International Food Research Journal 18: 677-681 (2011)

Occurrence of Norovirus GI in green and red onion

[1*]Noor Hidayah, M.S., [1,2]Tuan Zainazor, C., [1]Pui, C. F., [1]Noorlis, A., [2]Noor Eliza, M.R., [2]Naziehah, M.D., [1]Ghazali, F.M., [3]Cheah, Y. K., [4]Nakaguchi, Y., [4]Nishibuchi, M. and [1]Son, R.

[1]Center of Excellence for Food Safety Research, Department of Food Science, Faculty of Food Science and Technology, University Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia
[2]National Public Health Laboratory, Ministry of Health Malaysia, Lot 1853, Kg. Melayu Sungai Buloh, 47000 Sungai Buloh, Selangor, Malaysia.
[3]Department of Biomedical Science, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia
[4]Centre for Southeast Asian Studies, Kyoto University, Kyoto 606-8501, Japan

Abstract: Several Norovirus cases due to consumption of green onions have been reported during recent years but reports on red onions are not found. Onions are one of the major tastes in Malaysian food which are sometimes consuming raw especially the green onion. Viral contamination in onions can occur due to planting condition and not properly prepared food. This situation can pose the human health risk. A method was developed to detect the Norovirus that might present on different type of onions. In this study, 60 samples were collected from local market. Elution by Tryptose Phosphate Glycine broth and concentration steps using negatively charge filter were applied to enhance the detection of virus in food due to low copies of virus on food surface. The viral RNA was extracted using Qiagen Rneasy Mini kit before further detection using One-step RT-PCR. The total incidence of Norovirus in green onion and red onion was 13.33% (4/30) and 3.33 % (1/30) respectively. This is the first report of the detection of Norovirus in red and green onions in Malaysia. Based on the results, it is concluded that this method is reliable to detect Norovirus on onions and vegetables surface and hence can be applied in the laboratories for routine or food borne outbreak investigation.

Keywords: Norovirus, green onion, red onion, negative charge filter, one-step RT-PCR

Introduction

Previous studies indicate that enteric viruses, especially Norovirus are the major etiology of food borne illness in the world (de Wit *et al*., 2007). Comparing with other enteric virus which can cause gastroenteritis such as Hepatitis A virus, Rotavirus, Astrovirus and Adenoviruses, Norovirus is by far the most causative agent (Mead *et al*., 1999). Norovirus is a member of a *Caliciviridae* family and are diverse genetically (Boga *et al*., 2004). It consists of five genogroups (G1, GII, GIII, GIV and GV) (D'Souza and Jaykus, 2006). GI and GII is the most common genogroup that infect human (Ho *et al*., 2007). Human Norovirus is transmitted via person-to-person contact or through fecal oral route (Hewitt *et al*., 2007). Outbreak related with Norovirus normally occurred in public settings, including restaurants, hospitals, schools, catered events and cruise ships (Kim *et al*., 2008). Small dose of virus can trigger the infection (Bucardo *et al*., 2008). Consumption of raw or improperly handled food can become a major risk factor for food borne outbreak.

Vegetables including green onion have also been associated with enteric virus contamination and gastroenteritis. Contamination of food and vegetables could be from the soil, water irrigation and infected food handlers who harvest and handle the fresh produce (Papafragkou *et al*., 2006). There was also a probability for green onion to become contaminated with norovirus (Dentinger *et al*., 2001). Three deaths were reported among 601 cases related with hepatitis A virus due to the ingestion of contaminated green onion (Butot *et al*., 2007).

The information about Norovirus contamination of food is limited due to lack of appropriate detection methods and inability to culture the virus (Butot *et al*., 2007). The presence of Norovirus particles on food surface is low (Park *et al*., 2008). Methods develop must capable to concentrate and detect the virus. Adsorption-elution using micropore filter seem to be the most accurate methods to concentrate viruses, especially waterborne (Villar *et al*., 2006). PCR method is one of the most sensitive methods to detect viral in the environmental samples (Villar *et al*., 2006). Rapid and sensitive characteristics are the key advantages of Reverse transcription-PCR (RT-PCR) to become as an ideal tool to investigate an outbreak of suspected viral origin (Parshionikar *et al*., 2003)

*Corresponding author.
Email: *noorhidayah_ms@yahoo.com*
Tel: +6012-3100304

In this study, three steps were used to achieve the reliable results. The steps included eluting the Noroviruses from food surface, using Tryptose Phosphate Broth-Glycine, concentration of the virus using negatively charged filter (HA, Milipore), and extraction of the viral RNA using Plant RNeasy Mini Kit (Qiagen, Gmbh) for further detection by RT-PCR. The aim of this study is to detect the presence of Norovirus in the red and green onions that becomes the major taste of Malaysian foods.

