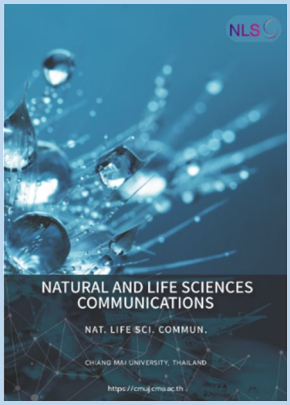
Research article



Production of Encapsidated RNA Particles as a Working Standard in Detecting Foodborne Viruses in Oysters

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ABSTRACT

Monitoring foodborne viruses via nucleic acid amplification tests rely on stable RNA standards to obtain reliable testing. This study aimed to produce RNA-based standard reagents for hepatitis virus (HAV) or norovirus detections which relies on viral-like particle (VLP) technology. Using a plasmid packaging system, plasmids containing DNA encoding Qβcoat protein (CP) monomer and the VP1 gene of viruses were co-transformed into *E. coli* host cells. In cell lysates, expressed CP was characterized by western blot and the whole icosahedral formation of VLPs was proved by electron microscope analysis. Encapsidated RNAs were measured and assessed as a standard by a two-step reverse transcription recombinase polymerase amplification (RT-RPA). Our results showed that CP has a distinguished protein band with a molecular weight of 14.5 kDa but a few variabilities of particle size were visualized. When adjusting the pH of the lysate to lower than 6, a more intense protein band and substantial particles with homogenous particle size were observed. These VLPs were found to enclose HAV and norovirus RNA contents to 1.2×10^7 copies/ng and 1.9×10^7 copies/ng, respectively. When analyzed by RT-RPA, linear regression analysis confirmed the alternative application of RNAs enclosed in VLPs to naked RNA synthesized from in vitro transcription. Using the *E. coli* expression system to produce Qβ VLPs allows cost-effective production and, therefore, can be implemented in laboratories with basic equipment. These encapsidated RNAs may become an ideal "standard" for detecting foodborne viruses via a molecular test in food and clinical samples.

Keywords: Molecular testing, Nanoparticles, Nucleic amplification, RNA standards, Viral-like particles

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INTRODUCTION

Foodborne viruses are predominantly transmitted to humans via fresh and minimally processed foods. They are present in low amounts yet threaten human health. Therefore, sensitive and specific methods are required to monitor viral contaminations in food. Due to their high sensitivity, nucleic acid amplification tests have been used to monitor foodborne viruses. However, molecular viral assays must contain dependable RNA controls or standards to obtain reliable testing. In addition, this type of RNA also serves as a quantitative "standard" or "calibrator" against which the samples are measured. These RNAs are commonly derived from inactivated infectious agents that raise safety concerns. Culturing pathogenic agents is also restricted to specialized laboratories. Alternatively, these RNAs are synthesized from a DNA plasmid by *in vitro* transcription. Nonetheless, RNA in its naked form is highly vulnerable to RNase, which is ubiquitous in the environment. Using long-term storage of RNA can lead to unreliable detection (Fleige and Pfaffl, 2006).

Naked RNA can be encapsidated to protect from RNase within virus-like particles (VLPs) that are self-assembled from one or several structural proteins. The particles mimic the conformation of a native virus, but without genetic materials. VLPs can be synthesized in cell-free systems, though the production yields are incredibly low (Lingappa et al., 2005). However, VLPs can be obtained in large quantities from a heterologous expression of the corresponding cloned genes. In recent years, VLPs have received noticeable attention to be used as a control for *in vitro* diagnostics (Beld et al., 2004; Eisler et al., 2004) along with vaccines (Khudyakov, 2008; Roldão et al., 2010; Liu et al., 2012), targeted drug or gene delivery, and nanotechnology (Soto and Ratna, 2010). With a good safety profile, VLPs were suitable as a nanoparticle to protect RNA (Fang et al., 2018). Simple bacteriophages, MS2 and Qβ, have mainly gained attention from researchers as they infect *Escherichia coli*. Their RNA genome can be encapsidated within their particles formed by 180 copies of capsid protein (CP) with diameters of roughly 28-30 nm (Golmohammadi et al., 1996; Machida and Imataka, 2015). The self-assembly of VLPs can be initiated by co-expressing recombinant RNAs and CPs containing Qβ hairpin (hp) from separately cloned plasmids in *E. coli*. VLPs were developed for applications in diagnostic procedures. RNAs encapsidated within MS2 VLPs are now used as controls or standards for the detection of various viruses, such as human immunodeficiency virus (Pasloske et al., 1998; Zhan et al., 2009), severe acute respiratory syndrome coronavirus (Drosten et al., 2001), enteroviruses (Beld et al., 2004; Donia et al., 2005), Avian influenza virus (Das et al., 2006), measles virus (D. Zhang et al., 2015; L. Zhang et al., 2015), and ebola virus (Wang et al., 2015). Fang and co-workers (Fang et al., 2017) previously demonstrated the packaging of non-viral RNA in Qβ VLPs. Notwithstanding, the synthesized VLPs required ultracentrifugation in CsCl or sucrose-gradients to purify VLPs, and therefore, they cannot be implemented in laboratories with essential equipment.

