Molecular diagnostic methods for detection and investigation of human Noroviruses- *Norwalk* virus from *Callista umbonella* (Bivalvia) in the Northern Persian Gulf (Iran)

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Received: August 2020 Accepted: December 2020

Abstract

Norwalk virus is the major cause of food borne gastroenteritis worldwide with the highest infections by GII genotype. The burden of *Norwalk* virus related disease is considerable, affecting all age groups around the world. *Norwalk* virus is extremely contagious pathogen which can simply transmitted by consuming contaminated food and lead to serious outbreak gastroenteritis disease. The aim of this study was to investigate the incidence of *Norwalk* virus contamination in Bivalvia in the Northern Persian Gulf (southwest of Iran). All samples which examined for *Norwalk* virus (norovirus) contaminations were negative as investigated and verified by reverse transcriptase- polymerase chain reaction (RT-PCR) tests. The results of PCR products on gel electrophoresis indicated that there were no norovirus contaminations in tested samples. Our investigation indicated that molluscs from southwest of Iran are not contaminated with norovirus, however, it would be wisely to eat molluscs with adequate cooked to avoid any contamination including norovirus.

Keywords: Norwalk Virus, Gastroenteritis outbreak, Seafood, Norovirus

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[DOI: 10.18331/SFS2021.7.2.15]

Introduction

The *Norwalk* virus, recently known as *norovirus*, is a small RNA virus and the most common cause of viral gastroenteritis. (Lopman *et al.*, 2016). Norovirus is one of the pathogenic agents that can be related to food borne disease (Iwamoto *et al.*, 2010). Norovirus is associated with approximately fifty percent of the gastroenteritis outbreaks because of its extremely transmittable nature which can result in serious illness such as diarrhea, nausea and vomiting (Sharma *et al.*, 2020). It has been reported that norovirus is the main cause of outbreaks worldwide with 18% of diarrheal illnesses along with 200,000 deceases per year (Lopman *et al*., 2016, Sharma *et al*., 2020). Apart from its periodical incidence, norovirus infectious is more widespread in colder months, autumn, and winter.

Norovirus is belong to a group of virus family named *Caliciviridae* (Katayama *et al.*, 2006). Based on molecular investigation, seven genogroups have been recognized which among them GI, GII and GIV are known to infect humans (Vinjé, 2015). One of which, GII genogroup has a predominant genotype namely GII,4 which has identified as an aetiology of global epidemics of gastroenteritis (Siebenga *et al.*, 2007, Siebenga *et al.*, 2009). It has been stated that norovirus related disease by GII,4 genotype has caused around 80% of all reported outbreak over the last two decades (Lu *et al.*, 2020).The genome of norovirus consists of three open reading frames (ORFs), of which ORF1 encodes a high polyprotein that is fragmented by the viral protease in mature non-structural proteins while ORF2 and ORF3 encode the major and minor capsid protein respectively (Thorne and Goodfellow, 2014; Mattison *et al.*, 2018). Norovirus may be recognised by reverse transcriptase PCR (RT-PCR) or electron microscopy of contaminated samples or by enzyme immunoassay for the discovery of viral antigen in serum samples.

Previous investigations have reported that gastroenteritis outbreak mostly occurred in crowded communities such as health care facilities, schools, hotels and restaurants (Girish *et al.*, 2002; Meakins *et al.*, 2003; Lu *et al.*, 2020). Numerous studies have demonstrated that norovirus can be transmitted via different routes such as person to person, through the fecal-oral path, or by digestion of contaminated water and food (Lopman *et al.*, 2006; Hewitt *et al.*, 2007; Wolf *et al.*, 2009). As a result, consumption of non-hygiene food and drink which are prepared by infected persons can cause gastroenteritis disease. Reported norovirus associated outbreaks are mostly due to contaminated vegetables, fruits, cereals and raw food (Sharma *et al*., 2020). It is worthy to mention that besides humans, norovirus can contaminate a wide range of hosts including aquatic animals such as mollusc (Le Guyader *et al*., 2009; Iwamoto *et al*., 2010).

