Real-Time Nucleic Acid Sequence-Based Amplification Assay for Detection of Hepatitis A Virus

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A nucleic acid sequence-based amplification (NASBA) assay in combination with a molecular beacon was developed for the real-time detection and quantification of hepatitis A virus (HAV). A 202-bp, highly conserved 5’ noncoding region of HAV was targeted. The sensitivity of the real-time NASBA assay was tested with 10-fold dilutions of viral RNA, and a detection limit of 1 PFU was obtained. The specificity of the assay was demonstrated by testing with other environmental pathogens and indicator microorganisms, with only HAV positively identified. When combined with immunomagnetic separation, the NASBA assay successfully detected as few as 10 PFU from seeded lake water samples. Due to its isothermal nature, its speed, and its similar sensitivity compared to the real-time RT-PCR assay, this newly reported real-time NASBA method will have broad applications for the rapid detection of HAV in contaminated food or water.

Outbreaks of acute gastroenteritis due to hepatitis A virus (HAV) have been attributed to consumption of drinking water and foods considered safe on the basis of bacterial standards (23). These outbreaks indicate that meeting bacterial standards does not always ensure the absence of infectious viruses, and more reliable approaches are needed to detect HAV and other enteroviruses in environmental samples.

Conventional methods for the detection of HAV are based on cell culture propagation, which is often difficult to perform and can take several weeks and continuous propagation in one or more cell lines before a sufficient amount of viral antigen or nucleic acid is produced to allow detection (5, 6). In addition, no single cell line is currently recommended for the detection of HAV (19, 22).

To date, molecular methods, such as the reverse transcription (RT)-PCR technique, are the most commonly studied, offering improved sensitivity, specificity, and the possibility of direct detection of HAV in environmental samples (11, 16). In RT-PCR, the viral RNA is first converted to a single-stranded complementary DNA in a reverse transcription step, followed by PCR amplification of the target complementary DNA sequences to a detectable level. However, RT-PCR procedures have the disadvantages of requiring a two-step amplification process and relying on the use of expensive thermal-cycling equipment, which add to the complexity and the cost of their implementation for routine testing programs.

Unlike RT-PCR, nucleic acid sequence-based amplification (NASBA) is a homogeneous, isothermal nucleic acid amplification method (17) that is particularly suited to RNA targets in a double-stranded DNA background (12). A cocktail of three

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MATERIALS AND METHODS

Virus strain and propagation. A cytopathic HAV strain, HM175, was obtained from the ATCC (Manassas, Va.). Fetal rhesus monkey kidney (FRhK-4) cells were used for the propagation and titration of HAV. HAV titers were measured by performing plaque assays. Briefly, cell monolayers were grown overnight in 12-well culture plates at 37°C in the presence of 5% CO₂. A 100-μl portion of the virus dilution was inoculated into each of three wells. The virus was allowed to be in contact with the cells for 90 min at 37°C in the presence of 5% CO₂, and

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10^8-fold without the use of expensive thermal-cycling equipment. NASBA has proved successful in the detection of various mRNAs (3, 9) and in the detection of both viral (17) and bacterial (21) RNAs. Diagnostic procedures based on NASBA methodology have been described for several viruses, including human immunodeficiency virus type 1 (10), cytomegalovirus (26), enterovirus (13), West Nile and St. Louis encephalitis viruses (17), parainfluenza virus (14), and hepatitis C virus (8).

Molecular beacons (MBs) are single-stranded nucleic acid sequences that possess a stem-loop structure that is double labeled with a fluorescent dye and a universal quencher at the 5’ and 3’ ends, respectively (25). By combining the standard NASBA technology with MB, a real-time detection system can be generated. During the NASBA reaction, the MB hybridizes to the target RNA, separating the reporter dye and the quencher and yielding a measurable fluorescence emission directly proportional to the concentration of the target sequence (20).

In the present study, the development of an MB-based real-time NASBA assay for the detection of hepatitis A virus was investigated. The sensitivity of the assay was evaluated using serial dilutions of HAV. The specificity of the procedure was tested against closely related viruses from the Picornaviridae. The possibility of combining such an assay with immunomagnetic separation (IMS) for the detection of the virus in surface water samples was also demonstrated.
then 2 ml of semisolid agarose was overlaid upon each well. The plates were incubated at 37°C in the presence of 5% CO₂ for 8 days. This was followed by fixation and staining for plaque counting as described previously (7). Other viruses used in this study (e.g., coxsackievirus B6, echovirus 11, bacteriophage φX174, poliovirus 1 LscAb [ATCC], rotavirus SA11, and echovirus 19) were grown separately on their specific mammalian or bacterial host cells.