In this study, we aimed to produce RNA-based standard reagents for HAV or norovirus detections which rely on VLP technology. The study was also conducted to optimize the final VLP yields. Enclosed RNA was also assessed for use as the standard reagent in oyster sample. Unlike amid reverse transcription (RT)-polymerase chain reaction (PCR), RT-recombinase polymerase amplification (RPA) assay was unaffected by inhibitors found in food and carried out only with fundamental apparatus to maintain a constant temperature. For these reasons, we chose a two-step RT-RPA assay to detect RNA extracted from VLPs and asses for use as a standard for application in clinical laboratory tests and diagnostics.

MATERIALS AND METHODS

Samples

Fresh oysters from local markets were thoroughly cleaned by scrubbing their outer surface and rinsing with water. The whole forms were pooled into a 2-g individual sample and homogenized for nucleic-based analysis.

Primer designs

In this study, all primers were designed to specifically bind the conserved region of the coat protein gene, VP1 of all HAV, and norovirus strains known to date. Primers with a length of 35-bp were synthesized (Macrogen, Seoul, South Korea) and screened for amplification efficiency on agarose gel electrophoresis. The primer pairs of HAV and norovirus (Table 1) amplify 203- and 232-bp products from the former and the latter. Their specificity was also assessed against non-target enteric viruses.

Table 1. Primer sets used in this study.

Construction of Qβ coat protein (CP) and RNA expression plasmids

Oligo name	Oligo sequence (5' to 3')	Tm (°C)
Amplification reactions		
HAV_F4	TCTACTGAGCAGAATGTTCCTGATCCCCAAGTCGG	76.9
HAV_R1	CTGATGTATGTCTAAACTCTCCAGGTTTCAATTCA	70.9
NOV_F2	GCACCTGTAGCGGGCCAACAAAATGTAATTGACCC	76.9
NOV_R2	CTGTGAACGCGTTCCTCACTAGAATTACCTGCACT	74.9
VLP construction		
Qβ_CP F	AGTTCCATGGATGGCTAA	51.4
Qβ_CP_R	AACTCCTAGGTCAGTGAT	51.4
VLP F	AGTTTCTAGAAATATATA	40.3
VLP R	AACTGCTAAGCATGCTGT	51.4

A two-plasmid expression system was used to produce VLPs (Figure 1). pCDF-CP was initially generated by amplifying synthetic DNA encoding Qβ CP monomer (NC_001890) including via PCR with the Qβ CP primer pair (Table 1). The resultant DNA fragment including 3' hexahistidine (6X) Tag was digested with *Nco*I and *Avr*II and cloned into pCDF-1b vector (Addgene, Watertown, Massachusetts, USA). Transformants were selected on Luria agar plates (LA) containing 50 µg/mL streptomycin (Sm). The RNA expression plasmids, pET-HAV, and pET-NoV, were also produced through PCR using primers specifically bound to 5'spacer region and 3'Qβ hairpin of synthetic DNA (Table 1). Amplified fragments of 422 bp and 352 bp containing the VP1 gene of HAV (Accession No. EF207320) and norovirus (Accession No. MG78678), respectively were cloned at 5'*Xba*I and 3'*Blp*I restriction sites into pET-28b(+) (SnapGene, San Diego, California, USA). Transformants growing on an LA plate with 50 µg/mL kanamycin (KAN) were selected. Prior to expression, all transformants were screened for insert identity through double-enzyme digestion, PCR, and DNA sequencing.

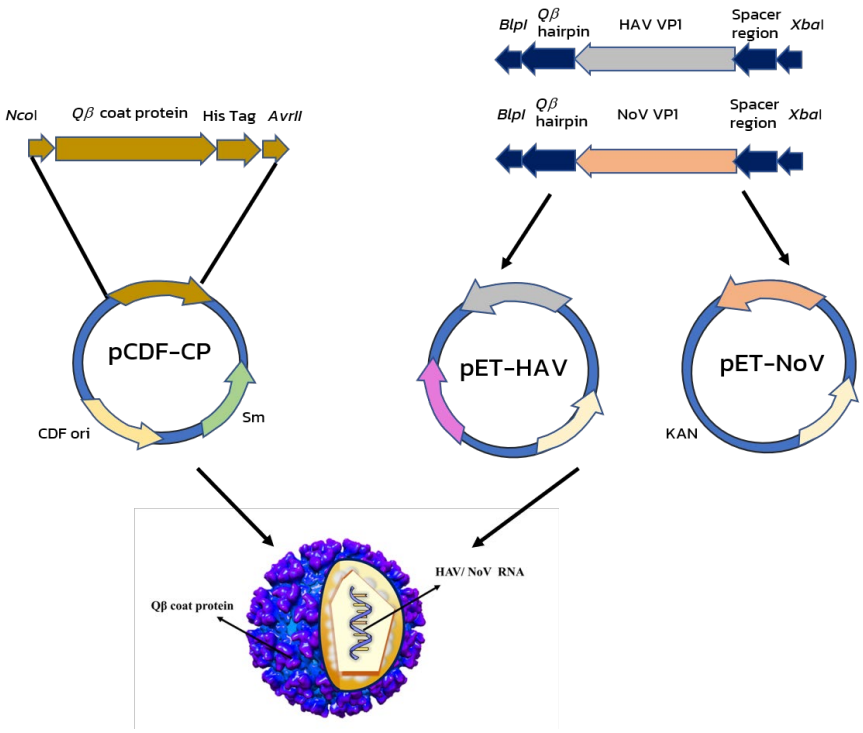

Figure 1. Schematic Flow diagram of methods used to construct the hepatitis virus- and norovirus viral-like particles (VLPs).