Callista umbonella (Lamarck, 1818) classified to the Veneridae or venerids,

[DOI: 10.18331/SFS2021.7.2.15]

common name, venus clams, are a very large family of minute to large, saltwater clams, marine bivalve molluscs (Carpenter, 1997; Jousseaume, 1888). Nevertheless, along with the advantages obtained from seafood consumption, there is a possible risk for transmission of norovirus-contaminated seafood to humans (Iwamoto *et al*., 2010). Norovirus is extremely contagious pathogens which can simply transmitted by consuming contaminated food and lead to serious outbreak gastroenteritis disease. Due to its minuscule infectious dose and its high outbreak rates, norovirus can persevere in the environment and contaminate huge numbers of people (Iwamoto *et al*., 2010). Therefore, this study aimed to investigate the contamination of molluscs to norovirus in Ahvaz. Ahvaz is a capital city of Khuzestan province and is in the southwest of Iran nearby Persian Gulf. Bivalve mollusc is one of the most favoured food in Khuzestan accompanying with other Iranian cuisine.

Materials and methods

Specimens collection and processing

A total of 110 molluscs, were collected from main fish market in Ahvaz and Mahshahr during the colder months of autumn to winter 2019. After collecting samples, placed them on sterile containers and transferred to the workplace while kept them on ice box. In laboratory, all samples were washed by deionised water, then dissected by sterile scalpel and their digestive tissues separated for further usage. Later, digestive tissues from all molluscs were separately homogenized, then PBS buffer with an equal volume of each sample added and centrifuged for 10 min at 5,000 rpm at 4°C and the supernatant were used as a possible source of norovirus. Figure 1 shows the processing of molluscs in the laboratory.

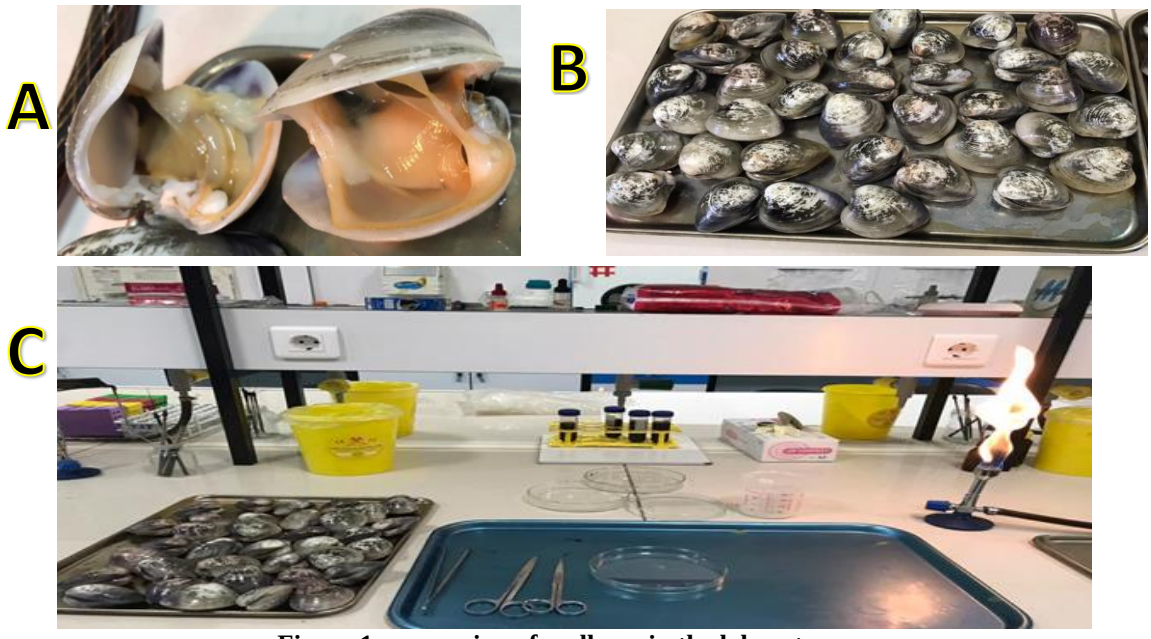

Figure 1: processing of molluscs in the laboratory.

