Development of virus concentration methods for detection of rotavirus in bivalve shellfish

การพัฒนาวิธีทำให้ไวรัสเข้มข้นสำหรับตรวจไวรัสโรตาในหอยกาบคู่

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ABSTRACT

Detection of enteric viruses in foods is of great importance for virological analysis in food safety. The present study aimed to develop virus concentration methods for detection of rotavirus in various kinds of bivalve shellfish by reverse transcription-nested polymerase chain reaction (RT-nested PCR). Virus concentration by Methods A (adsorption - twice elution - extraction), B (adsorption - twice elution - precipitation - twice extraction), and C (adsorption-twice elution-twice precipitation-twice extraction) gave the same sensitivity of RT-nested PCR and could detect rotavirus at the lowest concentration of 5.69 copies/ml in concentrates of oysters, cockles, and mussels. In seeding experiments, the detection limit of Methods A and B was equal (2.78 copies/4 g of each shellfish) and lower than that of Method C. These findings suggest the most suitable Method A for rapid concentration of rotavirus from bivalve shellfish.

บทคัดย่อ

การตรวจไวรัสก่อโรคในอาหารมีความสำคัญสำหรับการวิเคราะห์ทางไวรัสวิทยาในด้านความปลอดภัยของอาหาร การศึกษานี้มีวัตถุประสงค์เพื่อพัฒนาวิธีการทำให้ไวรัสเข้มข้นสำหรับตรวจไวรัสโรตาในหอยกาบคู่ชนิดต่างๆ ด้วยวิธี reverse transcription - nested polymerase chain reaction (RT-nested PCR) การทำให้ไวรัสเข้มข้นด้วยวิธี A (adsorption - twice elution - extraction) วิธี B (adsorption - twice elution - precipitation - twice extraction) และวิธี C (adsorption - twice elution - twice precipitation - twice extraction) เมื่อตรวจไวรัสโรตาด้วย RT-nested PCR ให้ผลความไวเท่ากันและสามารถตรวจพบไวรัสโรตาน้อยที่สุด 5.69 copies/ml ในหอยนางรมปากจีบ หอยแครงและหอยแมลงภู่ที่ทำให้เข้มข้นแล้ว การทดลองเติมไวรัสโรตาในเนื้อเยื่อทางเดินอาหารก่อนผ่านกระบวนการทำให้ไวรัสเข้มข้นพบว่า วิธี A และ B สามารถตรวจไวรัสโรตาได้น้อยที่สุดเท่ากัน (2.78 copies/4 g ของหอยแต่ละชนิด) และน้อยกว่าวิธี C ผลการศึกษานี้แนะนำวิธี A เป็นวิธีที่เหมาะสมที่สุด สำหรับการทำให้ไวรัสโรตาเข้มข้นอย่างรวดเร็วจากหอยกาบคู่

Key Words: Rotavirus, Bivalve shellfish, RT-nested PCR
คำสำคัญ: ไวรัสโรตา หอยกาบคู่ พีซีอาร์

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Introduction

Rotavirus is the leading cause of severe diarrhea in infants and young children worldwide (Umesh et al., 1998). Globally, acute diarrhea caused by rotavirus is responsible for 527,000 deaths and 2.4 million admissions to hospital each year among children under 5 years (Umesh et al., 2006). In Thailand, the prevalence of rotavirus infection is in the range of 27-50% among hospitalized children (Pattara et al., 2007; Utcharee et al., 2008) and 42% of all ages (Thitiluck, 2011). Rotavirus, belonging to the family *Reoviridae* has been classified into seven groups (A to G) whereas group A rotavirus comprises of important pathogens of humans (Ulrich et al., 2005). The virus is shed in feces in high numbers and transmitted via fecal-oral route and may contaminate surface water and foods (Walda et al., 2006). Food-borne outbreaks caused by rotavirus are uncommon, however, the findings of rotavirus present in various kinds of bivalve shellfish may imply a health risk to humans (Françoise et al., 2000; Leera et al., 2008). Viruses in sewage-containing water can accumulate in bivalve shellfish through filter-feedings. Rotaviruses have been detected in oysters, and mussels (Rosanna et al., 2007; Françoise et al., 2008; Dongying et al., 2008). Although rotavirus mainly infects young children, the rotavirus infected adults can spread the virus through direct or indirect to children and surrounding environments. After consumption of shellfish contaminated with rotavirus, the infected adults may transmit the virus to children, leading to an outbreak.