Materials and Methods

Norovirus positive control

Three throat swab samples were collected from patients infected with enteric virus. Throat swab samples were diluted in phosphate-buffered saline (PBS) to obtained 10% throat swab suspension. The mixture was vortex to separate the solids material with supernatant. The supernatant was further analyzed by using RT-PCR with two pairs of degenerate primers for Norovirus. The results proved that the samples were positive with Norovirus. The positive samples were then stored at -80ºC until further use as a template for positive control.

Red and green onion samples

Onion samples were purchased at local market around Selangor, Malaysia. A total of 30 samples of Red onions and 30 samples of Green onion were used in this study. All samples were stored at 4ºC until testing. The method described by Simard *et al*., 2007 was used to concentrate and extract viruses with minor modification.

Concentration methods

Each sample was weighed approximately 25 g into the sterile filtered stomacher bag. $MgCl_2$ with 25 µM final concentration was added. Viruses were released from the food surface by gentle shaking with Tryptose Phosphate Broth Glycine buffer pH 9.0 (2.9% Tryptose Phosphate Broth 6% Glycine) at 90 rpm for 15 minutes at room temperature. The elution buffer was then transferred into 50 ml centrifuge tube to separate the solid material with supernatant. An HA negatively charged membrane filter (Milipore) with a pore size 0.45 µm was placed into the vacuum pump. The supernatant was poured inside the glass funnel to absorb the virus on the membrane filter. The membrane filter was rinsed with H_2SO_4. Viruses on the membrane filter were released using elution buffer agitated at 60 rpm for 15 minutes at room temperature. The elute was then adjusted with 1N HCl solution to pH 7.0±0.2. The adjusted elution buffer was centrifuged at 4000 rpm for one hour. The supernatant was then transferred into microfuge tube and SDS 10% was added to achieve a final concentration of 1% SDS. 20 mg/ml Proteinase K was added together to improve the detection before further incubation for one hour at 37ºC. The solution was then proceeding to extraction stage.

Viral RNA extraction

Viral RNA was extracted from 150 µl elute using the Qiagen RNeasy Plant Mini Kit (QIAGEN, Gmbh) according to the manufacturer's instructions with slightly modification. The samples were added with 450 µl of RLT-β-Mercaptoethanol and subjected to vortex for 15 min. Samples were heated for 2 min at 56ºC plus another additional incubation for 5 min at room temperature. The samples were added with 0.5 ml volumes of pure ethanol and loaded into the RNeasy spin column. Further washing with guanidine salts and ethanol will remove contaminants. Silica based spin column membrane will promote binding of viral RNA and inhibitors will be separated. Finally, the high quality RNA was eluted into 50 µl RNase free water. The RNA extract was stored at -80ºC until further analyzed by Reverse Transcription RT-PCR.

Detection of Norovirus by Reverse Transcription PCR (RT-PCR)

Reverse transcription was performed using two sets of degenerate primers (MON 431-MON 433 and MON 432-MON434) with a final concentration 0.6 µM for each primer. The RT-PCR mixture consisted of 5 µl of RNA and a final volume of 20 µl of a reaction mixture containing 10 µl of RNase free water, 5 µl of 5X Qiagen One-step RT-PCR buffer, 1 µl of dNTP, and 1 µl of Qiagen one-step RT-PCR enzyme (QIAGEN, Gmbh) These sets of primer generating PCR products of 213 bp. The viral was reverse transcribed for 30 min at 50ºC then incubated for 15 min at 95ºC. The PCR condition consisted of 40 cycles of 45 sec at 94ºC, 30 sec at 52ºC and 45 sec at 72ºC. Final elongation at 72ºC for 10 min. Positive control and negative control were included in each RT-PCR run. The results were analyzed using ethidium bromide on 2% agarose gel.