Expression of Qβ CP and viral RNA

The recombinant plasmids were expressed under T7 promoter/*lac* operon control in *E. coli* BL21 (DE3). pCDF-CP was co-transformed with either pET-*NoV* or pET-HAV into *E. coli* host and selected on 50 µg/mL Sm and KAN. A group of single colonies were inoculated into 5 mL NYZ medium [1% selected peptone (N.Z. amine)], 0.5% sodium chloride, 0.5% yeast extract) with the corresponding antibiotics. After being incubated overnight at 37°C Incubator (Contherm, Wellington, New Zealand), one milliliter of the bacterial culture was transferred into 100 mL of ZYM-5052 auto-induction medium (Studier, 2005) and allowed to continue growing for 24 h. After centrifuging at 6,500g, 4ºC for 30 min, the cell pellets were collected and resuspended in an equal volume of Qβ buffer (10 mM $MgCl_2$, and 20 mM Tris-HCl, pH 7.5). Then 10 µL of 10X protease inhibitor was added to the cell suspension in the final 1%. The VLPs were released from host cells by sonication (Sonicator Probe: UP50H (Hielscher, Teltow, Germany) with 10-s on/off intervals on ice for 10 min or until the crude lysates were clear. The crude lysates were adjusted to pH between 3 and 5. Non-pH adjusted (pH 6) and pH-adjusted lysates were centrifuged (KITMAN-T24, TOMY, Tokyo, Japan) at 1600g for 5 min. After discarding cell debris, the supernatant containing RNA enclosed VLPs was collected. Empty Qβ VLP extracted from cells harboring only pCDF-CP was used as a control.

Protein purification by nickel -nitrilotriacetic acid (Ni-NTA) chromatography

VLPs in the crude lysates were isolated using Ni-NTA chromatography (HisPur™ Ni-NTA Chromatography Cartridge, Thermo Scientific, Waltham, Massachusetts, USA) based on the 6XHis Tag design at the carboxyl-terminal of Qβ CPs. The cartridge was equilibrated with 5–10 column volumes of an equilibration/binding buffer (20 mM sodium phosphate, 300 mM sodium chloride, and 10 mM imidazole at pH 7.4) at a flow rate of 1–2 mL/minute for 1-mL cartridge. Bacterial lysate (10 mL) was mixed with the equilibration/binding buffer

at a ratio of 1:1 and applied to the cartridge. Subsequent to washing the resin with 10–15 column volumes of a wash buffer (20 mM sodium phosphate, 300 mM sodium chloride, and 20–40 mM imidazole at pH 7.4), the coat proteins of VLPs were eluted with an elution buffer (20 mM sodium phosphate, 300 mM sodium chloride, and 300 mM imidazole at pH 7.4). Purified VLPs (2 mL) were assessed for the protein concentrations using a Pierce BCA protein assay kit (Thermo Scientific, Waltham, Massachusetts, USA) and compared with a bovine serum albumin standard curve. The proteins were stored at −80°C until ready to be employed amid experimentations.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analyses

SDS-PAGE was utilized to analyze the molecular weight of Qβ CP monomers with 12% separating gel compared with the Precision Plus Protein Dual Color Standard protein marker (Bio-Rad, Hercules, California, USA). The protein gels were stained resultant of continuous shaking in solution (0.1% (w/v) Coomassie blue R-250 (CBB), 20% (v/v) methanol, 10% (v/v) acetic acid) for 30 min and destained in solution (50% (v/v) methanol and 10% (v/v) acetic acid) until the gel background was clear. The protein bands on a replica gel were transferred onto a nitrocellulose membrane in transfer buffer (192 mM glycine, 25 mM Tris 10% (v/v) methanol) at 25 V overnight. The membranes were soaked in blocking solution for 4 h at room temperature. The anti-Histidine tag, which was the primary antibody (1:2000), was added and incubated for 2 h at room temperature. Post-washing, the protein complexes were bound with the secondary antibody (optimized horseradish peroxidase reagent working dilution 1:5000) for 1 h and detected with KPL 1-component TMB Membrane Peroxidase substrate (Gaithersburg, Massachusetts, USA) until protein bands of the expected 14.5 kDa were visualized.

Transmission electron microscopy

Ten microliters of samples were deposited on a formvar film-coated copper grid and incubated for 2 min. After drying on a grid for 12 h, the grid was stained in 15 µL of 2% aqueous uranyl acetate for 2 min. The dried grids were kept at 25°C prior to imaging with a Phillips Tecni20 transmission electron microscope.