**RNA purification.** Total viral RNA was extracted by the phenol-chloroform method (22, 24). Briefly, 100 μl of hepatitis A virus-infected cell culture was mixed with 100 μl of phenol-chloroform (1:1) and vortexed vigorously before centrifugation. The RNA-containing aqueous top layer was mixed with a mixture of 4.0 M LiCl and ice-cold 100% ethanol and placed at −20°C overnight. Following the recovery of RNA pellets by centrifugation, the pellets were washed with 70% ethanol and dried under a vacuum. The dried RNA pellets were dissolved in 100 μl RNase-free water and kept at −20°C.

**Primers and molecular beacon.** The 5′ noncoding region (5′ NCR) of HAV, which is conserved among different HAV isolates (1), was chosen as the target region for amplification. The primer pair UC1 (5′-AATGGATCCGGTAAAGTCTCAATGTTGGGAAGGGACGGCGG-3′) and T7KH2 (5′-AATCTAATACGACCTATAGGGAGACGGCGTTGAATGGTTTTT-5′) was used to amplify a 202-bp region of the 5′ NCR of the HAV genome. The 3′ antisense NASBA primer was elongated with the preferred transcription initiation sequence at the 5′ end (underlined) and indicated with the prefix T7.

A molecular beacon (HAV MB: 6-FAM-5′-CTTGGCGGATAGGTGAG ACCGGCGGGGAAG-3′-DABCYL [the stem sequence of the MB is underlined, and the target sequence of HAV is in bold]) (Midland Certified Reagent Co., Midland, TX), which is perfectly complementary to a 20-bp region of the noncoding region (5′-DABCYL [4′-dimethylaminophenylazo] benzoic acid) was used as the quencher at the 3′ end.

**Real-time NASBA reaction.** A Bio-Rad (Hercules, CA) iQcycler IQ Real-Time PCR Detection System was used to perform the real-time NASBA assay. The NASBA reactions were performed as described previously (2) with some modifications. The final reaction mixture volume was 25 μl. An 18-μl pre-reaction mixture was prepared to give a final concentration in 25 μl of 40 mM Tris-HCl (pH 8.5), 50 mM KCl, 12 mM MgCl₂, 1.0 mM (each) deoxyribonucleoside triphosphate, 2.0 mM (each) ribonucleotide-5′-triphosphate, and 15% (vol/vol) dimethylsulfoxide, 0.2 μM of each cartridge-purified primer, and 100 nM of HAV MB. Five microliters of purified viral RNA was added to the 18 μl of pre-reaction mixture in a 0.5-ml Bio-Rad PCR plate, which was incubated for 5 min at 65°C in order to disrupt any secondary structure in the target RNA. The plate was then placed at 40°C. After 5 min, 2 μl of an enzyme mixture containing 2.6 μg of bovine serum albumin (Promega Corp.), 40 international units (IU) of T7 RNA polymerase (Novagen Inc., Madison, WI), 8 IU ofavian myeloblastosis virus reverse transcriptase (Seikagaku, Ijamsville, Md.), 0.2 IU of RNase H (New England Biolabs, Beverly, MA), and 12.5 IU of RNAse Inhibitor (Promega Co.) were added to each well, followed by incubation at 40°C for 5 min. This was followed by 10 min at 42°C to allow the template to anneal with the MB, the amplicon was detected by the MB as a significant fluorescent signal that could be detected (data not shown), and the probe moiety of the beacon, both of which were based on the 5′ NCR of HAV strain HM-175. The presence of other microorganisms or viruses in food and water samples could potentially affect the specificity of the assay. To test this, NASBA assays were conducted with other potential pathogens (e.g., coxsackievirus B6, echovirus 11, and echovirus 19) and indicator microorganisms (e.g., bacteriophage φX174, Escherichia coli, and rotavirus SA11) commonly found in contaminated food or water. None of these species produced significant fluorescence that could be detected (data not shown), demonstrating the specificity of the assay. To investigate whether the presence of other pathogens could affect the sensitivity of HAV detection, similar assays were conducted by mixing 10⁵ PFU of HAV with 10⁵ PFU of other viruses or phages. No noticeable differences in the fluorescent signal were detected, again indicating the highly specific nature of the primers and the molecular beacon (data not shown).