A detection method for virus in food consists of the concentration step and identification by polymerase chain reaction (PCR) based assay (David and Gary, 2001; Leera et al., 2008). However, a low viral load of contamination and an inefficient recovery of virus during the concentration process pose a problem for detection of virus in food. The aim of this study was to develop virus concentration methods for testing rotavirus in bivalve shellfish such as oysters (*Saccostrea commercialis*), cockles (*Arca granulosa*), and mussels (*Perna viridis*).

Materials and methods

Rotavirus positive control

A rotavirus-positive stoolsample determined by quantitative real-time RT-PCR (5.69×10^6 copies/ml) was used as a positive control.

Concentration of virus from bivalve shellfish

The viruses in digestive tissues from oysters, cockles, and mussels were concentrated by three different methods. Method A (adsorption - twice elution -extraction) includes the steps of acid adsorption, alkaline elution, re-elution, and chloroform extraction. Method B (adsorption - twice elution - precipitation-twice extraction) includes the steps of acid adsorption, alkaline elution, re-elution, polyethylene glycol (PEG) precipitation, chloroform extraction, and re-extraction. Method C (adsorption-twice elution - twice precipitation-twice extraction) includes the steps of acid adsorption, alkaline elution, re-elution, PEG precipitation I, PEG precipitation II, chloroform extraction, and re-extraction. The complete steps of Method C were as follows: in brief, digestive tissues from bivalve shellfish were removed by dissection. Chilled and sterilized distilled water (150 ml) was added to digestive tissues (4 g) and homogenized by blender at

high speed twice for 45 sec each. Acid adsorption: the homogenate was adjusted to pH 5 with 1 N HCl, shaken at 200 rpm for 15 min on ice, and centrifuged at 2,900 × g for 15 min at 4°C. Alkaline elution: the pellet was suspended with 2.9% tryptose phosphate broth containing 6% glycine, pH 9.0, shaken at 215 rpm for 15 min on ice and centrifuged at 10,000 × g for 15 min at 4°C. Re-elution: the supernatant (S_1) was collected and the pellet was resuspended with one volume (wt/vol) of 0.5 M arginine-0.15 M NaCl, pH 7.5. The suspension was shaken at 230 rpm for 15 min on ice and centrifuged at 10,000 × g for 15 min at 4°C. The supernatant (S_2) was decanted, combined with S_1 and adjusted to pH 7.5 with 1 N HCl. PEG precipitation I: the virus was precipitated by adding PEG 8000, 50% (wt/vol) solution in 7.5% NaCl; 1:4 to obtain a final concentration of 12.5% PEG and 1.9% NaCl to the supernatant. The mixture was shaken at 120 rpm for 2 hr on ice, refrigerated overnight, and then centrifuged at 10,000 × g for 1 hr at 4°C. The pellet was resuspended in 0.05 M phosphate-buffered saline (PBS), pH 7.5. PEG precipitation II: the supernatant was precipitated again with PEG-NaCl solution. The mixture was shaken at 120 rpm for 2 hr on ice and then centrifuged at 10,000 × g for 10 min at 4°C. Extraction: the pellet was dissolved in 0.05 M PBS, extracted with chloroform to a final concentration of 30%, and mixed by vortex. Then, the tube was centrifuged at 3,000 × g for 15 min at 4°C. The top layer of the aqueous phase (A_1) was collected. Re-extraction: the pellet was re-extracted with 0.5 volume of 0.5 M arginine- 0.15 M NaCl, pH 7.5, and centrifuged. Then, the top layer of the aqueous phase (A_2) was collected, and combined with the A_1. The volume of concentrates (A_1 + A_2) were reduced further to approximately 0.8 ml using speedVac centrifugation at 4°C for 4-5 hr and stored at -80°C until nucleic acid extraction. Methods A and B were similar to Method C but shorter processing steps were carried out.

