Performance of concanavalin A-immobilized on polyacrylate beads for the detection of human norovirus and hepatitis A virus in fecal specimens

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Abstract Quantitative reverse transcription PCR (qRT-PCR) is a sensitive method for the detection of foodborne viruses in fecal samples. However, the performance of qRT-PCR depends on the efficiency of virus concentration methods. In this study, the effect of Concanavalin A (Con A)-immobilized on polyacrylate beads (Con A-PAB) on the qRT-PCR performance, in terms of sensitivity and specificity to detect foodborne viruses in human fecal specimens was compared with commercial viral RNA extraction kit (VRNA). The detection of foodborne viruses by qRT-PCR was validated by viral genome sequencing. Both Con A-PAB and VRNA methods were equally sensitive and specific for detecting hepatitis A virus in fecal specimens. Even though both methods showed high specificity (100% vs. 100%) for detecting human norovirus (HuNoV), Con A-PAB method exhibited higher sensitivity (100% vs. 42.9%) and accuracy (100% vs. 73.3%) compared to VRNA method. In conclusion, the application of

Con A-PAB would improve the performance of qRT-PCR for the detection of HuNoV in fecal samples.

Keywords Concanavalin A · Hepatitis A virus · Human norovirus · Sensitivity · Specificity

Introduction

Foodborne pathogens are recognized as a significant food safety hazard and represent the most reported cause of illness outbreaks worldwide (WHO, 2015). Human norovirus (HuNoV) and hepatitis A virus (HAV) are the most concerned pathogens due to high incidence of morbidity and mortality, and potential for transmission via contaminated food (FAO/WHO, 2012). HuNoV and HAV are responsible for viral gastroenteritis and viral hepatitis outbreaks worldwide, respectively. The human gastrointestinal tract is a reservoir of these viruses, (Randazzo et al., 2018) and human feces remains a potential source of

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spreading the virus. Therefore, the detection of the viruses in fecal sample is indicative of probable foodborne virus infection.

Several detection methods, such as enzyme immune, immunochromatographic, microfluidic, nanomaterial- and biosensor-based assays, and antibody-based magnetic separation, DNA aptasensor, reverse transcription PCR (RT-PCR), and quantitative RT-PCR (qRT-PCR) are available for the detection of different foodborne viruses (Kumthip et al., 2017; Neethirajan et al., 2017; Park et al., 2008; Schwab et al., 2001; Siqueira et al., 2011; Wu et al., 2019). These methods have different levels of sensitivity, specificity, and accuracy to detect the viruses. Among the detection methods, qRT-PCR is extensively used for the detection of the viruses in fecal specimens due to high sensitivity and simultaneously test a large number of samples (Hoehne and Schreier, 2006; Kageyama et al., 2003). However, the detection of the viruses in fecal samples is challenging due to the presence of PCR inhibitors, which interfere the polymerase activity (Schrader et al., 2012). Consequently, viral extraction and concentration are critical to overcome such hindrance in the detection of the viruses present in fecal samples.

For extraction and concentration of viral nucleic acids from fecal samples prior to PCR, a remarkable number of manual, semi-automated, and automated viral RNA extraction kits are commercially available; however, low and inconsistent sensitivity and specificity of extraction kits for different viral RNA imply a significant problem (Brassard et al., 2009; Iker et al., 2013; Yoon et al., 2016). Although paramagnetic silica-based materials, ion exchange resins, Sephadex, Chelex, and cetrimonium bromide have been used to remove a wide range of PCR inhibitors (Arnal et al., 1999; Kemp et al., 2006; Sur et al., 2010), the efficiency is strongly dependent on the composition of sample matrix and no general recommendations can be established for different viruses and their genotypes (Schrader et al., 2012). Even though immunomagnetic separation (IMS) has been used to concentrate HAV and HuNoV from artificially contaminated food (Park et al., 2008), the application of IMS remains limited because a specific antibody used in IMS would not be suitable for novel HuNoV strains due to the antigenic diversity of HuNoV (Lindesmith et al., 2008). Therefore, non-specific ligands, such as histo-blood group antigens (HBGA) were used as attachment factors to capture HuNoV (Cannon and Vinjé, 2008; Tian et al., 2011). However, other foodborne viruses could not recognize HBGA, and not all HuNoV genotypes interact with HBGA (Almand et al., 2017; Singh et al., 2016). Alternatively, concanavalin A (Con A), a carbohydrate-binding lectin, showed a potential for concentrating HuNoV (Kim et al., 2017). Even though the Con