Results

Norovirus was detected by targeting the ORF1 gene using two sets of primer (Table 1). These primers showed the same amplicon size which is 213 bp (Trujillo *et al*., 2006). PCR analysis included both the positive and negative control (Figure 1). From the

samples analyzed, green onion showed the highest prevalence rate about 13.33%. From the thirty samples of red onion that were analyzed, only one showed a positive result for Norovirus contamination that contribute to 3.33% prevalence rate (Table 2).

The contamination of Norovirus GII was not detected. No PCR band appeared during the visualization of the gel. The positive control used was confirmed with PCR analysis and sequencing. Due to similar amplicon size showed by both genogroups, comparison of the sequence by using BLAST program is important to differentiate both genogroups.

Table 1. Primer used for conventional RT-PCR for Norovirus detection

Primer	Sequence[a]	Location[b]
MON 431	5' TGG ACI AGR GGI CCY AAY CA 3'	5093-5305
MON 432	5' TGG ACI CGY GGI CCY AAY CA 3'	5093-5305
MON 433	5' GAA YCT CAT CCA YCT GAA CAT 3'	5093-5305
MON 434	5' GAA SCG CAT CCA RCG GAA CAT 3'	5093-5305

[a] R=A or G, Y=C or T.
[b] Nucleotide positions for conventional RT-PCR based on Norwalk virus (Genebank accession no. M87661) and Hawaii virus (Genebank accession no. U07611).

Table 2. Detection of Norovirus genogroup I and II in two types of onion

Common name	Scientific name[a]	No. of samples	No. of positive samples		Total prevalence (%)
			Genogroup I	Genogroup II	
Red onion (bulb)	*Allium atrorubens S. Wats*	30	1	NA	3.33
Green onion (leaves)	*Allium wakegi*	30	4	NA	13.33

[a] Scientific name based on Integrated Taxonomic Information System

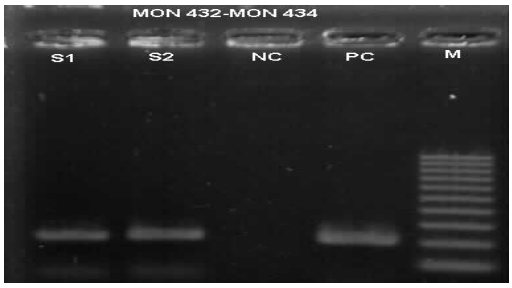

Figure 1. Results shows a band at 213bp by using primer MON 432-MON434 which represents Norovirus GI

Discussions

Norovirus infection in human has been emerged in developed and developing countries (Kroneman *et al*., 2008). The incidence of Norovirus contamination in food is quite low and limited. Several cases related with contamination of Norovirus in green onion had been reported in Ohio and Pennsylvania based on the clinical sample analysis (Lashley *et al*., 2007). Norovirus is an important aetiology for foodborne virus; therefore detection of this organism in food is essential.

In this study, a total of 60 samples of red and green onions were analyzed. Results showed that green onion was highly contaminated with Norovirus GI. The possible reason for the higher incidence rate of Norovirus in green onion can be attribute to the food surface condition. Compared with red onion, green onion surface is moist and easy for the fecal matter or organic matter such as soil to hold firmly on the onion surface.

The growth conditions of onion also add to contamination factors. Onion is a cold season plant. It is grow in the soil with the entire bulb implanted. Therefore, the probability for Norovirus to attach on its surface is high. Harvesting and processing storage also contribute to contamination due to infected handlers (Ward *et al*., 1982). One of the transmission routes is spreading from person to food (Hewitt *et al*., 2007)

Extraction of Norovirus from food is quite tedious. Availability of this virus on food surface is low (D'Souza and Jaykus, 2006). This is because Norovirus cannot replicate without a host but highly stable on food surface (Lamhoujeb *et al*., 2008). Elution and concentration are important steps in extracting Norovirus in food due to low copies number. So, elution by Tryptose Phosphate broth-glycine was used in this study to detach the virus from food surface.

