Comparison of virus concentration methods for detection of norovirus in bivalve shellfish

การเปรียบเทียบวิธีทำให้ไวรัสเข้มข้นสำหรับตรวจไวรัสโนโรในหอยกาบคู่

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ABSTRACT

Norovirus is the most prevalent causative agents of viral foodborne diseases associated with shellfish consumption. The objective of this study was to compare three different methods for concentrating and detecting norovirus in bivalve shellfish. Norovirus genogroup (G) II was inoculated into concentrates from oysters, cockles, and mussels and identified by reverse transcription-nested polymerase chain reaction (RT-nested PCR). The sensitivities of RT-nested PCR for detection of norovirus GII in oyster and cockle concentrates from all three methods were not different at 0.22 copies/ml. However, for mussel concentrates, RT-nested PCR gave a lower sensitivity of 2.2×10^3 copies/ml. Among those concentration methods, the most appropriate method composed of adsorption, twice elution, and extraction steps has high sensitivity, simplicity and rapid processing time.

บทคัดย่อ

ไวรัสโนโรเป็นสาเหตุหลักทำให้เกิดโรคติดเชื้อไวรัสที่เกี่ยวข้องกับการบริโภคหอยกาบคู่ การศึกษานี้มีวัตถุประสงค์เพื่อเปรียบเทียบวิธีทำให้ไวรัสเข้มข้นแตกต่างกัน 3 วิธี และตรวจไวรัสโนโรในหอยกาบคู่ โดยเติมไวรัสโนโรจีโนกรุ๊ป II ในหอยนางรม หอยแครง และหอยแมลงภู่ที่ทำให้เข้มข้นแล้ว และตรวจพิสูจน์ด้วยวิธี reverse transcription-nested polymerase chain reaction (RT-nested PCR) พบว่าวิธี RT-nested PCR มีความไวในการตรวจไวรัสโนโรจีโนกรุ๊ป II ในหอยนางรมและหอยแครงที่ทำให้เข้มข้นทั้ง 3 วิธี ไม่แตกต่างกันที่ 0.22 copies/ml อย่างไรก็ตามสำหรับหอยแมลงภู่ วิธี RT-nested PCR มีความไวต่ำกว่าโดยตรวจไวรัสโนโรจีโนกรุ๊ป II ได้ที่ 2.2×10^3 copies/ml ในบรรดาวิธีทำให้ไวรัสเข้มข้นทั้งหมดเหล่านั้น วิธีที่เหมาะสมที่สุดซึ่งประกอบด้วยขั้นตอนการดูดซับ ชะสองครั้ง และสกัด มีความไวสูง ทำได้ง่าย และรวดเร็ว

Key Words: Norovirus, Bivalve shellfish, RT-nested PCR
คำสำคัญ: ไวรัสโนโร หอยกาบคู่ พีซีอาร์

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Introduction

Noroviruses have been recognized as the major causative agent of non-bacterial acute gastroenteritis in humans and play a role in epidemic and sporadic gastrointestinal illnesses worldwide (Roger et al., 2009). In Thailand, the viruses cause 8-45% of hospitalized patients of all ages with acute gastroenteritis (Pattara et al., 2007; Rungnapa et al., 2008; Leera et al., 2010). Noroviruses, previously known as Norwalk-like viruses, belong to the family *Caliciviridae* and are classified into five genogroups (GI-GV), of which GI, GII, and GIV are associated with gastroenteritis in humans, whereas GIII is found in bovine and GV in mice (Kim, 2007). Norovirus genogroup II, genotype 4 is a common genotype associated with most outbreaks (Li et al., 2009; Leera et al., 2010). Norovirus outbreaks have occurred in healthcare settings (Marion, 2009), cruise ships (Marc-Alain et al., 2004), and school and university (Robert et al., 2008). The viruses are transmitted mainly via fecal-oral route from person to person contact and through contaminated water and food (Roger et al., 2009). Among food items implicated in these outbreaks, shellfish are recognized as a potential vehicle because these animals are filter feeders and concentrate viruses present in water (David, 2000). Norovirus can bind specifically to digestive tissues of oyster (Françoise et al., 2006). Consumption of raw or slightly cooked shellfish can lead to gastrointestinal infection in humans and are associated with outbreaks (Samara et al., 2007). The detection methods of enteric viruses in bivalve shellfish consist of concentration of viruses from shellfish and identification of viruses by molecular techniques such as reverse transcription-polymerase chain reaction (RT-PCR) and real time RT-PCR (Mette et al., 2004; Grant et al., 2008). However, the identification of viruses in shellfish is problematic because of the low density of contamination and the presence of PCR inhibitors (Anna et al., 2007). Recently, a virus concentration method has been developed in our laboratory and used for detection of noroviruses in oysters *(Crassostrea belcheri)* (Leera et al., 2011). However, this virus concentration method is time-consuming and requires several processing steps. The present study aimed to compare three different virus concentration methods for molecular detection of norovirus from various kinds of bivalve shellfish including oysters (*Saccostrea commercialis*), cockles (*Arca granulosa*), and mussels (*Perna viridis*).