A has successfully concentrated HuNoV in artificially contaminated food, it has not been tested for other foodborne viruses on naturally contaminated fecal samples (Kim et al., 2017).

Hence, the aim of the present study was to evaluate the applicability of newly developed Con A immobilized on polyacrylate beads (Con A-PAB) combined with qRT-PCR (hereafter denoted as Con A-PAB method) for the detection of foodborne viruses in fecal samples from gastroenteritis cases. The sensitivity and specificity of the method were compared with a commercial QIAamp Viral RNA Extraction Mini Kit (VRNA) combined with qRT-PCR (hereafter denoted as VRNA method). The performance of the method to identify the viruses in fecal samples was verified using viral genomic sequencing.

Materials and methods

Sample collection and preparation

Fecal specimens were collected from volunteer participants of all age and sex groups with gastroenteritis cases from different locations in Texas (Bryan, College Station, and Houston areas, TX, USA). Sample collection was based on the standard protocol approved by the Texas A&M University Institutional Review Board (IRB2017-0308D). Participants or parental permission and minor assent forms were used whenever necessary, according to the regulatory requirements of the U.S. Department of Health & Human Services. The sample was prepared by dispersing an approximately 20% (w/v) feces suspension in phosphate buffered saline (PBS) using a vortex mixer. The suspension was clarified by centrifugation at $12,000 \times g$ for 10 min at 4 °C and the supernatant was stored at -80 °C until use.

Viral RNA preparation

Viral RNA was extracted from fecal supernatant using a Con A-PAB employed with magnetic beads as described previously (Kim et al., 2017). Con A isolated from *Canavalia ensiformis* (Jack bean, C7275, Sigma, St. Louis, USA) and carboxylic acid functionalization of the polyacrylate bead (WK-60L, Samyang Co., Seoul, Korea) were purchased to prepare Con A-PAB. The supernatant of the fecal specimen diluted in 2 mL PBS transferred to the Con A-PAB, and incubated at 23 °C for 15 min. Thereafter, 2 mL of lysis buffer (4 M guanidine thiocyanate, 0.1 mM EDTA, and 10 mM Tris-HCl adjusted to pH 7.5) was added to the Con A-PAB and incubated at 23 °C for 3 min. The RNA-binding magnetic bead suspension (50 μ L, Goshenbio, Seoul, South Korea) was then mixed with the lysate in a separate 5 mL tube. Isopropyl alcohol (1 mL)

was added and inverted to mix with the magnetic beads. The beads were collected by magnetic separation and the supernatant was discarded. Elution buffer (100 µL) was added and the samples were incubated at 65 °C for 2 min. Afterwards, the beads were precipitated using a magnetic bar and the extracted total RNA supernatant was collected. For comparison, a commercial VRNA (QIAGEN, Hilden, Germany) was used to extract the virus according to the manufacturer's protocol. Briefly, 20% (w/v) of fecal supernatant was used for each samples. A viral lysis buffer (560 µL, AVL buffer, QIAGEN) was added to the fecal supernatant and incubated at 23 °C for 10 min. Absolute ethanol (560 µL) was added to the sample and the contents were transferred to a spin-column. The samples were washed, added QIAamp virus elution buffer (100 µL, AVE buffer, RNase-free water containing 0.04% sodium azide, QIAGEN) and incubated at 23 °C for 1 min. The total RNA was collected into a sterile tube.