The recovery efficiency of an electronegative filter for Norovirus was evaluated previously in sewage treatment plant, mineral water, tap water, river and seawater (Rigotto *et al*., 2009). Norovirus is a positive sense RNA (Boga *et al*., 2004). Therefore, concentration of Norovirus using negatively charged filter is reliable method used to capture virus. Onion is normally covering with soil especially green onion. In order for the virus on the sample surface successfully filtered, the soil must be separate to avoid the filter clog. Therefore, centrifugation of the sample is important before any elution-concentration steps take place. To enhance the absorption of virus to the filter, $MgCl_2$ was used. The highest recovery can be achieved with 25 mM $MgCl_2$ (Rigotto *et al*., 2009). The $MgCl_2$ was then released from the filter using H_2SO_4 and only Norovirus remain on the filter. Through this process, highest concentration of Norovirus from food surface can be achieved. Extraction of Norovirus was using Qiagen Rneasy Plant Mini Kit. This kit applies silica membrane method and was reported as the most reliable and straightforward method for purifying viral nucleic acids in water samples (Gentry *et al*., 2009).

Conventional RT-PCR is one of the reliable methods to detect the presence of Norovirus on food surface. This method is able to detect the low copies

number of RNA because of nucleotide amplication technique in RT-PCR through several cycles. In this study, partial Norovirus sequence for region B was amplified by using primers MON 432 and MON 434 for genogroup 1 and MON 431 and MON 433 for genogroup II. These primers amplify a small region within the 3' end of the ORF1 portion of the genome (Trujillo *et al*., 2006).

To our knowledge, this is the first reported methodology for the detection of Norovirus in different types of onions. This method has been proven to be a reliable technique to detect Norovirus in a broad range of raw vegetables. It can be applied in the laboratories and very useful for routine surveillance and during food borne outbreaks.

References

Boga, J. A., Melo´n, S., Nicieza, I., de Diego, I., Villar, M., Parra, F. and de On˜a, María. 2004. Etiology of sporadic cases of pediatric acute gastroenteritis in Asturias, Spain, and genotyping and characterization of Norovirus strains involved. Journal of Clinical Microbiology 42 (6): 2668–2674.

Bucardo, F., Nordgren, J., Carlsson, B., Paniagua, M., Lindgren, P., Espinoza, F. and Svensson, L. 2008. Pediatric Norovirus Diarrhea in Nicaragua. Journal of Clinical Microbiology 46(8): 2571-2580.

Butot, S., Putallaz, T. and Sanchez, G. 2007. Procedure for rapid concentration and detection of enteric viruses from berries and vegetables. Applied and Environmental Microbiology 73(1): 186-192.

D'Souza, D. H. and Jaykus, L. A. 2006. Molecular approaches for the detection of foodborne viral pathogens. In Maurer. J. (Ed). PCR Methods in Foods, p.91-117. United States: Springer US.

de Wit, M.A.S., Widdowson, M.A., Vennema , H., de Bruin, E., Fernandes, T. and Koopmans, M. 2007. Large outbreak of norovirus: The baker who should have known better. Journal of Infection 55: 188-193.

Gentry, J., Vinjé, J. and Lipp E. K. 2009. A rapid and efficient method for quantitation of genogroups I and II norovirus from oysters and application in other complex environmental samples. Journal of Virological Methods 156: 59–65.

Hewitt, J., Bell, D., Simmons, G.C., Rivera-Aban, M., Wolf, S. and Greening, G. E. 2007. Gastroenteritis outbreak caused by waterborne Norovirus at a New Zealand ski resort. Applied and Environmental Microbiology 73 (24): 7853–7857.

Ho, E. C. M., Cheng, P. K. C., Lau, A. W. L., Wong, A. H. and Lim, W. W. L. 2007. Atypical Norovirus epidemic in Hong Kong during summer of 2006 caused by a new genogroup II/4 variant. Journal of Clinical Microbiology 45 (7): 2205–2211.

Internet: Concentration of Hepatitis A virus and Rotavirus in spring or mineral bottled water samples and their detection by the reverse-transcriptase polymerase chain reaction 2007. Laboratory procedure. Downloaded from *http://www.hc-sc.gc.ca/fn-an/res-rech/anly-meth/microbiol/index_e.html.* on 5/12/2009.

Internet: Integrated Taxonomic Information System. Downloaded from *http://www,itis.gov/* on 22/09/2010.

Kim, H.Y. Kwak, I. S., Hwang, I. G. and Ko, G. P. 2008. Optimization of methods for detecting norovirus on various fruit. Journal of Virological Methods 153: 104–110.

