison, WI, USA) software.

Real time RT-PCR

To analyze viral copy number within each sample, NoVs were amplified with a one-step real time RT-PCR kit (Ambion, Austin, TX, USA) as previously described[17]. The real time RT-PCR reaction mixture contained 5 μL of extracted RNA, 12 μL of 2× RT-PCR buffer (Ambion, Austin, TX, USA), 1 μL of each NoV GI and GII primer (10 pM), 0.5 μL of each fluorescent probe (10 pM), 0.5 μL of 25× enzyme mix, 1.5 μL of enhancer, and 3 μL of distilled water. 5 μL of extracted NoV sample was added to each well, and the final total volume was 25 μL (Table 2). The cycling conditions were: reverse transcription at 45°C for 30 min, predenaturation at 95°C for 10 min, and 45 cycles of denaturation at 95°C for 15 sec and annealing, and extension at 56°C for 1 min.

Results and Discussion

Among 2,729 food handlers comprised of 764 male and 1,965 female, asymptomatic infection was detected in 29

Table 1. Primers used for NoV detection by conventional PCR

Genogroup	Primer	Sequence (5' → 3')	Size (bp)	Application
I	GI-FIM	CTG CCC GAA TTY GTA AAT GAT GAT	313	One-step RT PCR
	GI-RIM	CCA ACC CAR CCA TTR TAC ATY TG		One-step RT PCR/Semi-nested PCR/ Sequencing
	GI-F2	ATG ATG ATG GCG TCT AAG GAC GC		Semi-nested PCR/Sequencing
II	GII-FIM	GGG AGG GCG ATC GCA ATC T	310	One-step RT PCR
	GII-RIM	CCR CCI GCA TRI CCR TTR TAC AT		One-step RT PCR/Semi-nested PCR/ Sequencing
	GII-F3M	TTG TGA ATG AAG ATG GCG TCG ART		Semi-nested PCR/Sequencing

Table 2. Primers and probes used for NoV detection by real-time RT-PCR

Genogroup	Primers/probes	Sequence (5' → 3')	Position
I	COG1F	CGY TGG ATG CGN TTY CAT GA	5291
	COG1R	CTT AGA CGC CAT CAT CAT TYA C	5375
	RING1(a)-TP	FAM-AGA TYG CGA TCY CCT GTC CA-TAMRA	5340
II	BPO-13	AIC CIA TGT TYA GIT GGA TGA G	5007
	BPO-13N	AGT CAA TGT TTA GGT GGA TGA G	
	BPO-14	TCG ACG CCA TCT TCA TTC ACA	5101
	BPO-18	VIC-CAC RTG GGA GGG CGA TCG CAA TC-TAMRA	5044

Table 3. The number of detection (per months)

Month	The number of detection		
	Number of samples	Number of detections	Detection rate
Oct	15	0	0%
Nov	1340	8	0.60%
Dec	177	0	0%
Jan	131	8	6.11%
Feb	536	9	1.68%
Mar	72	1	1.39%
Apr	458	3	0.66%
Total	2729	29	1.06%

(1.06%) by conventional nested RT-PCR. The prevalence of NoV in asymptomatic food handlers was 58.62% during winter season (from December to February). Especially, there was a tendency that the prevalence of asymptomatic NoV infection in January has higher detection than other months (Table 3). Among 29 samples, 5 GI-positive samples and 24 GII-positive samples were identified by real time RT-PCR. Mean viral load in stool specimens for GI NoV was 6.1×10^7 viruses/g (range, 2.1×10^2 to 3.0×10^8 viruses/g) and was 6.1×10^4 viruses/g (range, 1.5×10^1 to 9.7×10^5 viruses/g) for GII (data not shown). Sequencing and phylogenetic analysis showed that the 5 GI-positive samples were genotyped as GI-4, GI-6, GI-7, GI-9 and 24 GII-positive samples were GII-2, GII-3, GII-4, GII-6, GII-16, GII-17. Our data demonstrated that mean viral load in stool specimens of GI NoV was higher than that of GII NoV. The genotypic distribution of the 29 NoV strains was as follows: GI-4, 6.89% (*n*=2); GI-6, 3.44% (*n*=1); GI-7, 3.44% (*n*=1); GI-9, 3.44% (*n*=1); GII-4, 41.37% (*n*=12); GII-17, 20.69% (*n*=6); GII-2, 10.34% (*n*=3); GII-3, 3.44% (*n*=1); GII-6, 3.44% (*n*=1); GII-16, 3.44% (*n*=1) (Table 4).

