Journal of Virological Methods 249 (2017) 161–164

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Short communication

Development of a fast and efficient method for hepatitis A virus concentration from green onion

Yan Zheng, Yuan Hu*

Northeast Regional Laboratory, Office of Regulatory Affairs, U.S. Food and Drug Administration, 158-15 Liberty Ave, Jamaica, NY 11433, USA

ARTICLE INFO

Keywords:
Hepatitis A virus
Concentration methods
Carboxyl beads
Green onions

ABSTRACT

Hepatitis A virus (HAV) can cause serious liver disease and even death. HAV outbreaks are associated with the consumption of raw or minimally processed produce, making it a major public health concern. Infections have occurred despite the fact that effective HAV vaccine has been available. Development of a rapid and sensitive HAV detection method is necessary for an investigation of an HAV outbreak. Detection of HAV is complicated by the lack of a reliable culture method. In addition, due to the low infectious dose of HAV, these methods must be very sensitive. Current methods rely on efficient sample preparation and concentration steps followed by sensitive molecular detection techniques. Using green onions which was involved in most recent HAV outbreaks as a representative produce, a method of capturing virus particles was developed using carboxyl-derivatized magnetic beads in this study. Carboxyl beads, like antibody-coated beads or cationic beads, detect HAV at a level as low as 100 pfu/25 g of green onions. RNA from virus concentrated in this manner can be released by heat-shock (98 °C 5 min) for molecular detection without sacrificing sensitivity. Bypassing the RNA extraction procedure saves time and removes multiple manipulation steps, which makes large scale HAV screening possible. In addition, the inclusion of beef extract and pectinase rather than NP40 in the elution buffer improved the HAV liberation from the food matrix over current methods by nearly 10 fold. The method proposed in this study provides a promising tool to improve food risk assessment and protect public health.

Hepatitis A virus (HAV) ranks ninth among identified causes of foodborne diseases (Havelaar et al., 2015). In spite of vaccine introduction in 1995, HAV continues to be the leading cause of viral hepatitis and remains a global public health problem. Multiple high-profile outbreaks occur in the U.S. and worldwide. One example was the 2003 large outbreak in Pennsylvania in which contaminated green onions were identified to be the infectious source. Totally more than 600 sick patients, 124 hospitalization and 3 deaths were reported in this outbreak (Wheeler et al., 2005). HAV is transmitted through the fecal-oral route (Pinto et al., 2010; CDC, 2009). The association of the fresh produce consumption and outbreaks demands regulatory agencies to be equipped with sensitive HAV detection method for both outbreak investigation and routine surveillance.

One of the major HAV detection challenges lies in the resistance of field strain in tissue culture propagation (Weilandt et al., 2014). As a result, the presence of HAV is identified using PCR based molecular detection methods. In addition, the minimal HAV infectious dose is predicted to be less than 100 infectious particles (Papafragkou and Kulka, 2016). Such a small amount of virus hidden in the complex food matrix further complicates viral detection. Continuous effort is needed to develop a robust method with high sensitivity and consistency. Typical HAV detection starts from food sample collection, followed by sample preparation and molecular detection. Common sample preparation procedure begins with an elution step, which separates intact virus from the food matrix (Papafragkou and Kulka, 2016). Several reagents including glycine, beef extract, soy proteins have been previously reported to improve virus liberation from the food matrix (Dubois et al., 2002; Rzezutka et al., 2006). After elution, the released virus particles are concentrated in a small volume to enable follow-up detection. Typical concentration approaches include polyethylene glycol (PEG) precipitation, affinity/immuno-concentration, ultracentrifugation, and ultrafiltration (Williams-Woods and Burkhardt, 2013; Coudray et al., 2013; Bidawid et al., 2000; Uchida et al., 2007). RNA from concentrated viruses is then purified and detected using real-time reverse transcription PCR (RT-qPCR) (Papafragkou and Kulka, 2016). Instead of isolating whole virus particles, alternative sample preparation approach is to lysis virus in the presence of food matrix and use of concentrated viral RNA for RT-qPCR reaction (Perrin et al., 2015; Baert et al., 2008; Hida et al., 2013).