Materials and methods

Norovirus positive control

A norovirus GII-positive stool sample was used as a positive control for determining the sensitivity of RT-nested PCR. The stool sample contained norovirus GII of 1.32×10^8 copies/ml determined by quantitative real-time RT-PCR.

Shellfish processing methods

Digestive tissues of bivalve shellfish were dissected, weighed, and processed by virus concentration methods. In brief, chilled, and sterilized deionized water (150 ml) was added to digestive tissues (4 g) and homogenized by a blender at high speed twice for 45 sec each. Then, the homogenates of shellfish were measured conductivity and processed according to three different concentration methods: Method A (adsorption - twice elution - extraction), Method B (adsorption - twice elution - precipitation -

twice extraction), and Method C (adsorption-twice elution - twice precipitation- twice extraction). The virus processing steps are shown in Figure 1. The volume of concentrates was further reduced to approximately 0.8 ml using speedVac centrifugation for 6-8 hr at 4°C.

Comparison of concentration methods for testing norovirus in shellfish

The sensitivities of RT-nested PCR in digestive tissue concentrates were determined. A norovirus-positive stool sample was serially diluted ten-fold in nuclease-free water to obtain 10^{-1} to 10^{-8} dilutions (2.2×10^{-2} to 2.2×10^{5} copies/ml) and seeded into digestive tissue concentrates from Methods A-C. Digestive tissue concentrate without inoculating norovirus was used as a negative control.

Extraction of nucleic acids and RT-nested PCR

Viral RNA in digestive tissue concentrates was extracted using the RNeasy® Mini kit (QIAGEN, GmbH, Hilden, Germany) following manufacturer's instruction. In brief, 200 µl of digestive tissues concentrate was added with lysis buffer and applied to a spin column followed by washing buffer. Viral RNA was eluted with 60 µl of warm RNase-free water.

The presence of norovirus RNA was determined using RT-nested PCR as described by Kittigul et al. (2011). Specific primers for norovirus GII were used for amplification of capsid region (region C): COG2F (5'-CAR GAR BCN ATG TTY AGR TGG ATG AG-3'), G2-SKF