Molecular epidemiological studies of NoV strains in asymptomatic food handlers have reported that GII-4 was dominant in transmissibility[1,16,18]. Likewise, in our study, the NoV GII-4 strain was more prevalent than others. This is similar to the previous Japanese study investigating NoV outbreaks, in which the NoV samples were collected from asymptomatic food handlers in a hotel and confirmed belonging to NoV GII[2,4]. Accordingly, it is possible to assume

Table 4. The genotypic distribution of detection strains

	Prevalence of genotypes										
	Total	GI-4	GI-6	GI-7	GI-9	GII-2	GII-3	GII-4	GII-6	GII-16	GII-17
Total	29	2	1	1	1	3	1	12	1	1	6

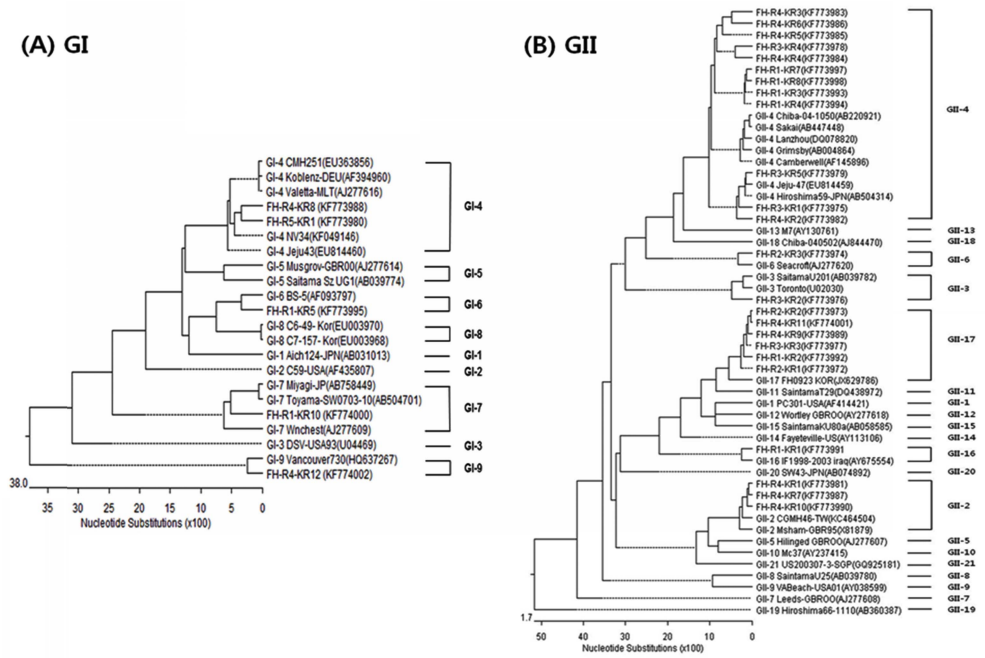

Fig. 1. Phylogenetic tree of NoV detected in asymptomatic food handler. Neighbor-joining phylogenetic tree based on nucleotide sequences of the capsid region of the NoV genome (A, norovirus GI; B, norovirus GII). The numbers in the branches indicate the bootstrap values. Reference strains of NoV selected from Genbank are indicated by accession numbers. The scaled indicates nucleotide substitutions per position.

that GII is the dominant genogroup in an asymptomatic food handler population. This might be related to the fact that genogroup II strains (especially GII-4) are more transmissible than the others[4,9]. The clinical manifestations (e.g., increased vomiting) of GII-4 strain infections or physical characteristics (e.g., environmental persistence) of this strain might facilitate spreading. In South Korea, previous study that the vehicle of transmission in this outbreak was dried radish salad prepared by the food handler and served to students in the elementary school[22]. Furthermore, approximately 50% of all NoV outbreaks in the United States are linked to ill food-handlers[20]. In this regard, asymptomatic carriers of Nov are main cause related to the occurrence of NoV outbreaks[9,15,21].