Preparation of RNA transcribed *in vitro*

A total of 20 µl reaction contains 2 µL of T7 RNA Polymerase Mix, 10 µL of NTP buffer, and 1 µg of plasmids in 8 µL of dH_2O. After incubation at 37°C for 4 h, DNase I was added to the reaction to eliminate the plasmid DNA. The transcribed RNA products were purified prior to analysis for quantity and quality by a nanodrop (ND-2800-ODJ Nano DOT Nucleic Acid Analyzer, Hercuvan Lab system, Malaysia) and denaturing RNA electrophoresis. All RNAs were aliquoted and stored at -80°C until use.

Preparation of denaturing gel electrophoresis for RNA analysis

Eight microliters of RNA transcribed product were first mixed with 2 µL of 10X MOPS Buffer (200 mM MOPS, 50 mM sodium acetate, 10 mM EDTA·$2H_2O$, 10 mM EGTA). The 10 µL formamide was then added to the mixture to obtain a total of 20 µL mixture. The mixtures were heated at 70°C for 10 min. After suddenly chilling with ice for ≥1 min, the RNA mixtures were added with 2 µL formaldehyde loading buffer (1 mM EDTA, pH 8.0, 0.4% bromophenol blue, xylene cyanol, and 50% glycerol). The RNAs were analyzed in 1% agarose gel electrophoresis containing 1X MOPS Buffer and 37% (v/v) formaldehyde at 50 V for 60 min. The RNA bands were visualized with 1 µg/mL ethidium bromide.

RNA extraction from VLPs

Fifty micrograms of viral VLPs were resuspended in 250 µL of extraction buffer (5% SDS, 25 mM DTT, 20 mM Tris-HCl, pH 7.5, and 50 mM NaCl). VLP suspension was boiled to release RNA at 70°C for 5 min or extracted with the TrizolTM reagent (Thermo Scientific, Waltham, Massachusetts, USA) following the manufacturer's instructions. In spiking experiments, 2 g of virus-free oysters were added with 5 µL of 10-fold diluted of HAV-VLPs or norovirus-VLPs and incubated at 37°C for 1 h. Each spiked sample was then added with 2 mL of 100 µg/mL proteinase K (Biotechrabbit, Hennigsdorf Germany). The mixtures were incubated at 37°C for 1 h and then at 60°C for 15 min under continuous agitation. The cell debris was separated via centrifugation at 14,000g for 5 min and filtered twice with filter papers. To obtain RNA, the flow-through was either boiled at 70°C for 5 min or extracted with the TrizolTM reagent (Thermo Scientific, Waltham, Massachusetts, USA). Nanodrop and agarose gel electrophoresis were used to check RNA integrity and concentration. RNAs were then applied to amplify cDNAs by RT.

Reverse transcription (RT)

The RNAs were reverse transcribed to obtain the single-stranded cDNA using a Random Hexamer RevertAid First Strand cDNA Synthesis kit (Thermo Scientific, Waltham, Massachusetts, USA). Following the manufacturer's instructions, a total volume of 20 µL reaction contains the total RNAs (0.1 ng–5 µg), 1 µL of oligo dT Random Hexamer primer, 4 µL of 5X Reaction Buffer, 1 µL of Ribolock RNase inhibitor (20 U/µL), 2 µL of 10 mM dNTPs mix, and 1 µL of RevertAid M-MuLV RT (200 U/µL). The mixture was initially incubated at 25°C for 5 min, followed by 42°C for 60 min. The reaction was stopped by shifting the temperature to 70°C for 5 min. The resulting single-stranded cDNAs were used as the templates for the downstream RPA reaction. The copy number was calculated according to the following Equation.

$$\text{DNA copies (molecules)} = \frac{mass\ of\ ssDNA\ (ng) * 6.0221\ x\ 10^{23}\left(\frac{molecules}{mole}\right)}{\left[\mathrm{N} * 330\left(\frac{\mathrm{g}}{\mathrm{mole}}\right)\right] * 1\ x\ 10^{9}\left(\frac{\mathrm{ng}}{\mathrm{g}}\right)}$$

Recombinase polymerase amplification (RPA)

Following the manufacturer's instructions (TwistAmp Ltd., Cambridge, UK), 1 µL of cDNA templates was added to 29.5 µL TwistAmp rehydrate buffer containing 0.42 µM forward and reverse primers. Two and a half microliters of 14 mM Mg $(Ac)_2$ were then added to start the 50 µL of reaction at 37°C for 20 min. Subsequent to purification using a GenepHlowTM Gel/PCR Kit (Geneaid, New Taipei City, Taiwan), the purified amplification products were analyzed on 1% (w/v) agarose gels containing 1X SYBR Safe (Invitrogen by Thermo Fisher Scientific, USA) in 1X Tris-Boric acid-EDTA (TBE) buffer.