Quantitative reverse transcription PCR (qRT-PCR)

The cDNA was synthesized from total RNA using iScript Reverse Transcription Supermix (Bio-Rad, Richmond, CA, USA) according to the manufacturer's protocol. qRT-PCR amplification was performed in 96-well blocks with a CFX-96 RealTime PCR System (Bio-Rad) using a TaqMan probe mix in a reaction volume of 20 µL, which contained 2 µL of the primary cDNA reaction mixture, 2 X Ex Taq HS Mix (Takara, Kusatsu, Shiga, Japan), and the primer pair. Primers used in this study are listed in Table 1. The PCR condition was applied as follows: activation at 95 °C for 3 min, followed by denaturation of 45 cycles at 95 °C for 10 s, annealing at 56 °C for 10 s and a final extension at 72 °C for 30 s. All qRT-PCR reactions were performed in

biological triplicate. Viral genomic copies in samples were quantified from the cycle threshold (Ct) values obtained from the qRT-PCR result and compared with the standard curves of HuNoV GII.4, HAV and rotavirus genome copies.

Viral genomic sequencing

PCR products of virus from fecal specimens were amplified using the same RT-PCR primer described above by an AllInOneCycler™ 384-well PCR system (Bioneer, Daejeon, South Korea), followed by purification using Accu-Prep Gel Purification Kit (Bioneer). Sequencing reactions were performed using a BigDye Terminator v3.1 sequencing kit (Applied Biosystems, Foster City, CA, USA) and sequenced using 3730XL DNA analyzer (Applied Biosystems, Foster City, CA). The sequence data was aligned and compared with different virus reference strains obtained from the GenBank database of the National Center for Biotechnology Information BLAST (<https://blast.ncbi.nlm.nih.gov>), and the percent identities between sequence pairs were compared.

Sensitivity, specificity, and positive and negative predictive values

Specificity, specificity, accuracy, and positive and negative predictive values (PPV and NPV) of both methods was evaluated with the viral genome sequence as the reference. The values were calculated by creating a 2 × 2 contingency table and using following equations (Greenhalgh, 1997):

Table 1 Sequence of primers used for qRT-PCR

Target	Primer	Primer sequence
HuNoV	COG1F	5'-CGY TGG ATG CGN TTY CAT GA-3'
	COG1R	5'-CTT AGA CGC CAT CAT CAT TYAC-3'
	RING1 ^a	FAM-AGA TYG CGA TCY CCT GTC CA-BHQ
	JJV2F	5'-CAA GAG TCA ATG TTT AGG TGG ATG AG-3'
	COG2R	5'-TCG ACG CCA TCT TCA TTC AC-3'
	RING2P	FAM-TGG GAG GGC GAT CGC AAT CT-BHQ
Rotavirus	JVKF	5'-CAG TGG TTG ATG CTC AAG ATG GA-3'
	JVKR	5'-TCA TTG TAA TCA TAT TGA ATA CCCA-3'
	JVKP	FAM-ACA ACT GCA GCT TCA AAA GAA GWGT-MGBNFQ-3
Hepatitis A virus	HAV68F	5'-CAG CAC ATC AGA AAG GTG AG-3'
	HAV240R	5'-CTC CAG AAT CAT CTC CAAC-3'
	HAV150	FAM-CCT GAA CCT GCA GGA ATT AA-MGBNFQ

^aGenome location of primers for GI is based on the sequence of Norwalk/68/US [GenBank: M87661] and for GII on the sequence of Lordsdale/93/UK [GenBank: AY032605]; Y ¼ C/T; I ¼ inosin; FAM ¼ fluorescein; BHQ ¼ black hole quencher

$$\text{Sensitivity} = \frac{\text{True - positive}}{\text{True - positive} + \text{False - negative}} \times 100$$

$$\text{Specificity} = \frac{\text{True - negative}}{\text{True - negative} + \text{False - positive}} \times 100$$

$$\text{Accuracy} = \frac{\text{True - positive} + \text{True - negative}}{\text{True - positive} + \text{False - positive} + \text{False - negative} + \text{True - negative}} \times 100$$

$$\text{PPV} = \frac{\text{True - positive}}{\text{True - positive} + \text{False - positive}} \times 100$$

$$\text{NPV} = \frac{\text{True - negative}}{\text{True - negative} + \text{False - negative}} \times 100$$