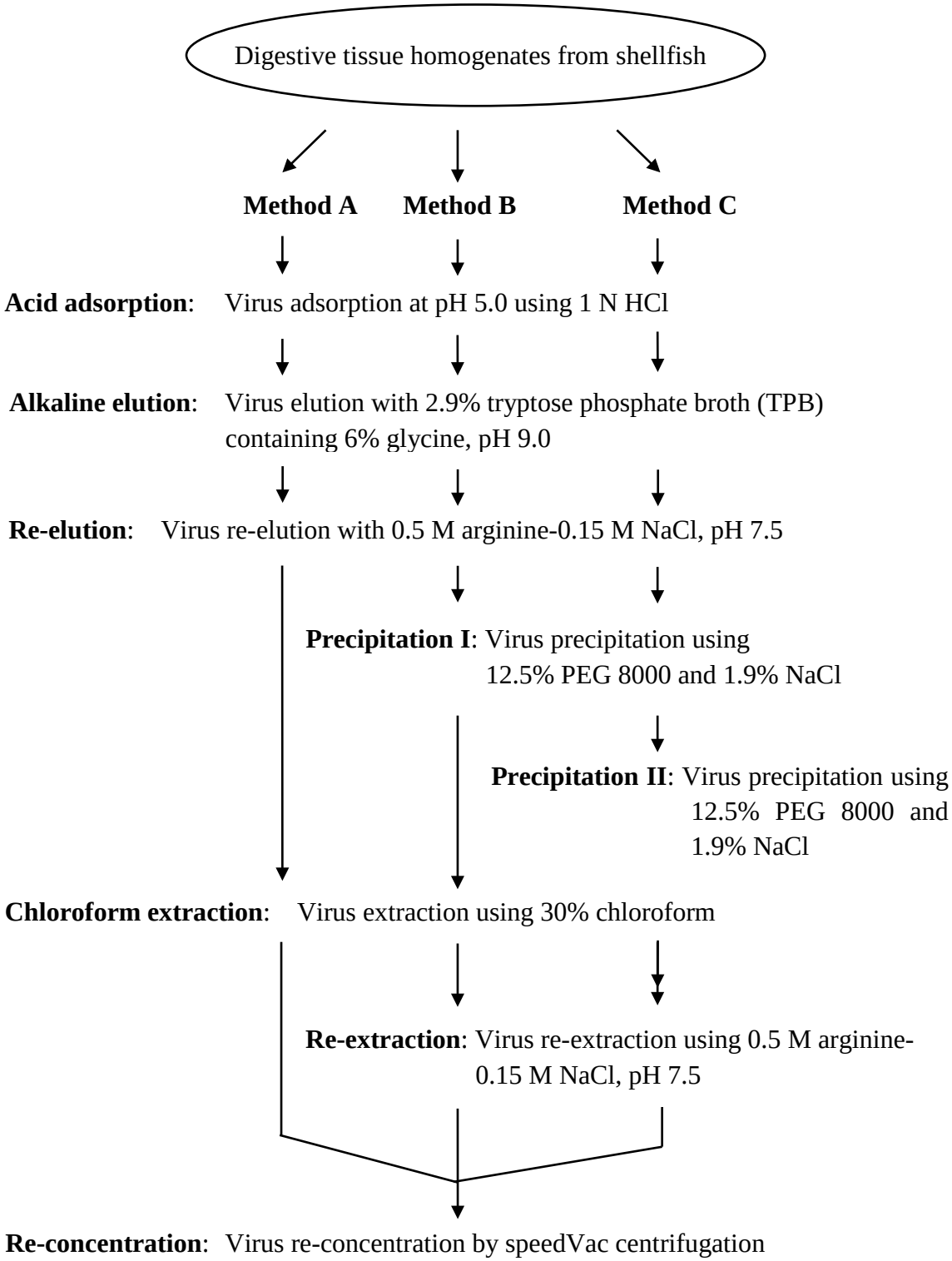

Figure 1 Three different virus concentration methods for concentrating norovirus from shellfish samples; Method A: adsorption - twice elution - extraction, Method B: adsorption - twice elution - precipitation - twice extraction, and Method C: adsorption - twice elution - twice precipitation - twice extraction.

(5'-CNT GGG AGG GCG ATC GCA A-3'), and G2-SKR (5'-CCR CCN GCA TRH CCR TTR TAC AT-3'); Y = C or T, R = A or G, B = C, G or T, H = A, C or T, N = A, T, G or C (Kojima et al., 2002; Kageyama et al., 2003). RT-PCR was performed in 50 µl of reaction volume using SuperScript™ III One-Step RT-PCR System with Platinum® *Taq* DNA polymerase (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. In brief, RNA extract (2 µl) was heated at 94ºC for 4 min, and spinned briefly, then placed on ice for at least 10 min. The denatured RNA was added to RT-PCR mixture (48 µl) consisting of a buffer containing 0.2 mM each of dNTP, 1.6 mM $MgSO_4$, SuperScript™ III RT/Platinum® *Taq* Mix, 0.33 µM each of primers COG2F and G2-SKR, and nuclease-free water. RT-PCR was carried out with following steps: RT at 42ºC for 60 min; initial denaturation at 94ºC for 2 min; PCR 35 cycles at 94ºC for 1 min, 50ºC for 1 min, 72ºC for 1 min; and final extension at 72ºC for 3 min. For nested PCR, the RT-PCR amplification product (2 µl) was added to reaction mixture (48 µl): 2.5 mM $MgCl_2$, 1X *Taq* buffer, 0.2 mM each of dNTP, 2.5 U *Taq* DNA polymerase, 0.33 µM each of primers G2-SKF and G2-SKR, and nuclease-free water. The cycling conditions were as follows: 94°C for 3 min, PCR 30 cycles; 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min; and final extension at 72°C for 15 min. Amplicons were eletrophoresed in a 1.5% agarose gel followed by staining with ethidium bromide (0.5 µg/ml). Norovirus GII showed DNA fragment of 344 bp.