Assessment of enclosed RNAs used as a standard

Serial 10-fold diluted cDNA templates were made. One microliter of each diluted template was employed in RPA reaction, and their amplified products were analyzed through agarose gel electrophoresis. Band intensities of these products on agarose gel were measured using the Image J program (developed by the National Institutes of Health and Laboratory for Optical and Computational instrumentation). Data were subsequently converted to molecules. In addition, RNA products from *in vitro* transcription were also applied as templates in RT-RPA. After gel electrophoresis, a graph was generated. Band intensity of the reaction products of RNA from VLP was set on the Y-axis and that of transcribed RNA on the X-axis. In this study, each dilution was tested in duplicate, and the whole assay was performed twice.

Statistical analysis

Standard curves were generated and linear regression was used to test for equivalence of of RNAs enclosed VLPs versus naked RNAs. Data with a correlation co-efficiency of $r^2 \geq 0.9$ was considered to be acceptable for analysis.

DNA verification by sequencing

Amplified RT-RPA products were purified from the agarose gel (GeneHlow Gel/PCR kit; Geneaid, New Taipei City, Taiwan). DNA sequencing was used to verify their sequence identities (Macrogen, Seoul, South Korea).

RESULTS

Expression of DNA plasmids and characterization of QβVLPs

Expression plasmids were tested for their expression ability by *in vitro* transcription. The resulting RNA were visualized on 1% denaturing agarose gel electrophoresis (data not shown). After *in vivo* assembly via co-expressing recombinant RNAs and CPs, the VLPs were extracted from *E. coli* BL21(DE3) through sonication and purified using Ni-NTA chromatography. SDS-PAGE and western blot analysis showed that pCDF-CP alone, or either with pET- HAV or pET-NoV expressed the CP monomer (Figure 2A and 2B, respectively). All purified constructs showed a distinguished CPs protein band with a molecular weight of 14.5 kDa without proteolytic cleavage. To ensure that the constructed VLPs remain intact, the morphology of VLPs was visualized via transmission electron microscopy. Low with variably-sized particles were observed (Figure 2C).

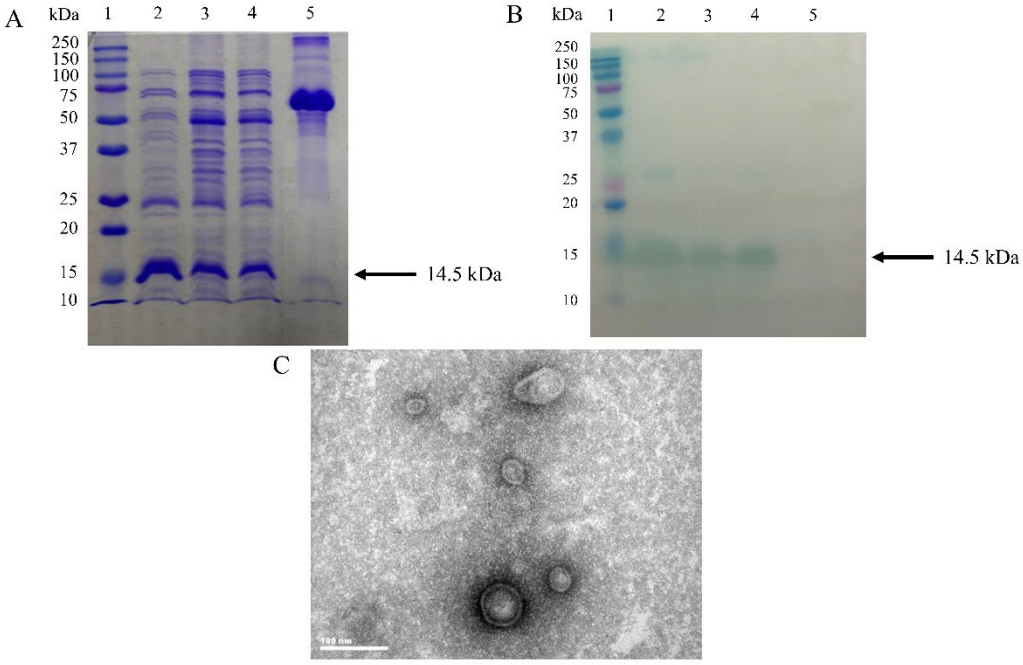

Figure 2. Characterization of viral-like particles (VLPs). (A) SDS-PAGE and (B) Western blot using anti-His Tag antibody. Lane 1: The Precision Plus Protein Dual Color Standard protein marker (Bio Rad, Hercules, California, USA). Lane 2: Qβ coat protein (CP) monomer. Lane 3: HAV-VLPs. Lane 4: NoV-VLPs. Lane 5: Bovine serum albumin was used as the negative control. The arrows on the right of Figures 2A and 2B indicate the expected molecular weight of Qβ CP monomer of 14.5 kDa. (C) Transmission electron microscope images of HAV-VLPs. Scale bar=100 nm.

The production of RNA enclosed VLPs

Following sonication, the crude lysates were adjusted to a pH between 3 and 5. When adjusting pH to lower than 6, SDS-PAGE and western blot displayed a more intense protein band than at pH 6 (Figure 3A and 3B, respectively). We selected crude lysates at pH 4 which seems to be the optimal condition for protein expression to finally prove the whole icosahedral formation of the VLPs. Transmission electron microscopy revealed substantial particles with homogenous particle size at an average diameter of 30 nm (Figure 3C). To ensure the packaging of RNA in particles, VLPs were heated at 70°C for 5 min. Under such conditions, RNA can be quickly released from its packaging by which its yield is roughly comparable to that of the TrizolTM reagent (Figure 4). The production yield of HAV and norovirus packaged RNA is about 68.89 ng and 126 ng of RNA for every 50 ug VLP (protein) measured through spectrophotometry. The Equation can convert these RNA contents to $1.2x10^7$ copies/ng of the former and $1.9x10^7$ copies/ng of the latter.