Affinity concentration commonly uses magnet beads coated with

* Corresponding author.
E-mail address: Yuan.hu@fda.hhs.gov (Y. Hu).

http://dx.doi.org/10.1016/j.jviromet.2017.09.013
Received 27 March 2017; Received in revised form 18 July 2017; Accepted 13 September 2017
Available online 14 September 2017
0166-0934/ Published by Elsevier B.V.

HAV specific antibodies to purify virus particles from eluents. However, the binding between HAV and the antibody may weaken due to immunogenetic drift caused by rapid viral mutation, leading to a long-term risk of false-negative detection (Stals et al., 2012). Cationic beads exemplified by polyethyleneimine (PEI) coated beads, which hypothetically bind to the negatively HAV capsid proteins nonspecificly and are thus less sensitive to immunogenetic drift, were explored for HAV concentration (Uchida et al., 2007; Papafragkou et al., 2008). Using automated magnetic capture system, one group has reported that cationic beads consistently detect as low as 10^{-1} pfu HAV/25 g food sample, although only 7×10^2 pfu HAV/25 g sample is detected in a second lab using similar method (Papafragkou et al., 2008).

Technological advances have also been made in other HAV sample preparation methods. For example, ultrafiltration has been reported to achieve a recovery rate of 60%, whereas direct RNA isolation method has achieved a detection limit of 300 pfu/25 g food matrix (Perrin et al., 2015; Hida et al., 2013; Lee et al., 2012). Recently, an ultracentrifugation-based method has been adopted by the U.S Food and Drug Administration (FDA). This method can consistently detect HAV contamination at a 50 PFU/g (1250 pfu/25 g) when green onions are used as a fresh produce (Papafragkou and Kulka, 2016; Williams-Woods and Burkhardt, 2013).

The goal of this study is to develop a foodborne HAV sample preparation method for improved sensitivity and reduced sample manipulation. More specifically, different elution buffer used for virus separation from the food matrix and specific beads used for affinity concentration approach will be evaluated.

To optimize elution buffer, the effect of adding 1% beef extract and pectinase (Dubois et al., 2002; Stals et al., 2012) 2 or membrane solubilizing 1% NP40 to the standard FDA adopted glycine elution buffer (0.75 M Glycine and 0.15 M NaCl, pH 7.6) (Williams-Woods and Burkhardt, 2013) was tested. A whirl-pak bag containing 25 green onions inoculated with specified amount of HAV (HAV HM175/18f from ATCC) were treated with either of the following elution buffers. 1) glycine buffer (0.75 M Glycine, 0.15 M NaCl, pH 7.6). 2) Glycine buffer + 1% NP40 (0.75 M Glycine, 0.15 M NaCl, 1% beef extract, pH 7.6). 3) Glycine buffer + beef extract + pectinase (Sigma-Aldrich, St. Louis, MO) (0.75 M Glycine, 0.15 M NaCl, 1% beef extract, 100U pectinase pH 7.6). 4) TGBF buffer + 100U pectinase (100 mM Tris, 50 mM Glycine, 1% beef extract, 100U pectinase, pH 9.5). After shaking at room temperature for 30 min, viruses liberated into the elution buffers were concentrated by ultracentrifugation at 36,000 rpm for 1 h, followed by RNA extractionusing QIAamp® viral RNA extraction kit and RT-qPCR detection with 3 µl RNA in a 25 µl Qiagen OneStep RT-PCR reaction system (Williams-Woods and Burkhardt, 2013). The RT-qPCR reaction was carried out by a Smart Cycle II. A dual-labeled (Cy5 and a quencher) hydrolysis probe, which enables fluorescent HAV detection, and a RNA internal amplification control, which monitors matrix-derived inhibition, were both included in the RT-PCR reaction. Mock HAV infected green onions were used as a negative control in every experiment. A standard curve using RNA at specific concentration dilutions is generated along with each RT-qPCR experiment. The correlated coefficiency of the standard curve in a single experiment ranged from 0.96 to 1. Threshold cycle (Ct) with Cy5 emission at 10 fluorescence units was recorded and compared with standard curve for virus concentration calculation. Statistical analysis was performed using student's *t*-test.

