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Assessment of Noroviruses in selected *Ulam* from local market in Malaysia

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Abstract: Presence of Norovirus in food can cause viral gasteroenteritis. Recently, lots of reports relating to Norovirus in food have been published. Special attention must be paid to the raw foods as they are not subjected to further heat treatment. In this study, *pegaga, kesum*, *tauge* and *ulam raja* (popular salad vegetables in Malaysia) were investigated for Norovirus. A total of 32 samples from each type of salad vegetables were purchased from local market and analyzed using One-step RT-PCR (Reverse Transcriptase-Polymerase Chain Reaction**)** for both genogroups namely Norovirus Genogroup I and Genogroup II. Results showed that *tauge* had the highest contamination with Norovirus Genogroup I (15.6%) comparing to *pegaga* (9.4%), *kesum* (12.5%) and *ulam raja* (0%). Samples were free from Norovirus Genogroup II. The study showed that raw vegetables are high-risk foods and can be contaminated with Norovirus.

Keywords: Norovirus, *pegaga, kesum*, *tauge* and *ulam raja*, One-step RT-PCR

Introduction

Gastroenteritis is a major public health issue all over the world. Noroviruses are now considered as emerging pathogens and recognized as the leading cause of nonbacterial, acute gastroenteritis in humans. Norovirus was previously known as "Norwalk-like viruses" as the first outbreak occurred in an elementary school in Norwalk, Ohio, United States in 1968 (Hansman *et al*., 2004). Four years after the Norwalk outbreak, Norovirus coincidently identified by Kapikian in 1972. He analyzed the infected stools from the particular outbreak using Immune Electron Microscopy and realized that a 27 nm virus-like particle was similar to the viral particle from Norwalk gastroenteritis (Kapikian, 2000).

Based on the morphological identification by electron microscopy, Norwalk virus was initially explained as picornavirus or parvovirus. In 1980, this statement was changed as the reports on this virus were more consistent with the family of *Caliciviridae*. Using sequencing techniques, in 1990, this virus was found to be related to other "small round structured viruses" (SRSVs) and then grouped as *Caliciviridae* family (Lund and Lindqvist, 2004).

Noroviruses are classified in the family of *Caliciviridae* together with other 3 genera namely *Lagovirus*, *Vesivirus*, and *Sapovirus*. Sapoviruses and Noroviruses are normally related to human, but, Vesiviruses and Lagoviruses are principally of veterinary importance (Buchen-Osmond, 2003). Lagoviruses and Vesiviruses are grouped separately based on their morphology and genome sequence. Norovirus can be divided into at least 3 Genogroups (GI, GII and GIII) based on the genetic divergence of the RNA polymerase and in the capsid region. The GI and GII are important in human infections while GIII infects animals such as pigs and cows. Genogroup I comprise approximately seven clusters including the prototype Norwalk, Southampton and Desert Shield reference stains. GII comprise approximately ten genotypes including the Snow Mountain, Toronto, Bristol and Hawaii reference strains (Ando et al., 2000).

The consumption of contaminated food is the major cause of gastroenteritis outbreak reported all over the world. Large variety of bacteria, parasites and viruses are able to cause food related illnesses

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but in most of the cases, no causative agent can be identified. Recently, Norovirus has been listed as one of the food-borne pathogens in some countries. In Norovirus outbreaks, usually clinical specimen of patients (such as faeces, vomit, blood, serum, and throat swabs) are investigated. However, the investigation is not complete without considering the food eaten by the victims. Acute gastroenteritis outbreaks related to Noroviruses have been reported in cruise ships, nurseries, nursing homes, hospitals, hotels, military and holiday camps, and catering events (Cheesbrough *et al*., 2000; Anderson *et al*., 2001; Meakins *et al*., 2003; Fretz *et al*., 2005; Uchino *et al*., 2006; Verhoef *et al*., 2008; Braham *et al*., 2009). According to Vivancos *et al*. (2009), Norovirus outbreaks in Norfolk (East of England) are highly observed in the winter months and the majority of the cases occur in residential and nursing homes, and hospitals.