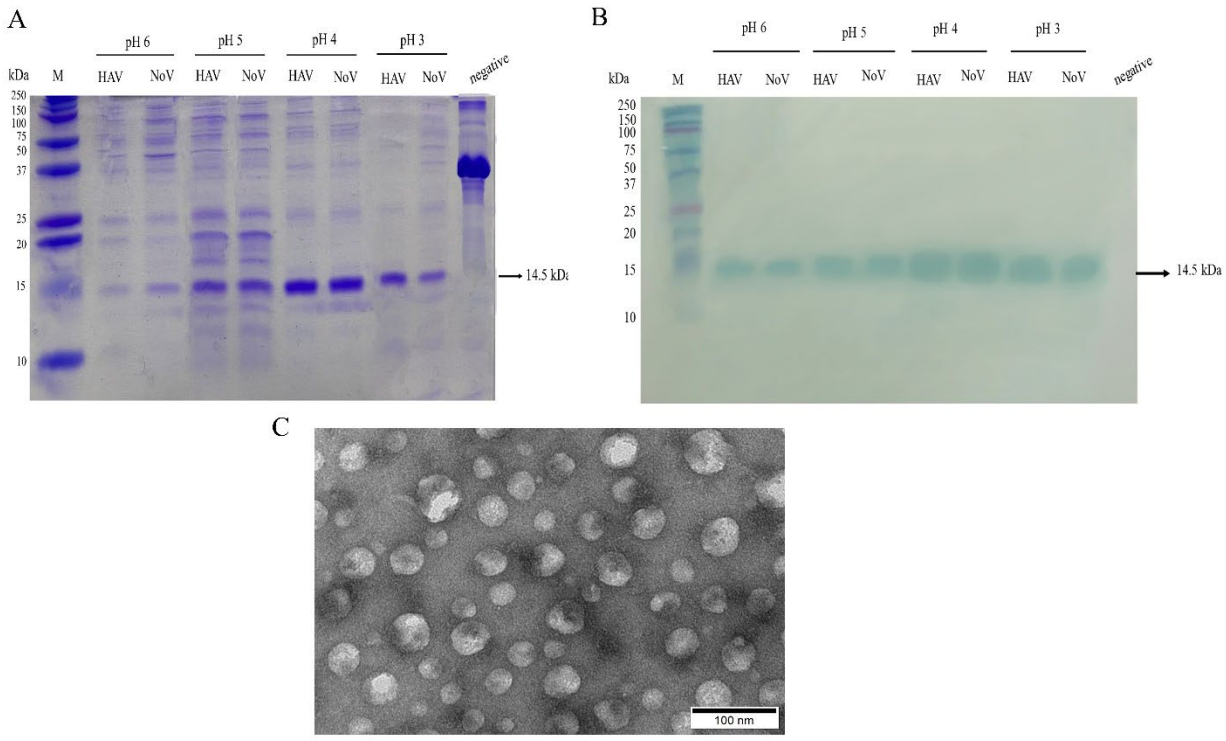

Figure 3. The effect of pH on the production of VLPs. (A) HAV-VLP and NoV-VLPs in the lysates at pH 3 to 6 were characterized by SDS-PAGE and (B) Western blot using anti-His Tag antibody. M: The Precision Plus Protein Dual Color Standard protein marker (Bio Rad, Hercules, California, USA). Bovine serum albumin was used as the negative control. The arrows on the right of Figures 2A and 2B indicate the expected molecular weight of Qβ CP monomer of 14.5 kDa. (C). Transmission electron microscope images of HAV-VLPs from the lysate at pH 4. Scale bar=100 nm.