[DOI: 10.18331/SFS2021.7.2.15]

Nucleic acid extraction and purification

The possible RNAs of norovirus were extracted and purified using RNA extraction Mini Kit (Dyna Bio viral, Cat #KI0025, Takapouzist, Iran) according to the manufacture's guidelines. The purified RNA then dissolved in RNAase free water and kept at - 80ºC till further use.

Primers

Two oligonucleotides, both for GII genogroup of norovirus, were purchased from the Takapouzist Company, Iran, and used for amplification in RT-PCR assays as follow:

P290 (5`-GATTACTCCAAGTGGGACTCCAC-3') and

P289 (5`-TGACAATGTAATCATCACCATA-3')

Reverse Transcription- Polymerase Chain Reaction (RT-PCR)

For reverse transcription (RT) reaction, in a total volume of 10 µL, added the subsequent components into an Eppendorf tube. A volume of 2 µL 10 x PCR buffer, 0.5 µL random hexamer primers, 1 µL dNTPs, 0.5 µL Super Script II reverse transcriptase, 3 µL extracted RNA, 0.25 µL RNase inhibitor, and 2.25 µL water. Then all samples were incubated at 42ºC for 1 hour and after that the cDNA denatured at 95ºC and kept it on ice.

For PCR reaction, firstly prepared and mixed the following components in a mini centrifuge PCR tube for each sample. A volume of 2.5 µL 10 x PCR buffer, 1 µL primer 1 (P289), 1 µL primer 2 (P290), 0.2 µL Taq DNA polymerase, 2 µL cDNA product, 17.8 µL water. Secondly, performed PCR starting at 94ºC for 3 minutes, 40 cycles of denaturation for 30 seconds at 94ºC, then annealing for 80 seconds at 53ºC followed by 60 seconds at 72ºC and 10 minutes at 72ºC for final extension. All materials were from Gen Fanavaran company, Iran. The PCR products were then demonstrated by 1% agarose gel electrophoresis and visualised by Gel Documentation (Vilber Lourma).

We used confirmed norovirus that were prepared from Razi vaccine and serum Institute as positive control and DW as negative control in all test stage of study, including extraction of genomes, cDNA synthesis and PCR.

Statistical analysis

A Chi-square test and fisher's exact test with two-tailed analysis were done using SPSS version 16.0 statistical software. All differences were considered significant when the values were $p<0.05$.

Results

After biometry (Ave. L. 5.8 cm; weight 64.5 g), all mollusc samples which examined for norovirus contaminations were negative as investigated and verified by RT-PCR tests. The results of PCR products on gel electrophoresis indicated that there were no norovirus contaminations in our samples. The

[DOI: 10.18331/SFS2021.7.2.15]

Figures 2 and 3 shows DNA bands of all samples together with DNA ladder which 319 bp band determined the presence of norovirus.

Table 1: Shows the results of PCR products on gel electrophoresis and their corresponding figures.

Samples	Presence of Norovirus	Figure
S1 , S2, S3, S4	-------	2A
C1, C2, C3, C4, C5, C6. C7	Only controls 4 and 6 (C4, C6)	2B
S5 , S6, S7, S8, S9, S10, S11, S12, S13, S14	-------	3A
S15 , S16, S17, S18, S19, S20, S21, S22,	-------	3B