**RESULTS AND DISCUSSION**

**Molecular-beacon-based real-time NASBA assay for HAV.** The ability of the MB-based real-time NASBA assay to detect HAV was investigated. The sensitivity of the assay was assessed using 10-fold serial dilutions of HAV samples. Any fluorescent signal that was 10-fold higher than the standard deviation of the mean baseline emission was indicative of a positive detection. A 202-bp fragment was correctly amplified by the primers, and the amplicon was detected by the MB as a significant increase in fluorescence (Fig. 1A). As little as 1 PFU was detected within 10 min using the real-time NASBA assay. This is substantially shorter than the 210 min reported recently for HAV detection using a conventional NASBA assay (14), which is based on end point detection of amplification products by electrophoresis. The substantial improvement in analysis time is primarily due to the early detection of fluorescent signal afforded by the use of MB.

The reproducibility of the assay was evaluated by comparing the detection times from four different NASBA assays. The results showed less than 5% variability among the assays (Table 1). A linear standard curve from 1 to 1,000 PFU was obtained by plotting the time required for the signal detection versus PFU (Fig. 1B).

**Specificity of the real-time NASBA assay.** The specificity of the molecular-beacon-based NASBA assay depends on the selected sequence of the primer set (UC1 and T7KH2) and the probe moiety of the beacon, both of which were based on the 5′ NCR of HAV strain HM-175. The presence of other microorganisms or viruses in food and water samples could potentially affect the specificity of the assay. To test this, NASBA assays were conducted with other potential pathogens (e.g., coxsackievirus B6, echovirus 11, and echovirus 19) and indicator microorganisms (e.g., bacteriophage φX174, Escherichia coli, and rotavirus SA11) commonly found in contaminated food or water. None of these species produced significant fluorescence that could be detected (data not shown), demonstrating the specificity of the assay. To investigate whether the presence of other pathogens could affect the sensitivity of HAV detection, similar assays were conducted by mixing 10⁵ PFU of HAV with 10⁵ PFU of other viruses or phages. No noticeable differences in the fluorescent signal were detected, again indicating the highly specific nature of the primers and the molecular beacon (data not shown).
RT-PCR assay for environmental samples was tested using surface water samples from Lake Elsinore in southern California seeded with HAV. When different dilutions of HAV were added to the lake water, no visible increase in fluorescence was observed from the real-time NASBA assay, indicating severe interference from the inhibitors present in the samples. Although many pretreatment methods are available for the concentration of virus from other inhibitory substrates presented in water samples, IMS is particularly attractive because of the potential for detecting intact and infectious viruses. As little as 10 PFU was detected in the seeded lake water samples (Fig. 2). These results demonstrate the potential of the combined IMS–real-time NASBA assay for rapid and quantitative detection of HAV in contaminated water and food.

**Conclusions.** A rapid and quantitative technique to detect the presence of HAV in contaminated food and water samples is essential to provide the required rapid diagnostic capability and to allow an evaluation of the possible health risk. Although traditional methods based on multiplication of HAV in cell culture have been replaced by modern molecular techniques, such as RT-PCR and NASBA, quantification is labor-intensive and typically requires hybridization with a radiolabeled probe. In this study, we have developed a molecular-beacon-based real-time NASBA assay for the detection of HAV. Introduction of a target-specific molecular beacon to the NASBA assay enables simultaneous amplification and real-time detection of RNA molecules in a closed-tube format (20). This is substantially better than other methods of detecting NASBA products, such as Northern blotting and the electrochemiluminescence technique, which require opening of the tubes after amplification and carry the risk of contamination. In addition, NASBA has a much higher inherent amplification capability than RT-PCR, resulting in faster detection (~100 min) than TaqMan RT-PCR (~180 min) (4) and molecular-beacon-based RT-PCR (1) assays. These benefits, combined with the isothermal nature (at 40° ± 1°C) of NASBA, allow high-throughput detection of HAV in a real-time manner.

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REFERENCES