Results

All shellfish samples collected were screened for the presence of naturally occurring noroviruses and the norovirus-negative samples were used for further experiments. For oyster (*Saccostrea commercialis*) concentrates, the application of concentration procedures (Methods A, B, and C) in 3-6 repeated experiments showed that all three methods were able to detect norovirus GII from stool dilution 10^{-7} or 0.22 copies/ml by RT-nested PCR (Table 1). Obviously, with norovirus GII from stool dilutions 10^{-4}-10^{-7} in cockle (*Arca granulosa*) concentrates, negative results were obtained. However, after the RNA extracts from those stool dilutions were diluted 1:2 in nuclease-free water prior to RT-nested PCR assay, the positive results of 344-bp norovirus were found and gave the same sensitivity as norovirus GII in oyster concentrates (Table 1, Figure 2). For mussel (*Perna viridis*) concentrates, all three methods could not detect

Table 1 Sensitivities of RT-nested PCR for detection of norovirus GII in bivalve shellfish concentrates from Methods A, B, and C.

Bivalve shellfish	Method	Sensitivity of RT-nested PCR: norovirus GII	
		Dilution	copies/ml
Oyster	A	10^{-7}	0.22
	B	10^{-7}	0.22
	C	10^{-7}	0.22
Cockle	A	10^{-7}	0.22
	B	10^{-7}	0.22
	C	10^{-7}	0.22
Mussel	A	10^{-3}	2.2×10^{3}
	B	10^{-3}	2.2×10^{3}
	C	10^{-3}	2.2×10^{3}

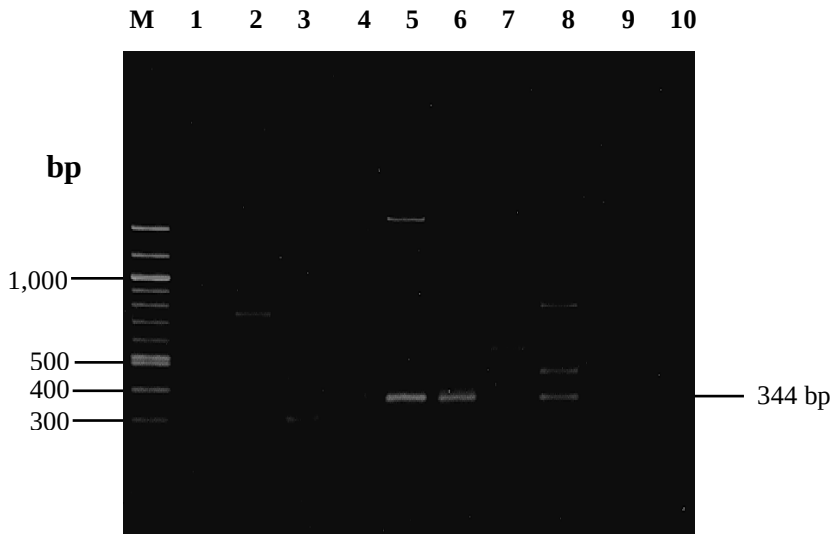

Figure 2 Amplicons of norovirus GII determined by RT-nested PCR in cockle concentrates from Method A (adsorption-twice elution-extraction). Lane: M, DNA marker (100-bp DNA Ladder); 1-4, undilute RNA extract; 5-8, RNA extract diluted 1:2: norovirus GII from stool dilutions 10^{-4}-10^{-7}, respectively; 9, RT-PCR negative control; 10, nested PCR negative control. Gel electrophoresis of the RT-nested PCR product of norovirus GII showed 344-bp band.

norovirus GII from stool dilutions 10^{-4}-10^{-7}. RT-nested PCR gave positive results of norovirus at end point stool dilution of 10^{-3} or 2.2×10^{3} copies/ml (Table 1, Figure 3). Although RNA extracts of norovirus GII from mussel concentrates were diluted two folds, negative results were found (data not shown).