Even consumption of low numbers of Norovirus particles can cause disease. Approximately 10-100 viral particles are the capable dose to cause infection. According to Lindesmith *et al*. (2003), the infectious dose of Norovirus is estimated to be between 1 and 10 viral particles. Infected individuals can spread high concentration of Norovirus through their feces and vomit. The levels of Norovirus particles shed in feces were estimated to be about 10^6 -10^{11}/g and approximately 10^7 per vomiting incident (Lund *et al*., 2004). According to Marks *et al*. (2000), aerosolization of vomit can result in droplets which contaminate surfaces of dish or cutlery and foods. Due to the absence of a robust tissue culture system, no drug or vaccine exists for treating Norovirus infections (Duizer *et al*., 2004; Straub *et al*., 2007). Rapid and selective identification of microorganisms can be achieved using polymerase chain reaction (PCR) which amplifies a specific fragment of DNA through an enzyme-catalyzed reaction in a thermocycler. Repetition of this process produces a copy of DNA in exponential manner (Scheu *et al*., 1998).

Virus is consists of single stranded RNA. For detection of viruses, the viral particle can not be directly applied to the PCR system unless the RNA is converted to DNA. To do so, reverse transcription-plymerase chain reaction (RT-PCR) should be used to complimentary the RNA to form cDNA. The RT-PCR method is the best tool for detection of Norovirus due it rapidity and sensitivity. Combination of the method with sequencing method provide valuable information about Norovirus RNA that help to investigate suspected viral origin (Parshionikar *et al*., 2003) and relate the epidemiological studies to obtain more genetic information about Norovirus genotype (Rosa *et al*., 2008; Patel *et al*., 2008). According to Park *et al*., 2008 and Kroneman *et al*., 2006, RT-PCR is a reliable method that has been widely used for Norovirus detection and surveillance studies. The aim of this study was to detect Norovirus in raw vegetables (*ulam* such as *pegaga, kesum*, *tauge* and *ulam raja*) using RT-PCR method.

Materials and methods

Positive control

Positive control was derived from throat swab samples from infected patients with gastroenteritis. Phosphate-buffered saline (PBS) was used to dilute throat swab samples to get 10% throat swab suspension. The suspension was kept at -80ºC as a template for positive control.

Oligonucleotide primers

A pair of primers (forward and reverse) was chosen from conserved regions in detecting Norovirus genogroup I and genogroup II. These primers consists of MON 431 (5' TGG ACI AGR GGI CCY AAY CA 3') and MON 433 (5' GAA YCT CAT CCA YCT GAA CAT 3') for genogroup II; MON 432 (5' TGG ACI CGY GGI CCY AAY CA 3') and MON 434 (5' GAA SCG CAT CCA RCG GAA CAT 3') for genogroup I, where R=A or G and Y=C or T. The location of nucleotide position was from 5093-5305 for conventional RT-PCR based on Norwalk virus (GeneBank accession number M87661) and Hawaii virus (GeneBank accession number U07611) (Simard *et al*., 2007).

Sample collection

Pegaga, kesum, *tauge* and *ulam raja* were collected from local markets in Selangor, Malaysia (Table 1). A total of 32 samples for each type of salad vegetables were analyzed in this study. To maintain the integrity of the samples, all samples were put on ice and analyzed within 24 hours.

Sample treatment

To concentrate and extract viral RNA, the modified method by Simard et al., 2007 was used. Briefly, samples were chopped into 2-3 cm pieces and 25g each sample was added with Tryptose Phosphate Broth Glycine buffer pH 9.0 (TPBG) (2.9% Tryptose Phosphate Broth 6% Glycine) and $MgCl_2$ (25 μM) solution in a sterile stomacher bag with filter. This step eluted the viral RNA from the surface of sample through gentle shaking at 90 rpm

for 15 min. The washing solution was transferred into a 50 mL polypropylene centrifugation tube and centrifuged at 4000 rpm (4ºC) for 10 min to separate any solid particles that may clot the membrane filter. The supernatant was filtered through a HA negatively charged membrane filter (Milipore) (pore size 0.45 µM) and 8 mL of 0.5 mM H_2SO_4 solution was added. Viral RNA from membrane filter was eluted using 6 mL of TPBG buffer solution with vigorous agitation at 60 rpm for 15 min at room temperature. The elution buffer was transferred into 12 mL polypropylene centrifugation tubes, neutralized with 1N HCl (pH7.0±0.2) and centrifuged at 4000 rpm (4ºC) for 1 h. volume of 150 µL of supernatant was transferred to a 2 ml microfuge tube and added with 10% SDS solution and Proteinase K (20 mg/ml) and was incubated at 37ºC for 1 h extraction step.