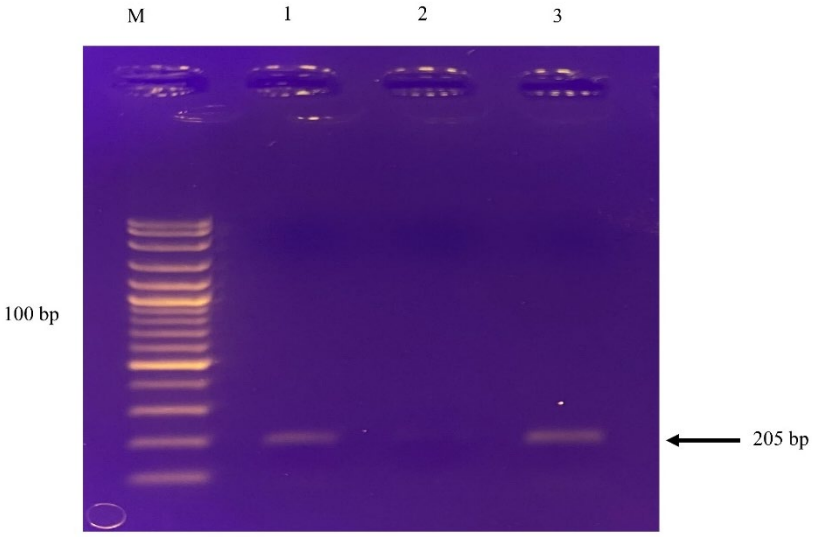

Figure 4. The packaging of RNA in VLPs. RNAs were extracted from VLPs and compared with TrizolTM reagent. RNA samples were used as templates in the two-step RT-RPA. The amplified products from heated VLPs (Lane 1), unheated VLPs (Lane 2), and TrizolTM reagent (Lane 3) were visualized on 1% agarose gel electrophoresis.

Assessment of VLPs used as a standard

RT-RPA yielded a clear signal when RNA extracted from purified VLPs was at a minimum of 4.54×10^4 molecules. In the spiking experiment, a minimum of RNA was detected by RT-RPA at 1.34×10^{10} molecules. To study the relationship between transcribed RNA and RNA extracted from VLP applied as templates of RT-RPA, the R^2 value in the linear regression model showed excellent linearity (R^2 = 0.9333) (Figure 5).

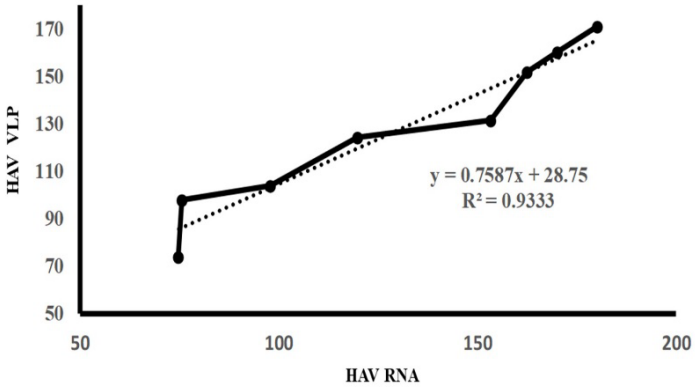

Figure 5. The equivalence of RNA enclosed viral-like particles to naked RNAs. Ten-fold serial dilutions of transcribed RNA and RNA extracted from HAV-VLPs were amplified by RT-RPA. Their band intensities of amplified products of RT-RPA on agarose gel were measured by program J (developed by the National Institutes of Health and Laboratory for Optical and Computational instrumentation). Linear Regress was used to test for the equivalence with R^2 = 0.9333.

DISCUSSION

In this study, we proposed a simple and economical method for synthesizing RNA-based standard reagents for HAV or norovirus detections via RT-RPA. Most molecular testing depends on using RNA synthesized by *in vitro* transcription for positive controls and external standards. Although transcribed RNAs are easier to prepare for detection, long-term storage affects their stability, thus leading to unreliable testing. Based on VLP technology, RNA was enclosed in a viral protein shell composed of Qβ CP monomers which are self-assembled in their precise and repeated structures. The thermodynamic stability of the icosahedral structure of VLPs protects enclosed RNA against RNases (WalkerPeach et al., 1999) and small diffusible species (Fang et al., 2018). In addition, RNA is more compact in the relatively large cavity space of VLPs and less dynamic than RNAs free in solution (Dent et al., 2013), thus making it less vulnerable to RNases.

Native Qβ bacteriophages lyse *E. coli* host and release particles into the culture media. However, synthesized VLPs were localized in the cytoplasmic fraction of *E. coli* and required disruption of cells by sonication. During a purification process, the extraction buffers are supplemented with reducing and chelating agents and protease inhibitors to protect the VLPs from oxidation and host proteases. After clarification through low-speed centrifugation, the VLP-containing solutions have to be concentrated by filtering to decrease the volume of the extract. Further VLP purification reduces the amount of host-derived impurities. Instead of using ultracentrifugation in CsCl or sucrose gradients, adding 6XHis-Tag at the carboxyl-terminal of Qβ CPs facilitates purification through affinity chromatography. The Tag design also aids in identifying the VLP structural proteins with western blot analysis using an anti-His tag antibody. However, amino acid substitutions could interfere with CP folding and result in VLP instability (Stonehouse and Stockley, 1993). In this study, the introduced Tag directly into the VLP structure did not lead to proteolytic cleavage as seen as a distinct band of 14.5 kDa on the western blot. Our result agreed with a previous report of including the His-tag at the N-terminal part of the CP of potato virus Y-like particles baculovirus (Kalnciema et al., 2012). For complete characterization, an electron microscope analysis is necessary as the visual proof of the newly synthesized VLPs. In our study, the added Tag did not interfere with the whole icosahedral formation of the VLPs. These particle sizes of about 30 nm were observed. Nevertheless, the yield of VLP production was low with variably-sized particles, which probably resulted from large, unstructured protein aggregates.