Results and discussion

The newly developed Con A-PAB method was evaluated in comparison with commercial VRNA method by detecting foodborne viruses in fecal samples. Currently, several automated and semi-automated virus concentration kits are commercially available; however, VRNA is a widely used and similar manual operation as for Con A-PAB. The kit was also reported to be highly sensitive, affordable, and convenient, along with rapid extraction and concentration of viral RNA (Fransen et al., 1998). Therefore, the kit was selected in the study for comparison. Among fifteen fecal samples, seven and three samples showed the presence of HuNoV by Con A-PAB and VRNA methods, respectively (Fig. 1). Con A-PAB method showed the presence of HuNoV genotypes, including GI.2, GI.4, GI.5, GII.1, GII.4, and GII.13; however, VRNA method could only detect GI.2, GI.4, and GII.13 (Fig. 1). Both methods adopt a solid

phase extraction method but different extracting phases are used in the two methods. The silica-based membrane under high chaotropic salt condition is applied in the VRNA method for binding and purifying RNA (Hourfar et al., 2005). The membrane was useful for binding viral RNA from a wide variety of viruses, but the binding was not possible for all viruses. The membrane could not effectively eliminate the PCR inhibitors present in fecal samples, such as complex polysaccharides, bilirubin, bile salt, lipids, and urates (Esona et al., 2013; Rådström et al., 2004; Schrader et al., 2012). However, Con A-PAB method is based on an interaction of Con A and viruses by specifically binding to either metal coordination or carbohydrate binding regions of Con A (Kim et al., 2017). Therefore, the presence of HuNoV in a high number of samples identified by the Con A-PAB method could be due to either highly specific binding of HuNoV to Con A or the ability to remove PCR inhibitors from the fecal samples. Similarly,

Fig. 1 Comparison of foodborne virus copy number detected by Concanavalin A immobilized on polyacrylate beads (Con A-PAB) and QIAamp Viral RNA Extraction Mini Kit (VRNA) methods. The cut-off for samples was set at Ct-value of 40. Hepatitis A virus strains 14 and 18f; HuNoV strains GI.4, GI.2, GI.5, GII.1, GII.4, and GII.13; Rotavirus strain G2; ND, not detected

previous studies reported that an immunomagnetic separation is effective to minimize inhibitors and concentrate HuNoV in fecal samples (Gilpatrick et al., 2000) but the method required specific antibody to capture particular genogroup of HuNoV. Furthermore, human histo-blood group antigen-conjugated to magnetic beads was reported to concentrate HuNoV but the method was only applied to detect HuNoV GII.4 from artificially contaminated food sample (Tian et al., 2011). Several manual kits, including RIDA[®]QUICK, Generon and AnDiaTec are commercially available to detect HuNoV; however, those kits are reported to have less capability to detect some genotypes of HuNoV GI in fecal specimens (Bruggink et al., 2015; Butot et al., 2010). As mentioned before, even though the difference in number of HuNoV-positive sample detected by Con A-PAB and VRNA methods, the methods showed equal number of HAV-positive fecal samples (Fig. 1). Apart from HAV, rotavirus in a fecal sample was detected by the Con A-PAB method but not detected using VRNA method. Consistently, other study also showed that the less ability of VRNA method to detect rotavirus in fecal sample due to the carryover of PCR inhibitors in the extract (Esona et al., 2013). However, reference rotavirus strains in absence of fecal matter were reported to be detected by the VRNA method (Kim and Kim, 2016).