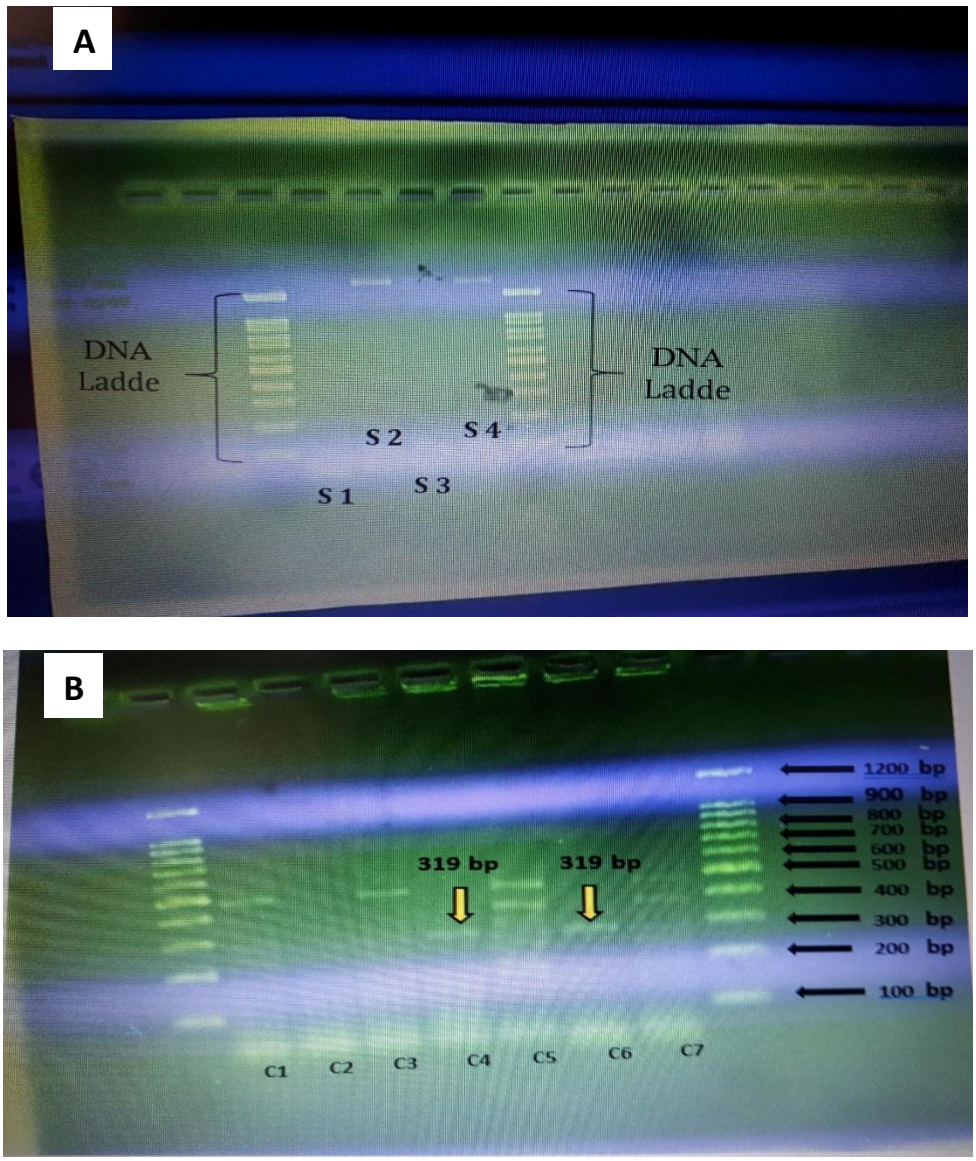

Figure 2: (A-B) indicate no DNA bands of four samples S1, S2, S3, S4 (Fig 2A) while DNA ladder with 319 bp band determined the presence of norovirus in controls C4 and C6 (Fig 2B).

[DOI: 10.18331/SFS2021.7.2.15]