Kroneman, A., Verhoef, L., Harris, J., Vennema, H., Duizer, E., van Duynhoven, Y., Gray, J., Iturriza, M., Bo¨ttiger, B., Falkenhorst, G., Johnsen, C., von Bonsdorff, C.H., Maunula, L., Kuusi, M., Pothier, P., Gallay, A., Schreier, E., Ho¨hne, M., Koch, J., Szu¨cs, G., Reuter, G., Krisztalovics, K., Lynch, M., McKeown, P., Foley, B., Coughlan, S., Ruggeri, F. M., Di Bartolo, I., Vainio, K., Isakbaeva, E., Poljsak-Prijatelj, M., Hocevar Grom, A., Zimsek Mijovski, J., Bosch, A., Buesa, J., Sanchez Fauquier, A., Hernande´z-Pezzi, G., Hedlund, K.O. and Koopmans, M. 2008. Analysis of integrated virological and epidemiological reports of Norovirus outbreaks collected within the foodborne viruses in Europe network from 1 July 2001 to 30 June 2006. Journal of Clinical Microbiology 46 (9): 2959-2965.

Lamhoujeb, S., Fliss, I., Ngazoa, S. E. and Jean, J. 2008. Evaluation of the persistence of infectious human Noroviruses on food surfaces by using real-time nucleic acid sequence-based amplification. Applied and Environmental Microbiology 74(11): 3349-3355.

Lashley, F. R. 2007. Categories and highlights of significant current emerging infectious diseases. In Lashley, F. R. and Durham, J. D. (Eds). Emerging Infectious Diseases. P. 49-83. New York: Springer Publishing Company.

Mead, P.S., Slutsker, L., Dietz, V., McCaig, L. F., Bresee, J. S., Shapiro, C., Griffin, P. M. and Tauxe, R. V. 1999. Food-related illness and death in the United States. Emerging Infectious Diseases 5 (5): 607-625.

Papafragkou, E., D'Souza, D.H. and Jaykus, L.A. 2006. Foodborne viruses: prevention and control. In Goyal, S. (Ed). Viruses in Food, p. 289-330. United States: Springer US.

Park, Y., Cho, Y. H., Jee, Y. M. and Ko, G. P. 2008. Immunomagnetic separation combined with real-time reverse transcriptase PCR assays for detection of Norovirus in contaminated food. Applied and Environmental Microbiology 74 (13): 4226-4230.

Parshionikar, S. U., Willian-True, S., Fout, G. S., Robbins, D. E., Seys, S. A., Cassady, J. D. and Harris, R. 2003. Waterborne outbreak of gastroenteritis associated with a Norovirus. Applied and Environmental Microbiology 69 (9): 5263-5268.

Rigotto, C., Kolesnikovas, C.K., Moresco, V., Simoes, C.M.O. and Barardi, C.R.M. 2009. Evaluation of HA negatively charged membranes in the recovery of human adenoviruses and hepatitis A virus in different water matrices. Memorias Inst Oswaldo Cruz 104 (7): 970-974.

Roberts, T.A., Cordier, J.L., Gram, L., Tompkin, R.B., Pitt, J.I., Gorris, L.G.M. and Swanson, K.M.J. 2005. Vegetables and vegetable products. In Roberts, T.A., Cordier, J.L., Gram, L., Tompkin, R.B., Pitt, J.I., Gorris, L.G.M. and Swanson, K.M.J. (Eds). Microorganism in Foods 6, p. 277-325. United States: Springer US.

Trujillo, A. A., McCaustland, K. A., Zheng, D. P., Hadley, L. A., Vaughn, G., Adams, S. M., Ando, T., Glass, R. I. and Monroe, S. S. 2006. Use of taqman real-time Reverse transcription-PCR for rapid detection, quantification, and typing of Norovirus. Journal of Clinical Microbiology 44 (4): 1405-1412.

Villar, L.M., de Paula, V.S., Diniz-Mendez, L., Lampe, E. and Gaspar, A.M.C. 2006. Evaluation of methods used to concentrate and detect hepatitis A virus in water samples. Journal of Virological Methods 137: 169-176.

Ward, B.K., Chenoweth, C.M. and Irving, L.G. 1982. Recovery of viruses from vegetable surfaces. Applied and Environmental Microbiology 44 (6): 1389-1394.