Extraction of viral RNA

Qiagen RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) was used to extract Viral RNA. Briefly, 450 µL of RLT-β-Mercaptoethanol (1 mL buffer RLT + 10 µL β-Mercaptoethanol) was added into the supernatant from previous step and mixed. The mixed solution was then incubated at 56ºC for 2 min using heating block. After incubation, the mixture was kept at room temperature for 5 min and 0.5 mL pure ethanol was added. After filteration and washing steps, 50 µL RNase free water was added and the RNA solution was transferred to -80ºC freezer.

Detection of Norovirus

The RT-PCR was carried out in 0.5 mL microcentrifuge tubes, with 25.0 µL of reaction mixture consisting of 10.0 µL RNase free water, 5.0 µL 5x Qiagen One-step RT-PCR buffer, 1.0 µL deoxyribonucleotide phosphate (dNTP), 1.5 µL of two sets of degenerate primers (forward and reverse), 1.0 µL Qiagen one-step RT-PCR enzyme mix, and 5.0 µL template RNA. The amplification condition for RT-PCR was as follows; reverse transcribed for 30 min at 50ºC, followed by 40 cycles consisting of initial PCR activation at 95ºC for 15 min, denaturation at 94ºC for 45 sec, annealing at 52ºC for 30 sec, extension at 72ºC for 45 sec and final elongation at 72ºC for 10 min. Ten µL of the amplification product was subjected to electrophoresis using 2.0% agarose gel. Amplified DNA fragments of specific sizes stained with ethidium bromide were visualized under UV fluorescence and recorded using a gel documentation system with the targeted amplicon size of 213 base pairs.

Results and Discussions

Norovirus for both genogroups (genogroup I and genogroup II) was detected using RT-PCR. Twelve samples out of 128 raw vegetables samples (*pegaga, kesum*, *tauge* and *ulam raja*) from local market were positive for Norovirus (9.4%). The results showed that Norovirus genogroup I was detected the samples except *ulam raja* (Figure 1,2).

Tauge had the highest prevalence (15.6%) of Norovirus. Prevalence of Norovirus in *pegaga* and *kesum* were 9.4% and 12.5%, respectively. *Ulam raja* was free from Norovirus genogroup I. Samples did not show any contamination with Norovirus genogroup II.

Raw vegetables were classified as high-risk foods that can be contaminated with pathogenic bacteria such as Escherichia coli (Ingham *et al*., 2004), Salmonella (Elexson *et al*., 2011), Listeria (Jeyaletchumi *et al*., 2010), Campylobacter (Chai *et al*., 2009) and also Vibrio (Tunung *et al*., 2010). As shown in this study raw vegetables were contaminated with Norovirus. Most vegetables are not subjected to heat treatment before consumption and if not cleaned properly, it will exposed consumer to the risk of food poisoning. Unlike most bacteria, viruses can contaminate food but are not able to replicate in food and only replicate in living cells. Food is considered a good vehicle for virus transmission (Sair *et al*., 2002). When food is contaminated with Norovirus (such as raw vegetables in this study), the virus can survive even in the refrigeration temperatures (Bidawid *et al*., 2003). So, the main point is to prevent the preliminary stage of contamination and to control the cross contamination throughout the food chain. According to the guidelines from Michigan Department of Community Health (2009) and Occupational Safety and Health Administration, United States (2008), transmission of Norovirus can be limited if the correct and good practices applied in daily life as usual food preparation practices. There have been some difficulties in investigation of viral food-borne diseases especially in identifying the true viruses from food and clinical specimens (Bresee *et al*., 2002). This study showed that RT-PCR is a good method to detect Norovirus in food samples.

Table 1. Type of vegetables analyzed in this study.