Extraction of nucleic acids

Viral RNA in concentrates from bivalve shellfish was extracted using the RNeasy® Mini kit (QIAGEN GmbH,Hilden,Germany) following the manufacturer's instruction**.** In brief, a 200 µl of shellfish concentrate was added with buffer RLT and 70% ethanol, and mixed by vortex. The samples were applied to an RNeasy mini spin column, and centrifuged. The RNeasy column was washed with buffer RW1 followed by two washes with buffer RPE. RNA was eluted from the RNeasy column using 60 µl of warmed RNase-free water, and stored at -80°C until further use.

RT-nested PCR

The presence of rotavirus RNA in bivalve shellfish samples were determined using RT-nested PCR as described by Leera et al. (2008). The primers specific to rotavirus were used for detection of VP7 (Michael et al., 1997). One-step RT-PCR was performed in 50 µl of reaction volume. 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 into RT-PCR mixture (48 µl) consisting of 1X Reaction Mix (a buffer containing 0.2 mM each of dNTP, 2 mM $MgSO_4$), SuperScript™ III RT/Platinum® *Taq* Mix (Invitrogen, Life technologies, Carlsbad, CA), 0.25 µM primer RV1 (5'-GTC ACA TCA TAC AAT TCT AAT CTA AG-3'), 0.25 µM primer RV2 (5'-CTT TAA AAG AGA GAA TTT CCG TCT G-3') and

nuclease-free water. The reaction tube was applied into a thermocycler (Thermo Hybaid, Frankin, MA). RT-PCR was carried out with following steps: RT at 41ºC for 60 min; initial denaturation at 94ºC for 2 min; PCR 25 cycles at 94ºC for 30 sec, 55ºC for 30 sec, 72ºC for 1 min; and final extension at 72ºC for 3 min. Nested PCR was performed in 50 µl of reaction volume. The RT-PCR amplification product (1 µl) was added into reaction mixture (49 µl): 1X PCR buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl), 3.5 mM $MgCl_2$, 0.2 mM each of dNTP, 1.25 U of *Taq* DNA polymerase (Invitrogen, Life technologies, Carlsbad, CA), 0.5 µM primer RV3 (5'-TGT ATG GTA TTG AAT ATA CCA C-3'), 0.5 µM primer RV4 (5'-ACT GAT CCT GTT GGC CAW CC-3'); W = A or T, and nuclease-free water. The reaction tube was applied into a thermocycler. The conditions were as follows: initial denaturation at 94°C for 1 min; PCR 40 cycles at 94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min; and final extension at 72°C for 3 min. DNA fragment of 346 bp was considered group A rotavirus.

Results

Naturally occurring rotaviruses in bivalve shellfish were screened by RT-nested PCR and the rotavirus-negative shellfish concentrates were inoculated with rotavirus from stool dilutions 10^{-3} - 10^{-6} (5.69×10^{-2} - 5.69 × 10 copies/ml). The rotavirus from oyster, cockle, and mussel concentrates by all three methods (A-C) could be detected by RT-nested PCR at the endpoint dilution of 10^{-4} or 5.69 copies/ml. The sensitivities of RT-nested PCR for detection of rotavirus in oyster concentrates are shown in Figure 1.