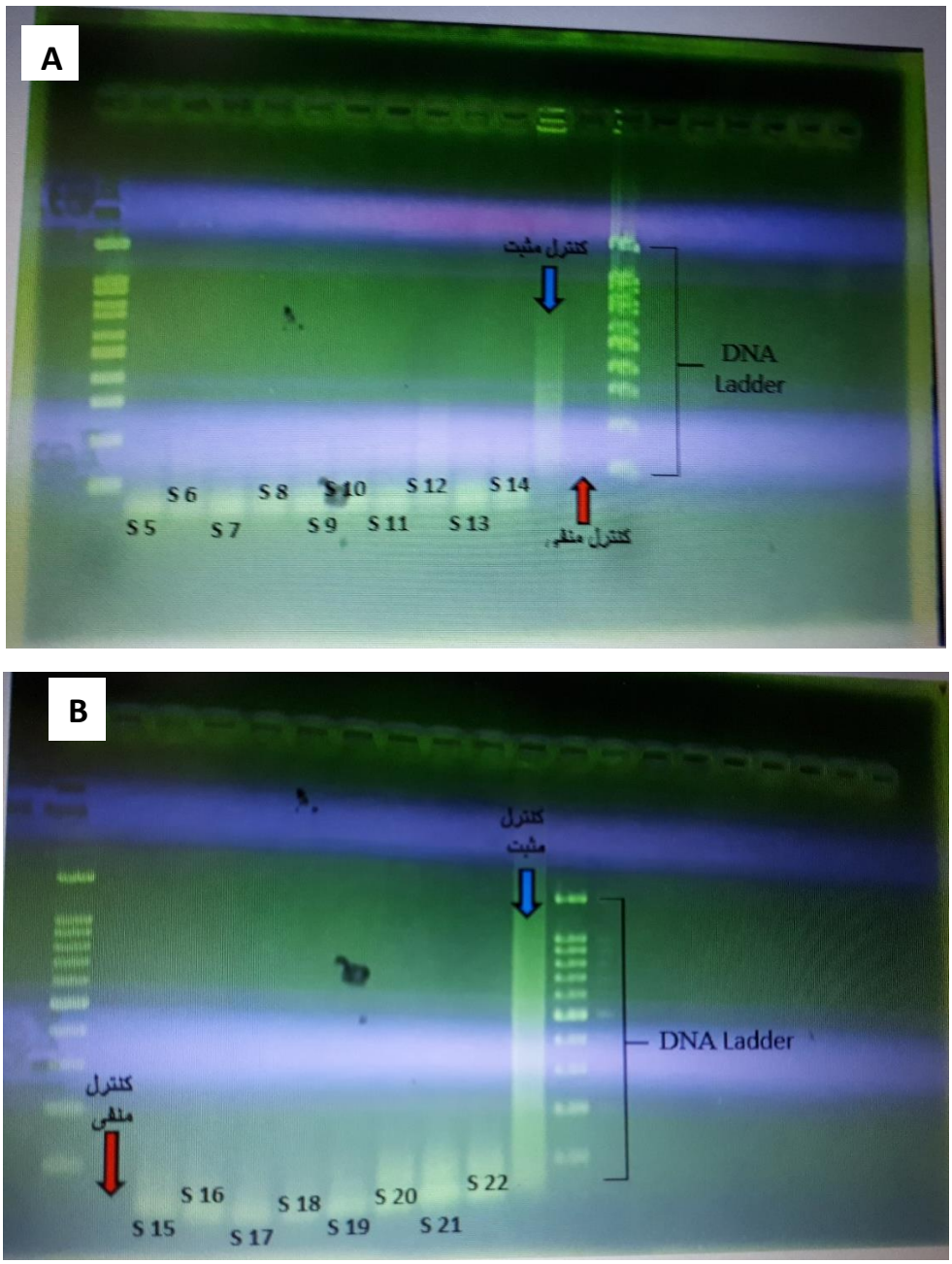

Figure 3 (A-B): indicate no DNA bands of ten samples, S5, S6, S7, S8, S9, S10, S11, S12, S13, S14 (Fig 3A), and for eight samples, S15, S16, S17, S18, S19, S20, S21, S22, (Fig 3B).

Discussion

Norovirus was reported as the third dominant cause of seafood outbreaks and the most frequent viral pathogen with two-third of all outbreaks viral disease associated with seafood (Iwamoto *et al.*, 2010). Up to date, many investigations have been done regarding norovirus transmission across person to person, through the fecal-oral route as well as contact with infected surfaces (Kapikian *et al.*, 1972; Meakins *et al*., 2003; Lu *et al*., 2020). However, little is known about transmission of norovirus contamination in seafood, particularly bivalve mollusc, to human. For instance, Mcleod *et al*, found that

[DOI: 10.18331/SFS2021.7.2.15]

molluscs contaminated with norovirus could be a high threat for human health even following cleaning in hygienic water (McLeod *et al.*, 2009). Another study, was reported the association between molluscs with norovirus contamination and possible hazard indicators, such as environmental temperatures and collecting region classifications to human health risk (Lowther *et al.*, 2012). It has been documented that molluscs with insufficient cooked could not inactivated norovirus and resulted human`s health in high risk (Alfano-Sobsey *et al.*, 2012). Results from two outbreaks investigations, using radioimmunoassay were identified norovirus as a main cause of gastroenteritis which related to consuming raw or inadequate cooked molluscs (Morse *et al.*, 1986).