Discussion

Different methods have been proposed for determination of virus contamination in oysters based on whole shellfish (Lee-Ann et al., 1996) or digestive tissues (Peter et al., 2005). On the basis of digestive tissues, the sensitivity of RT-PCR for detection of norovirus was consistently better than when the virus was extracted from whole shellfish (Robert et al., 1995). Norovirus binds specifically to histo-blood group antigens on gastrointestinal cells of oyster (Peng et al., 2006). The present study used dissected digestive tissues from bivalve shellfish to compare three virus concentration methods for testing norovirus by RT-nested PCR.

Method C (adsorption-twice elution-twice precipitation-twice extraction) is currently used in our laboratory for virus concentration (Leera et al., 2011) but the technique is time-consuming and requires a high amount of organic reagents. Methods A (adsorption - twice elution - extraction) and B (adsorption-twice elution - precipitation - twice extraction) were adapted from Method C to reduce processing steps. Norovirus GII could be detected by RT-nested PCR in oyster concentrates from all three methods spiked with the norovirus-positive stool sample at the same dilution of 10^{-7} or 0.22 copies/ml. Method A is superior as compared with Methods B and C since the virus processing time is rapid (11 hr) while Methods B and C spend 1 day 9 hr and 1 day 12 hr, respectively. It seems that there were no benefits by these extended processing steps in this study. The similar findings of no difference in sensitivities between Methods A, B and C were also observed for cockles and mussels. Of note, RNA extract of norovirus GII from cockle concentrates should be diluted two folds before detected by RT-nested PCR. It is likely that some PCR inhibitors were present in those concentrates. Additionally, the strong PCR inhibitors in mussel concentrates from all three methods reduced the sensitivities of RT-nested PCR to 10^{-3}. The extended processing steps of Methods B and C could not remove PCR inhibitors. PCR inhibitors including polysaccharides and organic compounds have been recognized to cause

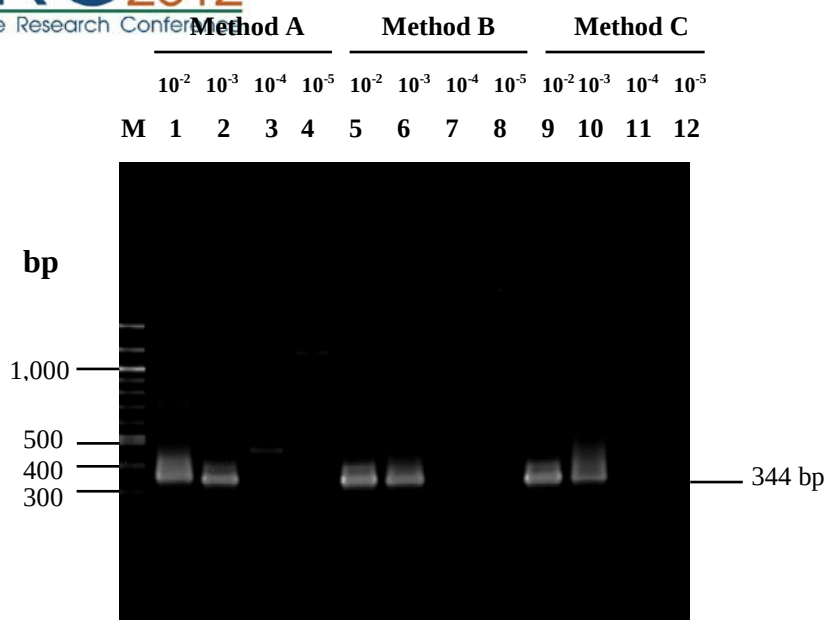

Figure 3 Amplicons of norovirus GII determind by RT-nested PCR in mussel concentrates. Lane: M, DNA marker (100-bp DNA Ladder); 1-4, Method A; 5-8, Method B; 9-12, Method C: norovirus GII from stool dilutions 10^{-2}-10^{-5}, respectively. Gel electrophoresis of the RT-nested PCR product of norovirus GII showed 344-bp band.

major problems in the application of RT-PCR to shellfish samples (Robert et al., 1993). The content of PCR inhibitors in shellfish may vary between different species and harvesting areas (Anna et al., 2007). The removal of PCR inhibitors in mussel concentrates needs to be further studied.

Conclusions

The present study demonstrates the three different methods for concentrating and detecting norovirus in bivalve shellfish samples by RT-nested PCR. Method A (adsorption - twice elution-extraction) is an appropriate method for concentration of norovirus in oysters, cockles, and mussels. This method may be useful for detection of norovirus in shellfish to guarantee the virological quality of shellfish products for human consumption.

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