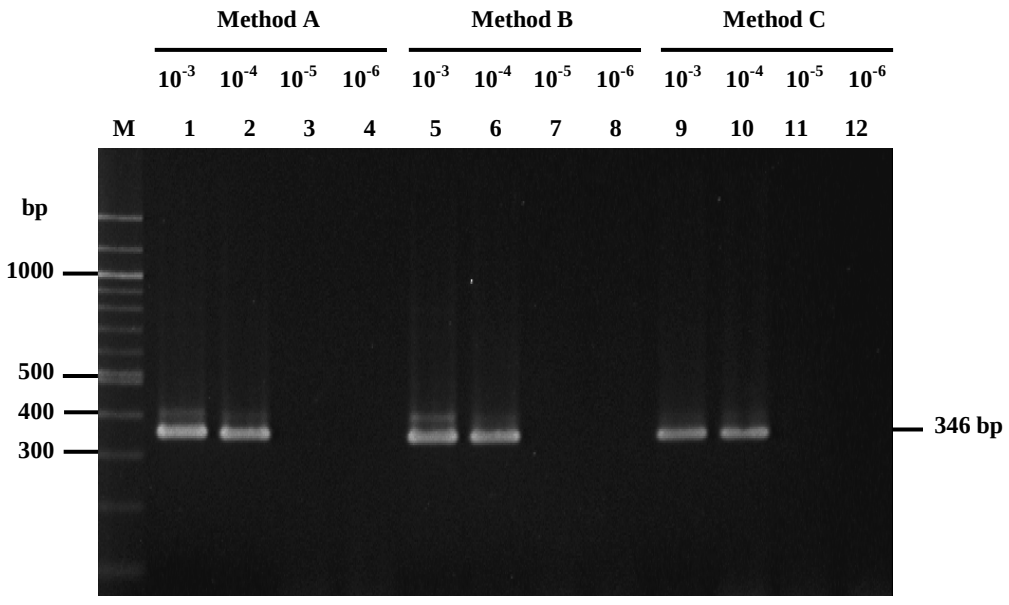

Figure 1 Amplicons of rotavirus detected in oyster concentrates by RT-nested PCR. Lane: M, DNA marker (100-bp DNA Ladder); 1-4, Method A; 5-8, Method B; 9-12 Method C: rotavirus in stool dilutions 10^{-3}-10^{-6}, respectively. Gel electrophoresis of the RT-nested PCR product of rotavirus showed 346-bp band.

Three different methods for concentrating rotavirus from oysters, cockles, and mussels were compared for detection of experimentally contaminated rotaviruses. Various dilutions of rotavirus-positive stool sample were seeded in digestive tissues of those bivalve shellfish, processed for concentration, and determined for rotaviral RNA using RT-nested PCR. The detection limit of rotavirus in oyster concentrates by Method A was equal to Method B (11.12 copies/4 g of digestive tissues) and lower two times than Method C (22.24 copies/4 g), as shown in Figure 2. In seeding experiments of cockles, Method A gave the lowest detection limit of 5.56 copies/4 g, whereas for mussels Methods A and B could detect rotavirus at the lowest detection limit of 2.78 copies/4 g. Method A took a shorter time to complete the virus concentrating process than Methods B and C (Table 1).

