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Development of a rapid total nucleic acid extraction method for the isolation of hepatitis A virus from fresh produce

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ABSTRACT

Recently, there have been increasing reports of foodborne illnesses associated with the consumption of fresh produce. Among these, hepatitis A virus (HAV) remains epidemiologically important and has been continually implicated in several outbreaks. We describe a rapid method (<8 h) for the isolation and subsequent detection with real-time quantitative PCR (RT-qPCR) of the HAV HM-175 cytopathic strain seeded onto baby spinach and sliced tomatoes using a total RNA extraction method, utilizing a high concentration (4 M) guanidine thiocyanate buffer. Consistent detection of HAV genome from both produce items was achieved at an inoculation level of 3×10^3 PFU/25 g of food, with less consistent detection achieved at 3×10^2 PFU/25 g. Initial studies revealed that a final precipitation of recovered RNA with potassium acetate to reduce carryover of polysaccharides and the addition of polyvinylpyrrolidone to remove polyphenolics in spinach were essential. For tomatoes, virus isolation was achieved with the incorporation of either an elution step with a high pH Tris-glycine-beef extract (TGBE) buffer or with an enzymatic digestion with pectinase. We also describe the development of a protocol for the detection of HAV from tomatoes utilizing a Luminex® microbead-based suspension array. The results correlated well with the RT-qPCR assay suggesting the feasibility of the Bioplex® as a detection platform for viruses isolated from foods.

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1. Introduction

Almost sixty percent of all foodborne illnesses in the United States are attributed to foodborne viruses, causing an estimated 5.5 million cases annually (Scallan et al., 2011). Although human norovirus remain the most prevalent agent among incidents of viral origin, hepatitis A virus (HAV) continues to be a global problem with higher rates of hospitalization and death compared to norovirus (Scallan et al., 2011). HAV infection is the leading cause of acute viral hepatitis and is transmitted mainly via the fecal–oral route with person-to-person contact being the most common mode of transmission (Pinto et al., 2010). The incidence of HAV infection varies considerably among countries with the illness being endemic in much of the developing world. However, in developed countries, the adult population remains highly susceptible to infection and thus to outbreaks of HAV. Although the HAV vaccination strategy in the U.S. has shifted the domestic epidemiological pattern of the virus from outbreak situations towards more sporadic incidents, the total percentage of HAV associated episodes where no potential source is identified is greater than 30% suggesting that the actual number of cases, including ones with a foodborne route, is likely to be higher (Klevens et al., 2010).

Consumption of fresh produce items in raw or minimally processed form has increasingly been associated with viral foodborne outbreaks (Berger et al., 2010; Lynch et al., 2009). Contamination of these products is thought to occur during either their production through contaminated irrigation water or during harvesting and packaging by food handlers (Carter, 2005). Increased globalization and mass production of produce items have and will continue to lead to an increase in number and scope of outbreaks. A wide spectrum of produce items has been implicated as the vehicle in HAV linked outbreaks including blueberries, strawberries, lettuce, green onions, raspberries, and semi-dried tomatoes (Calder et al., 2003; Donnan et al., 2012; Hutin et al., 1999; Reid and Robinson, 1987; Rosenblum et al., 1990; Wheeler et al., 2005). In most of these outbreaks, the route of contamination has not always been identified, but water quality and handling during post-harvest procedures may have played a role in disease transmission. In 2003, the largest documented HAV outbreak in the United States was associated with contaminated green onions consumed at a restaurant in Pennsylvania causing 601 cases of illness including 3 deaths and 124 hospitalizations (Wheeler et al., 2005). More recently, a multi-jurisdictional outbreak in Australia was linked to semi-dried tomatoes with over 562 reported cases (Donnan et al., 2012). The traceback investigation

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identified frozen tomatoes imported from countries endemic for HAV as the potential etiological agent (Donnan et al., 2012).

Due to the low infectious dose and the high stability of HAV in various environmental conditions (Butot et al., 2008; Koopmans and Duizer, 2004), a small amount of virus contamination on foods is thought to be sufficient to cause human infection. As wild type HAV remains difficult to culture, there continues to be a need for a robust method for the detection of low levels of viruses in foods that can be utilized for both surveillance and epidemiological investigations of foodborne viral outbreaks. Published methods typically utilize some combination of elution, concentration, nucleic acid extraction and removal of potential inhibitors for downstream molecular detection. For example, common sample preparations approaches include an elution of virus from the food matrix, followed by either polyethylene glycol (PEG) precipitation, immunomagnetic capture, filtration, or ultracentrifugation (Mattison and Bidawid, 2009; Stals et al., 2012).

In this study, we applied a total nucleic acid extraction approach with the subsequent detection of HAV by RT-qPCR to two distinct produce items, spinach and sliced tomatoes. These commodities were chosen due to their high likelihood of being implicated in outbreaks and for their very different matrix properties. Indeed, a recent analysis of high risk food commodity–pathogen pair ranked HAV associated with tomatoes and HAV with leafy greens in the 6th highest risk pair category (Anderson et al., 2011). Other direct RNA approaches have successfully identified norovirus in deli meats (Schwab et al., 2000), spareribs (Boxman et al., 2007), ready-to-eat foods (Stals et al., 2011), and oysters (Boxman et al., 2006) as well as HAV from cilantro and clams (Goswami et al., 2002). To our knowledge, the application of this direct nucleic acid approach has not yet been tested for HAV on any other fresh produce item other than cilantro, where the assay detection time exceeded three days. Here, we describe a rapid approach (<8 h) that can effectively detect, via real-time quantitative PCR (RT-qPCR), at least 3000 PFU/25 g of HAV in either spinach or sliced tomatoes. In this method, we opted to use sliced tomatoes rather than whole tomatoes as this is the form it is usually found in salad bars and restaurants and also due to the possibility of internalization of virus particles as shown for several viral and non-viral pathogens in various produce items (Dicaprio et al., 2012; Hirneisen et al., 2012).