Several physicochemical factors can affect VLP stability; pH is one such factor. Some studies demonstrated that bacteriophage MS2 could stay stable at pH 7 despite becoming less stable as pH decreases (Lago et al., 2001). In this study, when adjusting the pH of the lysate after sonication to lower than 6, the increased abundance of CP bands was visible on the western blot. The production yield of QβVLPs also improved at pH 4 probably due to the fact that, at a lower pH, non-VLP particles and aggregates were removed from filtration. Thus, the filtered solution substantially retains the complete and highly uniform VLP particles, as clearly seen with the EM. Besides pH factors, temperature also strongly impacts the stability of VLPs. Stonehouse and Stockley (Stonehouse and Stockley, 1993) found that the MS2 phage had a melting point of 66°C and can withstand temperatures as high as 68°C whereas Qβ VLPs are stable over a broad range of temperatures (Fiedler et al., 2010). In addition to those factors, the stability of VLPs can also be markedly raised by introducing disulfide bridges to crosslink the individual CPs (Bundy and Swartz, 2011).

Heating at 70°C for 5 min quickly released the packaged RNAs from the Qβ CP. The yield of RNAs was roughly comparable to that extracted from the TrizolTM reagent. In our study, the production yield of HAV and norovirus RNA was 68.89 ng and 126 ng for every 50 ug VLP (protein) measured by spectrophotometry or $1.2x10^7$ copies/ng and $1.9x10^7$ copies/ng, respectively. Sufficient RNA was

packaged in the particles to fully compensate for a cationic charge of Qβ CP (Muriaux et al., 2001). The packaging efficiency of our results was consistent with that in a plant cowpea mosaic virus (CPMV) in the range of 10^2-10^8 copies/ ng of VLP (Peyret et al., 2022). Still, it was lower than what was previously reported (5 µg) (Fang et al., 2018). The differences in packaging efficiency possibly resulted from the resistance markers of the expression plasmids and the composition of the cultivation medium that influenced the assembly of Qβ VLPs (Brown et al., 2009). The efficient packaging of RNA within VLPs *in vivo* may also depend upon the compactness of the target RNA structure, the absence of competing for binding motifs in the RNA, besides the presence of the Qβ hp (Fang et al., 2018). In this study, HAV, and norovirus RNAs presented a similar length of approximately 200 nucleotides. They could be encapsidated within VLPs with equal packaging efficiency. Notwithstanding, RNA length is not a solid predictor of packaging efficiency, though intrinsic RNA compaction affects the effectiveness of RNA wrapping within Qβ VLPs (Fang et al., 2018). Even in the same type of VLPs, we cannot assume that every particle is filled with the same amount of RNA or devoid of any RNA. If needed, density gradient ultracentrifugation can be used to distinguish Qβ VLPs based on their RNA contents. Since virus detection via RT-RPA in the food matrix is not anticipated, this was not performed for the current investigation.

Since the recombinant RNA in VLPs can be released from its packaging by low-temperature heating, VLPs can be added directly to the oyster samples in the spiking experiment. Our results revealed that both VLP-packaged and free recombinant RNA from *in vitro* transcription presented no difference in detection via RT-RPA. Accordingly, these results could arise from using newly synthesized RNA rather than RNA kept for an extended period. In addition, linear regression analysis strongly emphasizes the alternative use of encapsidated RNA to naked RNA.

The plasmid, a highly stable form of nucleic acid, can be transcribed into RNA through *in vitro* transcription. Although this method is effective, it can be time-consuming and expensive. In theory, RNA products from this reaction can be stored for extended periods at a temperature of -80°C. However, our observations, which were made using agarose gel electrophoresis and spectrophotometry detection, revealed that RNA samples from *in vitro* transcription tend to gradually degrade over time, with a degradation rate of approximately 25% after one week. This phenomenon may be attributed to fluctuations in temperature within the deep freeze. While freezers set to -20°C are more commonly found in laboratories with basic equipment, they are only suitable for short-term storage of RNA. Our experience indicates that RNA extracted from VLPs stored at -20°C for 15 months still exhibited visible results on an agarose gel, and hence, better amplifiability. In addition, it should be noted here that the utilization of HAV and norovirus Qβ VLPs presents numerous advantages over that of an intact virus. Production of a human virus relies on a cell culture system which is time-consuming. Culturing HAV and human norovirus can be even more challenging due to the lack of a cultivation system. Furthermore, norovirus has a high mutation rate, resulting in a non-homogenous sequence of a standard. Recombinant RNAs thus provide a well-characterized sequence less likely to contain variations.

CONCLUSION

VLP preparation methods are designed to be suitable for RNA storage in basic or minimally equipped laboratories. These encapsidated RNAs can be used as the working standards in detecting foodborne viruses. Moreover, the high yield of these VLP particles and stability of the RNAs allow for cost-effective production. Hence, this construct may become an ideal "control" and "standard" for application in clinical laboratory tests and diagnostics.

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AUTHOR CONTRIBUTIONS

Uraiwan Intamaso designed the experiments and wrote the manuscript. Palatip Chutoam performed the statistical analysis and data visualization. Supannee Lethochavalit collected specimen. Suthasinee Jinda conducted the experiments. All authors have read and approved of the final manuscript.

CONFLICT OF INTEREST

The authors declare that they hold no competing interests.

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