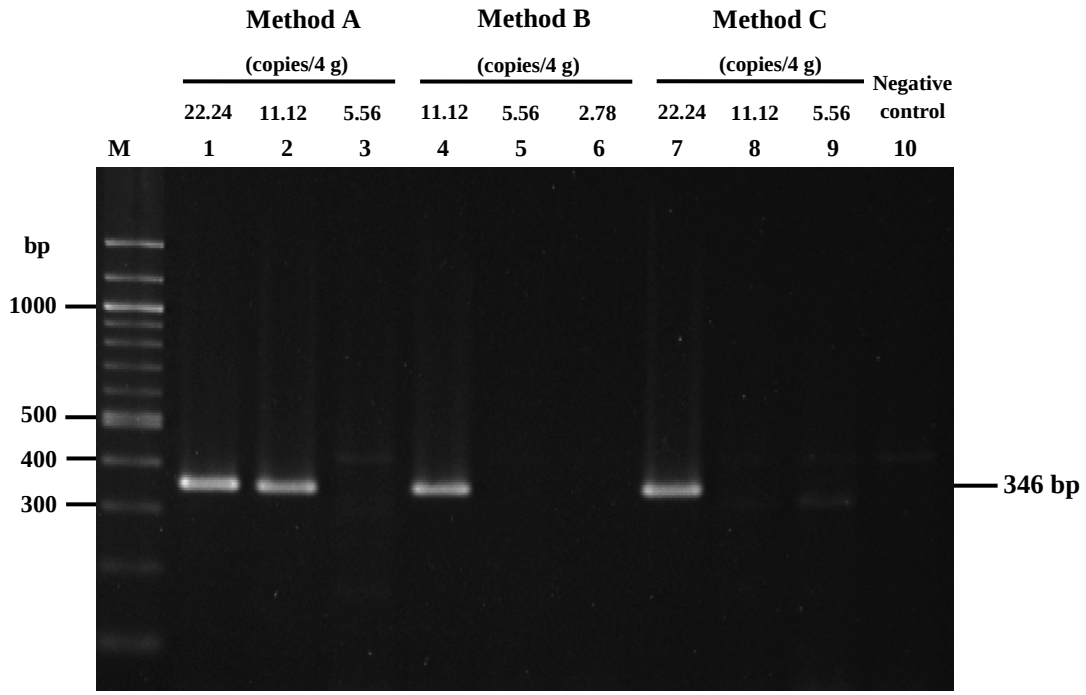

Figure 2 Amplicons of rotavirus seeded in digestive tissues from oysters processed by three concentration methods. Lane: M, DNA marker (100-bp DNA Ladder); 1-3, Method A seeded with rotavirus at 22.24, 11.12, and 5.56 copies/4 g; 4-6, Method B at 11.12, 5.56, and 2.78 copies/4 g; 7-9, Method C at 22.24, 11.12, 5.56 copies/4 g, respectively; 10, negative control of digestive tissues without seeding rotavirus. Gel electrophoresis of the RT-nested PCR product of rotavirus showed 346-bp band.

Table 1 Limits of detection of three concentration methods for rotavirus seeded in different bivalve shellfish samples

Method	Limit of detection*, copies/4 g			Processing time**
	Oysters	Cockles	Mussels	
A (adsorption-twice elution-extraction)	11.12	5.56	2.78	11 hr
B (adsorption-twice elution-precipitation-twice extraction)	11.12	22.24	2.78	1 day 9 hr
C (adsorption-twice elution-twice precipitation-twice extraction)	22.24	11.12	5.56	1 day 12 hr

*By RT-nested PCR.

**For virus concentration.

Discussion

Sewage - contaminated shellfish have been implicated in viral gastroenteritis outbreaks (Won, 2000). For methodological developments of virus detection in shellfish, a method consists of the concentration process and molecular identification of the virus. The present study attempted to develop virus concentration methods for detection of rotavirus in various kinds of bivalve shellfish. Previously, a method for concentrating and detecting rotavirus in oysters (*Crassostrea belcheri*) was successfully applied to examine rotavirus contamination (Leera et al., 2008). This method named adsorption-twice elution - twice precipitation - twice extraction or Method C in this study was compared with two modified methods (Methods A and B) with shorter processing time. These three virus concentration methods provided the same sensitivity for detection of rotavirus in all three kinds of bivalve shellfish including oysters (*Saccostrea commercialis*), cockles (*Arca granulosa*), and mussels (*Perna viridis*). PCR inhibitors have been shown to interfere the sensitivity of RT-PCR (Robert et al., 1993). Of interest, in the present study, there was no PCR inhibitors in all shellfish concentrates for detection of rotaviruses. In contrast, the study of norovirus demonstrated the presence of PCR inhibitors

in mussel concentrates (Anyarat, personal communication). It seems that PCR inhibitors present in mussel concentrates affected the reaction of norovirus amplification but not for rotavirus. The seeding experiments revealed the lowest detection limit of rotavirus processed by Method A. Among those virus concentration methods, Method A is the most efficient protocol for rotavirus recovery since the method is more rapid, simple, less time-consuming, and easy to handle. In addition, high concentrations of organic reagents as well as extended processing steps are not included using Method A. These findings indicate that the promising Method A is appropriate to be applied for detection of rotaviruses in naturally contaminated bivalve shellfish using RT-nested PCR.

Conclusions

This study highlights the virus concentration methods for detection of rotavirus in oysters, cockles, and mussels by RT-nested PCR. Methods A (adsorption - twice elution - extraction), B (adsorption - twice elution-precipitation - twice extraction) and C (adsorption - twice elution - twice precipitation - twice extraction) provided the same sensitivity for rotavirus (5.69 copies/ml). Additionally, Methods A and B showed the lowest detection limit of 2.78 copies/4 g of shellfish in seeding experiments. Taken together, Method A is the most appropriate method for concentration of rotavirus since the method has high sensitivity, rapid and simple to perform.