Although RT-qPCR is exquisitely sensitive with a capacity to be highly specific for its viral target sequence, thus providing a means for quantitative analysis, simultaneous detection of multiple targets (e.g., via multiplexed RT-qPCR) can be affected by the spectral overlap of fluorescent labels (on probes) and thus suffer from limited options for multiplexing. As an alternative to RT-qPCR with a greater capacity for multi-plex pathogen analysis, we have explored the use of a Luminex® assay on the Bioplex® platform. The Bioplex® is a flow cytometer based suspension array system reported to be able to simultaneously detect up to 100 different analytes (targets) within each well of a microplate (Dunbar, 2006). Nucleic acid from the pathogen of interest is amplified by RT-PCR with a biotin labeled primer, hybridized to capture probes conjugated on the microspheres and subsequently detected by the use of a reporter dye, streptavidin–phycoerythrin (SAPE). Each microsphere contains a unique fluorescence signature derived from the precise combination of two fluorophores and can be coupled to a distinct oligonucleotide probe. This suspension array method has been successfully used for the identification of a wide variety of bacteria and viruses in both clinical (Letant et al., 2007; Liu et al., 2011; Yu et al., 2011), and environmental samples (Baums et al., 2007). To date, the Luminex® assay has not been adapted for the identification of viruses extracted from food samples. We thus evaluated the feasibility of this detection platform for identification of HAV isolated from tomatoes as a first step in the development of a multiplex assay with other foodborne pathogens.

2. Materials and methods

2.1. Cells and viruses

The cytopathic strain of hepatitis A virus HM175/18f was purchased from ATCC (Manassas, VA), propagated and purified as previously described (Dotzauer et al., 2000; Kulka et al., 2009). Viral titer was determined to be 3×10^8 PFU/ml by plaque assay on Fetal Rhesus monkey Kidney cells (FRhK4) as described by Bidawid et al. (2000). All stocks were stored at −80 °C in aliquots prior to use. A fresh aliquot was thawed prior to each experiment to minimize any degradation due to repeated freeze–thaw cycles. The FRhK-4 cell line used was originally obtained from Dr. G. Kaplan (Center for Biologics Evaluation and Research, FDA) and cultured as previously described (Kulka et al., 2003).

2.2. Extraction of viral RNA from spinach and tomatoes

Produce samples were purchased from local retail stores and stored at 4 °C until use. Twenty five grams of bagged baby spinach or sliced tomatoes (without prior washing) was inoculated with 100 µl of serially diluted HAV in distilled water to give levels ranging from 3×10^6 to 300 total PFU and allowed to air dry in a laminar flow hood for 60 min. One hundred ml of guanidine buffer (4 M guanidine thiocyanate, 1× TE buffer, 0.55 M NaCl, 0.33 M Sodium Acetate, pH 6.8) and 2 g of polyvinylpyrrolidone (PVP) (Fisher, Pittsburgh, PA) was added directly to the spinach in a sterile filtered Whirl-Pak® plastic bag (Nasco, Fort Atkinson, Wisconsin) and homogenized in the stomacher for 1 min at 260 rpm. PVP was incorporated at 2% w/v in the lysis buffer as it is known to bind to polyphenolics and has been widely used in the plant science community for the isolation of plant nucleic acids for amplification (Jobes et al., 1995; MacRae, 2007). For tomatoes, phosphate buffered saline (PBS), pH 7.4, and other buffers as described below were used to elute the virus for 30 min on a shaking platform prior to the addition of 90 ml of guanidine buffer and homogenization in the stomacher. After homogenization, the eluate was collected and clarified by centrifugation at 13,000 ×*g* for 10 min at 4 °C to remove any gross solids (food particulates). The supernatant was collected and RNA was precipitated with the addition of 70% volume of ice cold isopropanol followed by a minimum of 30 min incubation at −20 °C. The RNA containing pellet was collected by centrifugation at 13,000 ×*g* for 10 min at 4 °C and re-suspended in the lysis buffer from the RNAqueous® Kit (Ambion, Grand Island, NY). RNA was further purified according to manufacturer instructions and eluted in a 50 µl final volume. An additional step was incorporated after the final virus RNA extraction to precipitate any remaining polysaccharides with 0.2 M potassium acetate (Wilkins and Smart, 1996). The RNA collected was stored at −80 °C or used immediately for RT-qPCR analysis. All experiments were done in triplicate (n = 3).

2.3. Use of elution buffer or enzymatic digestion for the recovery of HAV from tomatoes

As described above, an initial elution step was performed prior to the addition of guanidine buffer for the tomato samples. The following elution buffers were tested: i) 0.1 M Tris-HCl, 0.05 M glycine, 1% beef extract, pH 9.2 (TGBE), ii) 0.05 M glycine, 0.14 M NaCl, pH 9.5, iii) 0.05 M glycine, 0.14 M NaCl, pH 7.1, iv) PBS, 1 M NaCl, 1% Tween, pH 7.3, and v) PBS, pH 7.4. In addition, the use of pectinase and/or cellulase to improve recovery of HAV was further investigated as pectins and cellulose comprise a large proportion of the non-starch polysaccharides within a tomato (Reinders and Thier, 1999). The following enzyme combinations were added to PBS in the elution step: i) 300 µl cellulase (≥1000 units/g, Sigma, St. Louis, MO), ii) 300 µl pectinase (≥3800 units/ml, Sigma, St. Louis, MO), iii) 300 µl cellulase