Seafood is an important cause of outbreak food borne disease around the world which mostly attributed to consumption of raw bivalve molluscs. Among aquatic food, molluscs are in higher risk to be contaminated with various pollution in their surrounding environment compare to other seafoods, because they have inherently a filter feeding motion which lead to accumulation of pathogens such as norovirus (Doré *et al.*, 2010). Therefore, eating molluscs with improper cook may result in infection by this virus that can remain in the guts of poisoned living bivalve molluscs for long time (Le Guyader *et al.*, 2006). Because of this, we particularly target digestive tissue for investigation about norovirus.

In this study, samples were collected during cold months, as outbreaks of norovirus are more common, and performed RT- PCR as the most reliable method for norovirus detection. In addition, digestive tissues of bivalve molluscs were separated and used as the most sources of norovirus. Our investigation indicated that molluscs from southwest of Iran are not contaminated with norovirus, however, it would be wisely to eat molluscs with adequate cooked to avoid any contamination including norovirus.

The best management to avoid norovirus contamination is to consider all associated aspects, including serve qualified people who are involved in preparing, cooking and handling of food on strict hand / environment sanitation, as well as hold updated on hygienic codes. Moreover, avoid consuming raw or inadequate cooked seafood, particularly molluscs, in case of some limitations to perform assays for norovirus detection.

Conflict of Interest: None.

References

Alfano-Sobsey, E., Sweat, D., Hall, A., Breedlove, F., Rodriguez, R., Greene, S., Pierce, A., Sobsey, M., Davies, M. and Ledford, S., 2012. Norovirus outbreak associated with undercooked oysters and secondary household transmission. *Epidemiology & Infection,* 140**,** 276-282.

[DOI: 10.18331/SFS2021.7.2.15]

Carpenter, K.E., 1997. *Living marine resources of Kuwait, Eastern Saudi Arabia, Bahrain, Qatar, and the United Arab Emirates*, Food & Agriculture Org.

Doré, B., Keaveney, S., Flannery, J. and Rajko-Nenow, P. 2010. Management of health risks associated with oysters harvested from a norovirus contaminated area, Ireland, February–March 2010. *Eurosurveillance*, 15, 19567.

Girish, R., Broor, S., Dar, L. and Ghosh, D., 2002. Foodborne outbreak caused by a Norwalk-like virus in India. *Journal of Medical Virology*, 67**,** 603-607.

Hewitt, J., Bell, D., Simongs, G.C., Rrivera-Aban, M., Wolf, S. and Greening, G.E., 2007. Investigation of a waterborne norovirus outbreak in a New Zealand ski resort. *Applied and Environmental Microbiology*.

Iwamoto, M., Ayers, T., Mahon, B.E. and Swerdlow, D.L., 2010. Epidemiology of Seafood-Associated Infections in the United States. *Clinical Microbiology Reviews*, 23**,** 399-411.

Jousseaume, F., 1888. *Description des mollusques recueillis par M. le Dr. Faurot dans la Mer Rouge et le Golfe d'Aden*, Société zoologique de France.

Kapikian, A.Z., Wyatt, R.G., Dolin, R., Thornhill, T. S., Kalica, A.R. and Chanock, R.M., 1972. Visualization by immune electron microscopy of a 27-nm particle associated with acute infectious nonbacterial gastroenteritis. *Journal of Virology*, 10**,** 1075-1081.

Katayama, K., Hansman, G., Oka, T., Ogawa, S. and Takeda, N., 2006. Investigation of norovirus replication in a human cell line. *Archives of Virology*, 151, 1291-1308.