In this study, phylogenetic analysis was used to evaluate the relatedness of NoV strains detected and to compare their partial capsid sequence with those of GI and GII genogroup reference strains (Fig. 1). In phylogenetic analysis with GI genogroup, FH-R4-KR8 (KF773988) and FH-R5-KR1 (KF773980) strains were identified as genotype GI-4. 3 strains, FH-R1-KR5 (KF773995), FH-R1-KR10 (KF774000) and FH-R4-KR12 (KF774002) were analyzed as GI-6, GI-7 and GI-9, respectively. The FH-R4-KR8 (KF773988) and FH-R5-KR1 (KF773980) strains were clustered into the GI-4 NV34 (KF049146) with 97.0% and 89.9% identity, respectively. Sequence analysis revealed that strain FH-R1-KR5 (KF773995) shared the greatest identity with strain GI-6 BS-5 (AF093797) (92.5%). Strains FH-R1-KR10 (KF774000) and FH-R4-KR12 (KF774002) were found to be related most closely to GI-7 Miyagi-JP (AB758449) (90.3%) and GI-9 Vancouver730 (HQ637267) (94.5%), respectively. The NoV GII strains were 87.3 to 97.6% homologous with the reference GII-4, 89.9 to 92.1% with the reference GII-17, 90.0 to 96.7% with the reference GII-2, 91.6% with the reference GII-3, 93.5% with the reference GII-6 and 95.3% with the reference GII-16. FH-R4-KR3 (KF773983), FH-R4-KR6 (KF773986), FH-R4-KR5 (KF773985), FH-R3-KR4 (KF773978), FH-R4-KR4 (KF773984), FH-R1-KR7 (KF773997), FH-R1-KR8 (KF773998), FH-R1-KR3 (KF773993), FH-R1-KR4 (KF773994), FH-R3-KR5 (KF773979), FH-R3-KR1 (KF773975), and FH-R4-KR2 (KF773982) stains showed 87.3% to 97.6% sequence identity to the GII-4 genotype, suggesting that GII-4 is the most prevalent genotype in an asymptomatic Nov infection.

The results of this study showed that asymptomatic employees as well as symptomatic food handlers may contribute infection as a potential transmission source in NoV outbreaks. To reduce food contamination, strict general hygiene practices should be implemented. More attention should be paid to facilities for food workers to reinforce hand hygiene practices and prevent foodborne outbreak.

Nucleotide sequence accession numbers

The nucleotide sequence data have been submitted to GenBank and assigned accession numbers KF773975 to KF774002.

Acknowledgements

We thank the staffs at 5 centers for collecting stool specimens of food handler. This study was supported by a grant (121612KFDA033) from Ministry of Food and Drug Safety in 2013.

국문요약

노로바이러스는 전세계적으로 모든 연령에게 급성 장염을 일으키는 주요 원인체이다. 본 연구는 국내 식품업계의 식품종사자를 대상으로 무증상 노로바이러스 감염의 유행성을 조사하였다. 2,729명의 식품종사자에서 29명(1.06%)이 무증상 노로바이러스로 확인되었고 이 중 5명(17.24%)은 노로바이러스 GI 양성이었고 24명(82.76%)은 노로바이러스 GII 양성이었다. 특히 유전자 염기서열 분석과 계통 분석에서 GII-4 유전자형이 가장 유행성이 높은 것으로 나타났으며 많은 수의 무증상 식품종사자는 노로바이러스 GII-4 에 감염되어있었다. 따라서 본 연구의 결과는 무증상 식품종사자가 노로바이러스 감염에 잠재적인 전파 원인일 가능성이 있음을 보여준다. 또한 이러한 결과는 노로바이러스 예방에 대한 식품업체의 교육의 필요성을 강조한다. 무증상 노로바이러스 감염에 더 많은 주의를 기울여야 할 것이다.

References

1. Ozawa, K., Oka, T., Takeda, N., Hansman, G.S.: Norovirus infections in symptomatic and asymptomatic food handlers in Japan. *J. Clin. Microbiol*, **45**, 3996-4005 (2007).
2. White, P.A.: Evolution of norovirus. *Clin. Microbiol. Infect*, **20**, 741-745 (2014).
3. Trujillo, A.A., McCaustland, K.A., Zheng, D.P., Hadle, L.A., Vaughn, G., Adams, S.M., Ando, T., Glass, R.I., Monroe, S.S.: Use of TaqMan real-time reverse transcription-PCR for rapid detection, quantification, and typing of norovirus. *J. Clin. Microbiol*, **44**, 1405-1412 (2006).
4. Lu, J., Sun, L., Fang, L., Yang, F., Mo, Y., Lao, J., Zheng, H., Tan, X., Lin, H., Rutherford, S., Guo, L., Ke, C., Hui, L.: Gastroenteritis Outbreaks Caused by Norovirus GII.17, Guangdong Province, China, 2014-2015. *Emerg. Infect. Dis*, **21**, 1240-1242 (2015).
5. Cho M.G., Jeong H.M., Ahn J.B.: Detection of feline calicivirus as norovirus surrogate in food and water sources using filtration and real-time RT-PCR. *Food Sci. Biotechnol*, **20**,