As shown in Table 1, 1% beef extract and 100U pectinase significantly improved elution efficiency, probably because beef extract blocks non-specific virus-food adsorption (Stals et al., 2012) and pectinase solubilizes pectin and facilitates virus release (Hida et al., 2013). 100 mM Tris in TGBF buffer slightly improved virus separation, as evidenced by the recovery rate between buffer 3) and buffer 4), but the difference was not statistically significant. Although HAV is a non-enveloped RNA virus, about 80% of tissue-cultured virus particles are cloaked in a host-derived membrane (Williams-Woods and Burkhardt, 2013). The amount of virus released into the elution buffer was comparable with or without 1% NP40, suggesting that host-derived membranes did not prevent HAV liberation from the food matrix.

Table 1
HAV recovery rate with different elution buffers.

Elution buffer	Recovery at 5×10^3 inoculation[a]	Recovery% at 1×10^4 inoculation
Glycine Buffer	(0.3 ± 0.2)%	(1.0 ± 0.2)%
Glycine + 1% NP40	(0.3 ± 0.1)%	N/A[b]
Glycine buffer + beef extract + pectinase	(4.8 ± 1.9)%*	(3.9 ± 2.5)%
TGBF buffer + pectinase	(8.4 ± 1.4)%*	(9.6 ± 5.0)%*

* Statistically significant (Student's *t*-test, $p < 0.05$) compared to glycine buffer.
[a] Recovery was defined as total PFU detected/PFU inoculum. PFU detected was calculated based on the standard curve. The numbers were the (average ± standard deviation) of recoveries from three independent experiments.
[b] Not applicable.

After separating viruses from the food matrix, various concentration methods (Fig. 1) can be used to concentrate virus into a small volume for molecular analysis. Among these, bead-based concentration does not require expensive equipment and is relative easier in operation. To optimize bead-based concentration method, antibody-coated beads, cationic beads including PEI or amine group coated beads, and carboxyl derived magnet beads were compared in their binding affinity and operational simplicity. Antibody-coated beads were coated with a polyclonal antibody against HAV HM175 virions (ThermoFisher) at 20 ug/ml. PEI magnetic beads were generated by coating PEI to MagnaBind™ Carboxyl Derivatized Beads according to previously published report (Satoh et al., 2003). MagnaBind™ Amine or Carboxyl Derivatized Beads (Thermo Fisher, Waltham, MA) and MagPierce™ Protein G magnet beads (Thermo Fisher, Waltham, MA) were washed with PBS three times prior to usage. To compare their HAV binding affinity, virus elutes collected from 25 g green onions spiked with 5000 pfu HAV were separated evenly, and incubated with 10ul of antibody-coated, PEI-, amine- or carboxyl-derived beads for 1 h, respectively. After three washes with PBS, the beads were dissolved in 10 ul H_2O, heat-shock at 98 °C for 5 min, and analyzed by RT-qPCR (Williams-Woods and Burkhardt, 2013) to estimate the amount of viruses being concentrated. Alternative to heat shock, virus captured by beads were RNA-purified using QIAamp® viral RNA extraction kit prior to RT-qPCR analysis. As shown in Table 2, carboxyl derivatized beads demonstrated comparable HAV-binding affinity as polyclonal antibody coupled magnet beads. Surprisingly, no virus was detected with cationic amine or PEI coated beads when viruses were heat-shocked to release RNA. However, once the RNA was purified with commercial QIAamp® RNA extraction kit, positive HAV detection occurred (Table 2). The post RNA purification recovery rate was not statistically different between carboxyl beads and that of cationic beads (student's *t*-test). Although compared to kit extraction, heat-shock release RNA resulted in a slightly lower recovery rate. Due to the reduced volume of final RNA elution buffer, heat-shock released RNA was detected at a comparable sensitivity as kit extracted RNA (Fig. 2). For all subsequent carboxyl beads concentration studies, heat-shock was used to release RNA.

To test whether solubilizing the host-derived envelope (Feng and Lemon, 2014) to expose individual virus particle could improve affinity-based HAV concentration, eluents were treated with or without 1% NP40 prior to bead incubation. Indeed, treating eluents with 1% NP40 prior to adding beads increased the recovery rate of antibody-coated beads by (2.9 ± 0.3) fold and that of carboxyl beads by (3.3 ± 0.8) fold. It suggested that the exposure of individual virus particles was necessary for the affinity concentration of HAV, but not for viral liberation from the food matrix (Table 1). The ~3 folds increase in recovery rate also suggested that about 68% of virus particles were cloaked in membrane, which fell in the reported range of (79 ± 13)%