Le Guyder, F.S., Loisy, F., Atmar, R.L., Hutson, A.M., Eetes, M.K., Ruvoën-Clouet, N., Pommepuy, M. and Le Pendu, J., 2006. Norwalk virus–specific binding to oyster digestive tissues. *Emerging Infectious Diseases*, 12, 931.

Le Guyader, F.S., Parnaudeau, S., Schaeffer, J., Bosch, A., Loisy, F., Pommepuy, M. and Atmar, L., 2009. Detection and quantification of noroviruses in shellfish. *Applied and Environmental Microbiology*, 75, 618-624.

Lopman, B.A., Gallimore, C., Gray, J.J., Vipond, I.B., Andrews, N., Sarangi, J., Reacher, M.H. and Brown, D.W., 2006. Linking healthcare associated norovirus outbreaks: a molecular epidemiologic method for investigating transmission. *BMC Infectious Diseases*, 6, 108.

Lowther, J.A., Gustar, N.E., Powell, A.L., Hartnell, R.E. and Lees, D.N., 2012. Two-year systematic study to assess norovirus contamination in oysters from commercial harvesting areas in the United Kingdom. *Applied and Environmental Microbiology*, 78, 5812-5817.

[DOI: 10.18331/SFS2021.7.2.15]

Lopman, B.A., Steele, D., Kirkwood, C.D. and Parashar, U.D., 2016. The vast and varied global burden of norovirus: prospects for prevention and control. *PLoS Medicine*, 13.

Lu, Y., Ma, M., Wang, H., Wang, D., Chen, C., Jing, Q., Geng, J., Li, T., Zhang, Z. and Yang, Z., 2020. An outbreak of norovirus-related acute gastroenteritis associated with delivery food in Guangzhou, southern China. *BMC Public Health*, 20, 1-7.

Mattison, C.P., Cardemil, C.V. and Hall, A.J., 2018. Progress on norovirus vaccine research: public health considerations and future directions. *Expert Review of Vaccines*, 17, 773-784.

Mcleod, C., Hay, B., Grant, C., Greening, G. and Day, D., 2009. Localization of norovirus and poliovirus in Pacific oysters. *Journal of Applied Microbiology*, 106, 1220-1230.

Meakins, S., Adak, G., Lopman, B. and O'brien, S., 2003. General outbreaks of infectious intestinal disease (IID) in hospitals, England and Wales, 1992–2000. *Journal of Hospital Infection*, 53, 1-5.

Morse, D.L., Guzewich, J.J., Hanrahan, J.P., Stricof, R., Shayegani, M., Deibel, R., Grabau, J.C., Nowak, N.A., Herrmann, J.E. and Cukor, G., 1986. Widespread outbreaks of clam-and oyster-associated gastroenteritis. *New England Journal of Medicine*, 314, 678-681.

Sharma, S., Hagbom, M., Carisson, B., Öhd, J. N., Inslander, M., Eeriksson, R., Simonsson, M., Widerström, M. and Nordgren, J., 2020. Secretor status is associated with susceptibility to disease in a large GII. 6 norovirus foodborne outbreak. *Food and Environmental Virology*, 12, 28-34.

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. *Journal of Virology*, 81, 9932-9941.

Siebenga, J.J., Vennema, H., Zheng, D.P., Vinjé, J., Lee, B.E., Pang, X.L., Ho, E.C., Lim, W., Choudekar, A. and Broor, S., 2009. Norovirus illness is a global problem: emergence and spread of norovirus GII. 4 variants, 2001–2007. *The Journal of Infectious Diseases*, 200, 802-812.

Thorne, L.G. and Goodfellow, I.G., 2014. Norovirus gene expression and replication. *Journal of General Virology*, 95, 278-291.

Vinjé, J., 2015. Advances in laboratory methods for detection and typing of norovirus. *Journal of Clinical Microbiology*, 53, 373-381.

Wolf, S., Williamson, W., Hewitt, J., Lin, S., Rivera-Aban, M., Ball, A., Scholes, P., Savill, M. and Greening, G.E., 2009. Molecular detection of norovirus in sheep and pigs in New Zealand farms. *Veterinary Microbiology*, 133, 184-189.

[DOI: 10.18331/SFS2021.7.2.15]