1475 (2011).
6. García, C., DuPont, H.L., Long, K.Z., Santos, J.I., Ko, G.: Asymptomatic norovirus infection in Mexican children. *J. Clin. Microbiol*, **44**, 2997-3000 (2006).
7. Qi, R., Ye, C., Chen, C., Yao, P., Hu, F., Lin, Q.: Norovirus prevention and the prevalence of asymptomatic norovirus infection in kindergartens and primary schools in Changzhou, China: Status of the knowledge, attitudes, behaviors, and requirements. *Am. J. Infect. Control*, **43**, 833-838 (2015).
8. Sukhrie, F.H., Teunis, P., Vennema, H., Copra, C., Thijs, M.F., Bogerman, J., Koopmans, M.: Nosocomial transmission of norovirus is mainly caused by symptomatic cases. *Clin. Infect. Dis*, **54**, 931-937 (2012).
9. Gong Y.W., Oh B.Y., Kim H.Y., Lee M.Y., Kim Y.H., Go J.M., Lee J.M., Jeong H.S., Cheon D.S.: Molecular epidemiologic investigation of Norovirus infections in Incheon City, Korea, from 2005 to 2007. *J. Bacteriol. Virol*, **38**, 249-257 (2008).
10. Koh S.J., Cho H.G., Kim B.H., Choi B.Y.: An outbreak of gastroenteritis caused by norovirus-contaminated groundwater at a waterpark in Korea. *J. Korean Med. Sci*, **26**, 28-32 (2011).
11. Lowther, J.A., Gustar, N.E., Powell, A.L., Hartnell, R.E., Lees, D.N.: Two-year systematic study to assess norovirus contamination in oysters from commercial harvesting areas in the United Kingdom. *Appl. Environ. Microb*, **78**, 5812-5817 (2012).
12. Yu J.H., Kim N.Y., Lee E.J., Jeon I.S.: Norovirus infections in asymptomatic food handlers in elementary schools without norovirus outbreaks in some regions of Incheon, Korea. *J. Korean Med. Sci*, **26**, 734-739 (2011).
13. Ayukekbong, J.A., Andersson, M.E., Vansarla, G., Tah, F., Nkuo-Akenji, T., Lindh, M., Bergström, T.: Monitoring of seasonality of norovirus and other enteric viruses in Cameroon by real-time PCR: an exploratory study. *Epidemiol. Infect*, **142**, 1393-402 (2014).
14. Park D.J., Kim J.S., Park J.Y., Kim H.S., Song W., Hur M., Lee K.M.: Epidemiological analysis of norovirus infection between March 2007 and February 2010. *Korean J. Lab. Med*, **30**, 647-653 (2010).
15. Barrabeig, I., Rovira, A., Buesa, J., Bartolomé, R., Pintó, R., Prellezo, H., Domínguez, A.: Foodborne norovirus outbreak: the role of an asymptomatic food handler. *BMC Infect. Dis*, **10**, 269 (2010).
16. Gallimore, C.I., Cubitt, D., du Plessis, N., Gray, J.J.: Asymptomatic and symptomatic excretion of noroviruses during a hospital outbreak of gastroenteritis. *J. Clin. Microbiol*, **42**, 2271-2274 (2004).
17. Cheon D.S., Jeong H.S., Jeong A., Lee K.B., Lee M.H., Tahk H., Choi C.: Seasonal prevalence of asymptomatic norovirus infection in Korean children. *Foodborne Pathog. Dis*, **7**, 1427-1430 (2010).
18. Shinkawa, N., Noda, M., Yoshizumi, S., Tokutake, Y., Shiraishi, T., Arita-Nishida, T., Nishio, O.: Molecular epidemiology of noroviruses detected in food handler-associated outbreaks of gastroenteritis in Japan. *Intervirology*, **51**, 422-426 (2008).
19. Barreira, D.M., Ferreira, M.S., Fumia, T.M., Checon, R., de Sadovsky, A.D.: Viral load and genotypes of noroviruses in symptomatic and asymptomatic children in Southeastern Brazil. *J. Clin. Virol*, **47**, 60-64 (2010).
20. Widdowson, M.A., Sulka, A., Bulens, S.N., Beard, R.S., Chaves, S.S., Hammond, R., Salehi, E.D.: Norovirus and foodborne disease, United States, 1991-2000. *Emerg. Infect. Dis*, **11**, 95-102 (2005).
21. Chen, M.Y., Chen, W.C., Chen, P.C., Hsu, S.W., Lo, Y.C.: An outbreak of norovirus gastroenteritis associated with asymptomatic food handlers in Kinmen, Taiwan. *BMC Public Health*, **16**, 372 (2016).
22. Yu J.H., Kim N.Y., Koh Y.J., Lee H.J.: Epidemiology of foodborne Norovirus outbreak in Incheon, Korea. *J. Korean Med. Sci*, **25**, 1128-1133 (2010).