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References

Anyarat Thamjaroen. [Personal communication]

David H. Kingsley, Gary P. Richards. 2001. "Rapid and efficient extraction method for reverse transcription-PCR detection of hepatitis A and Norwalk-like viruses in shellfish." Appl Environ Microbiol 67: 4152-7.

Dongying Wang, Qiucheng Wu, Li Yao, Min Wei, Xiaojun Kou, Jianping Zhang. 2008. "New target tissue for food-borne virus detection in oysters." Lett Appl Microbiol 47: 405-9.

Françoise S. Le Guyader, Jean-Claude Le Saux, Katia A. Balay, Joanna Krol, Ophelie Serais, Sylvain Parnaudeau, et al. 2008. "Aichi virus, norovirus, astrovirus, enterovirus, and rotavirus involved in clinical cases from a French oyster - related gastroenteritis outbreak." J Clin Microbiol 46: 4011-7.

Françoise S. Le Guyader, Larissa Haugarreau, Laurence Miossec, Emeline Dubois, Monique Pommepuy. 2000. "Three-year study to assess human enteric viruses in shellfish." Appl Environ Microbiol 66: 3241-8.

Leera Kittigul, Kannika Pombubpa, Tippawan Rattanatham, Pornphan Diraphat, Fuangfa Utrarachkij, Supornvit Pungchitton, et al. 2008. "Development of a method for concentrating and detecting rotavirus in oysters." Int J Food Microbiol 122: 204-10.

Michael Gilgen, Daniel Germann, Jürk Luthy, Philipp Hübner. 1997. "Three-step isolation method for sensitive detection of enterovirus, rotavirus, hepatitis A virus, and small round structured viruses in water samples." Int J Food Microbiol 37: 189-99.

Pattara Khamrin, Supatra Peerakome, Supin Tonusin, Rungnapa Malasao, Shoko Okitsu, Masashi Mizuguchi, et al. 2007. "Changing pattern of rotavirus G genotype distribution in Chiang Mai, Thailand from 2002 to 2004: decline of G9 and reemergence of G1 and G2." J Med Virol 79: 1775-82.

Robert L. Atmar, Theodore G. Metcalf, Frederick H. Neill, Mary K. Estes. 1993. "Detection of enteric viruses in oysters by using the polymerase chain reaction." Appl Environ Microbiol 59: 631-5.

Rosanna Gabrieli, Alessia Macaluso, Luigi Lanni, Stefano Saccares, Fabiola D. Giamberardino, Barbara Cencioni, et al. 2007. "Enteric viruses in molluscan shellfish." New Microbiol 30: 471-5.

Thitiluck Swangsri. 2011. "Reverse transcription-multiplex polymerase chain reaction for genotyping of group A rotavirus." [Thesis in Public Health]: Mahidol University.

Ulrich Desselberger, Jim Gray, Mary K. Estes. 2005. "Rotaviruses". In: Brian W.J. Mahy, Volker T. Meulen, eds. Topley and Wilson's Microbiology and Microbial infections. New Jersey: ASM Press: 946-58.

Umesh D. Parashar, Christopher J. Gibson, Joseph S. Bresee, Roger I. Glass. 2006. "Rotavirus and severe childhood diarrhea." Emerg Infect Dis 12: 304-6.

Umesh D. Parashar, Joseph S. Bresee, Jon R. Gentsch, Roger I. Glass. 1998. "Rotavirus." Emerg Infect Dis 4: 561-70.

Utcharee Intusoma, Vorasith Sornsrivichai, Chuleeporn Jiraphongsa, Wandee Varavithaya. 2008. "Epidemiology, clinical presentations and burden of rotavirus diarrhea in children under five seen at Ramathibodi Hospital, Thailand." J Med Assoc Thai 91: 1350-5.

Walda B. van Zyl, Nicola A. Page, Wilhelm O. Grabow, Amber D. Steele, Maureen B. Taylor. 2006. "Molecular epidemiology of group A rotaviruses in water sources and selected raw vegetables in southern Africa." Appl Environ Microbiol 72: 4554-60.

Won Sang Lee. 2000. "Gastrointestinal infections in children in the Southeast Asia region: emerging issues." J Pediatr Gastroenterol Nutr 30: 241-5.