Vegetable (local name)	Common name	Scientific name
Pegaga	Indian pennywort	*Centella asiatica*
Kesum	Vietnamese coriander	*Poligonum minus*
Tauge	Mung bean sprout	*Vigna radiate*
Ulam Raja	Wild cosmos	*Cosmos caudatus*

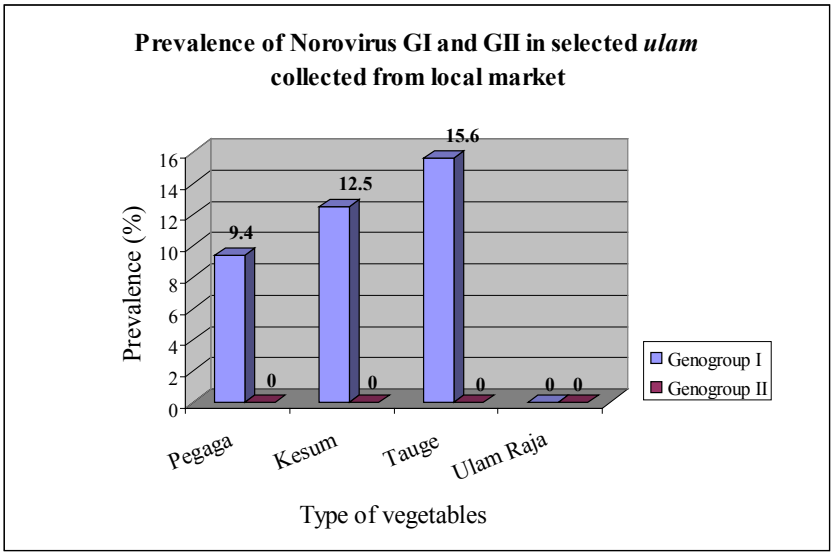

Figure 1. Prevalence of Norovirus GI and GII in selected *ulam* collected from local market.

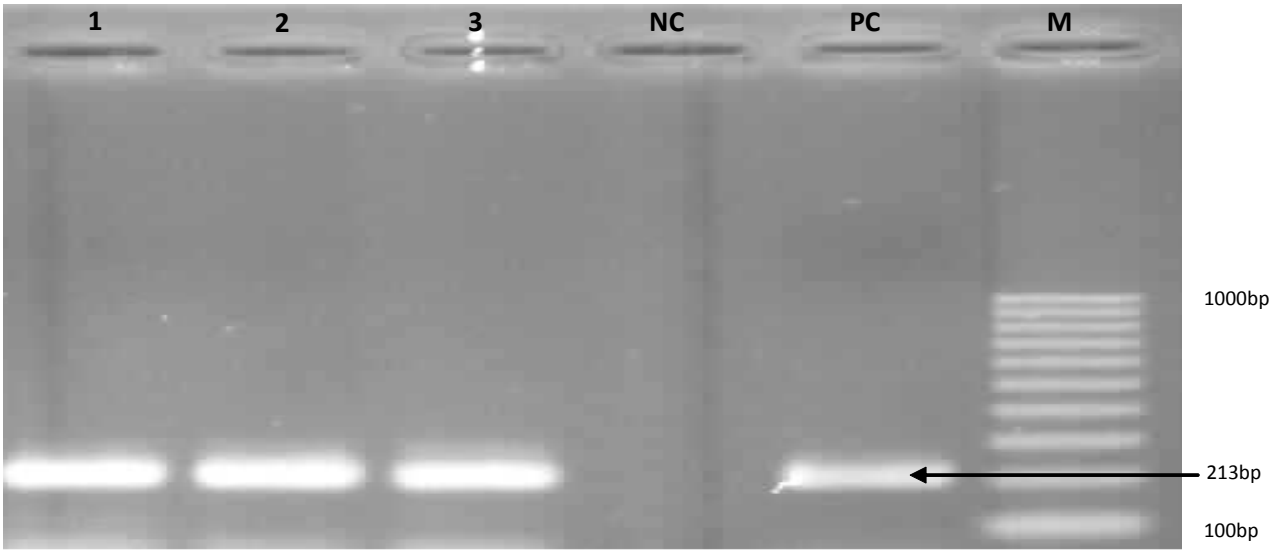

Figure 2. Representative agarose gel for Norovirus genogroup I detection in Indian pennywort (1-3). PC, positive control; NC, negative control; M, 100bp marker.

As a conclusion, it has proved that Norovirus is one of the contributing causes of acute gastroenteritis related to raw vegetables. Therefore, Norovirus should be added in the list of parameter analysis of acute gastroenteritis outbreak investigation. Not only clinical specimens should be taken during the investigation, but, suspected food and environmental surfaces should be considered as important points for sampling to have an appropriate measurement. Continuous monitoring program for Norovirus as food pathogenic organisms should be carried out to protect consumers.

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