Viral genomic sequencing, which authentically verified the presence of the viral genome, was performed to confirm the results of Con A-PAB and VRNA methods (Yang et al., 2017). The genomic sequencing validated the presence of all HuNoV genotypes GI.2, GI.4, GI.5, GII.1, GII.4, and GII.13 detected by Con A-PAB method in fecal samples with high level of sequence alignment (> 93%) (Supplementary Table 1). The identified two HAV genotypes (HAV47 and HAV18f) and rotavirus G2 using Con A-PAB method was validated by the genome sequencing with sequence alignment of > 98% (Supplementary Table 1). The high level of sequence alignment of virus genome

confirmed the presence of virus detected by Con A-PAB method.

The performance in terms of sensitivity, accuracy, specificity, PPV, and NPV of Con A-PAB and VRNA methods was compared with considering viral genome sequencing as confirmatory assay. Con A-PAB method showed a higher analytical sensitivity (100% vs. 42.9%) and accuracy (100% vs. 73.3%) to detect HuNoV than VRNA method (Table 2), indicating that Con A-PAB did not show false-negative results compared to VRNA method. The higher sensitivity of Con A-PAB method could be due to high binding affinity of Con A to bind with the virus (Kim et al., 2017). However, both Con A-PAB and VRNA methods showed high specificity (100%) to detect HuNoV, indicating that these methods did not show any false-positive results when validated with viral genome sequencing. Even though the specificity (100%) of different immunochromatographic assays, such as RIDA[®]QUICK, ImmunoCardSTAT[®], NOROTOP[®], and SD BIOLINE is high for the detection of HuNoV, the sensitivity (78%, 59%, 61% and 67%, respectively) of those assays is low and depends on virus genotypes (Ambert-Balay and Pothier, 2013). Moreover, the PPV was 100% for HuNoV detection using both methods, indicating that HuNoV-positive results from both methods are reliable to prove the presence of HuNoV in fecal samples (Table 2). However, with regard to NPV, the Con A-PAB method showed higher NPV than VRNA method, suggesting that the negative results from the Con A-PAB method could sufficiently prove the absence of HuNoV in fecal samples. Therefore, the Con A-PAB method could be a reliable method to confirm the absence of HuNoV infection.

In contrast to the performance of the method to detect HuNoV, both methods showed equal sensitivity, accuracy, and specificity (100% vs. 100%) for the detection of HAV in fecal samples (Table 2). Furthermore, PPV and NPV were also 100% for HAV detection using both methods. The results imply that the methods are equally reliable to

Table 2 Comparison between Con A-immobilized on polyacrylate beads and Viral RNA Extraction Kit methods with respect to their sensitivity, specificity, and predictive values

Parameters	Human norovirus		Hepatitis A virus	
	Con A-PAB method ^a (%)	VRNA method ^b (%)	Con A-PAB (%)	VRNA (%)
Sensitivity	100.0	42.9	100.0	100.0
Specificity	100.0	100.0	100.0	100.0
Accuracy	100.0	73.3	100.0	100.0
PPV ^c	100.0	100.0	100.0	100.0
NPV ^d	100.0	66.7	100.0	100.0

^aCon A-PAB, Concanavalin A immobilized on polyacrylate beads

^bVRNA, QIAamp Viral RNA Extraction Mini kit

^cPPV, Positive predictive value

^dNPV, Negative predictive value

detect HAV. Overall, Con A-PAB showed equal performance for detecting HAV and better performance for detecting HuNoV than VRNA method. The differences in the performance for detecting two viruses could be due to morphologic and genetic variations of viruses, which can alter the efficiency of inhibition of nucleic acid extraction and PCR (Haramoto et al., 2018; Hennechart-Collette et al., 2015). However, the constraint of study was the limited number of samples available for the study in the sampling area. Due to small sample size, the precision of sensitivity and specificity of the test could be affected.

In conclusion, even though Con A-PAB and VRNA methods were equally sensitive to detect HAV, the detection of HuNoV by the Con A-PAB method remarkably increased the sensitivity of diagnosis of the foodborne virus in fecal samples. The Con A-PAB based method could be an alternative to screening of different types of foodborne viruses in fecal samples.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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