

Development and Evaluation of a Broadly Reactive TaqMan Assay for Rapid Detection of Hepatitis A Virus

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Primers and a TaqMan probe for the 5'-untranslated region (UTR) of the hepatitis A virus (HAV) genome were designed and evaluated. The assay detected 0.5 infectious units of HAV and 40 copies of a synthetic transcript and provides an important screening tool for rapid quantitative HAV detection in clinical or environmental samples.

Hepatitis A virus (HAV) is the major cause of acute hepatitis in developed countries (27), while in developing countries infection is endemic. Person-to-person contact is the most common mode of transmission, and because HAV is transmitted by the fecal-oral route, improved hygiene and sanitation levels can reduce endemicity (1, 28, 30). In the United States, 48% of reported cases are of unknown origin (12), and contaminated food or water have been identified as a risk factor in only 4%. Currently, it is not known whether contaminated food or water is the source of infection for some of the cases with unknown origin (12). Contaminated food or water is usually recognized as a source when a cluster of infections occur (8, 10, 11, 13, 14, 21, 26).

HAV is a member of the genus *Hepatitisvirus*, which belongs to the family *Picornaviridae* (19). The nonenveloped virion contains a 7.5-kb positive-stranded RNA containing one open reading frame (ORF) of about 6,500 nucleotides. The genome is composed of five functional regions, the 5'-untranslated region (5'UTR), three regions that encode viral polypeptides (P1, P2, and P3), and the 3'-untranslated region (3'UTR). The 5'UTR is approximately 600 nucleotides in length and is the most conserved region of the genome and therefore is the most suitable region for design of primers that would detect most genotypes.

Although only one human serotype has been observed worldwide, HAV can be classified into six genotypes based upon differences in the 168-nucleotide sequence between 3024 and 3191 in the VP1-P2 region (17, 25). This more variable region has been used to distinguish genotypes. Genotypes I, II, and III were found in humans, while genotypes IV, V, and VI were recovered from simians (17, 20, 23, 29). Early investigations identified a seventh genotype (25), but recent analysis of the complete capsid genome region and complete genomes of additional isolates has indicated that genotype VII should be reclassified as genotype IIB (4, 17). Genotype I and III are each further classified into subtypes IA, IB, IIIA, and IIIB.

Based on existing information, Genotype I is the most common worldwide and Genotype 1A is more common than IB.

This report demonstrates the development and application of TaqMan real-time reverse transcription-PCR (RT-PCR) for the rapid detection of all human genotypes of HAV using highly conserved sequences within the 5'UTR. Two previous reports that describe real-time HAV assays used different primers and probes than those we have employed (5, 22). One of these assays required two reverse primers to theoretically detect all genotypes and reported a sensitivity of five RNA copies (5); the other assay used only one of the reverse primers and the same forward primer and probe as the first report, but it was not as sensitive as the first assay (22). A sensitive assay that can detect all human genotypes is needed. The investigation of outbreaks that may cross national and continental boundaries requires assays that will rapidly detect all genotypes of HAV in the clinical or environmental samples available for investigation by public health officials. In addition, routine testing should also be performed with a rapid, sensitive test that can detect all human genotypes.

The cell culture adapted cytopathic variant clone 24A HM-175 (6), a representative HAV genotype IB, was used to establish the sensitivity of the TaqMan assay for infectious virus. Virus propagation, purification, and plaque assay were performed as previously described (6), and a passage level 4 virus stock with titer of 2×10^7 PFU/ml was used in the experiments. Genotype IB RNA was extracted from this preparation. Wild-type genotype IA HAV RNA was obtained from a 20% stool preparation from a chimpanzee inoculated with a human wild-type virus (18). The genotype IIIA RNA was obtained from a human stool preparation (16). Prior to RNA extraction, the stool preparation was extracted with an equal volume of chloroform, and RNA was extracted from the aqueous fraction. Genotype VII (or IIB) RNA was obtained from a liver preparation stored at -70°C as previously described (3). The RNA from the representative IIA genotype was extracted from cell culture grown CF53/Berne (17). Viral RNA was extracted from preparations of each genotype or from serial 10-fold dilutions of the preparations using the QIAamp viral RNA mini-kit (QIAGEN, Valencia, CA) following the manufacturer's instructions. The extracted RNA was resuspended in Tris-EDTA buffer and used immediately in the assay. In addition to

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TABLE 1. Description of isolates evaluated with TaqMan assay^a

Virus sample	Source	Genotype	Reference
HLD-2	CDC	IA	18
HM-175, clone 24A	CDC	IB	6
CF-53	CDC	IIA	17
Sierra Leone	CDC	IIB	3
GA-76	CDC	IIIA	16
Quebec outbreak (7 samples)	CDC	IA	8
Stanislaus County, CA (3 samples)	CDC	IA	24

^a All samples were positive by real-time RT-PCR. CDC, Centers for Disease Control and Prevention.

these prototypes of each human HAV genotype, HAV-positive stool samples from two separate outbreaks were analyzed. Seven samples obtained during a well water outbreak (8) and three samples from a community-wide outbreak (24) were tested. All of these samples were previously characterized as genotype IA, the genotype most often found in North America. RNA was extracted from these outbreak samples (20% stool preparations) with the QIAamp MinElute Virus Spin kit using the manufacturers instructions (QIAGEN, Valencia, CA). Table 1 provides a summary of all the isolates tested.

The 5'UTR primers and TaqMan probe chosen for detection of HAV are shown in Fig. 1. The TaqMan probe was labeled with 6-carboxy-fluorescein (FAM) at the 5' and a with a black hole quencher (BHQ) at the 3'. Real-time RT-PCR was performed in the LightCycler (Roche, Indianapolis, IN) glass reaction capillaries containing sample RNA (2 µl each) in a 20-µl volume with 0.25 µM primers and 150 nM flourogenic probe. Nucleotides, reverse transcriptase, Taq DNA polymerase, and buffer were included in the QuantiTectTm Probe RT-PCR kit (QIAGEN, Valencia, CA). A typical protocol took approximately 90 min to complete. After a 30-min reverse transcription reaction and a hot-start denaturation step of 15 min, 45 cycles of 95°C for 10 s, 55°C for 20 s, and 72°C for 15 s were completed. The fluorescence was measured at the end of each annealing step. All amplifications reactions were carried out in duplicate. To confirm the correct amplicon size, products from each primer pair were subjected to agarose gel electrophoresis.

Synthetic RNA was synthesized in a two-step process: (i) the RT-PCR product of HM-175 24A (generated with primers described in Fig. 1) was ligated to T7 phage RNA polymerase promoter; (ii) PCR amplification was performed to generate a transcription template from which a large quantity of synthetic transcripts was made.

The PCR product from HAV (HM175-24A) was purified

with a PCR purification kit (QIAGEN, Valencia, CA) and ligated with the T7 bacteriophage promoter as described in the manufacturers protocol (Ambion Inc., Austin, TX). The resulting mixture was used immediately in the Lig'nScribe PCR with the forward primer from Ambion and the reverse HAV primer (Fig. 1). The resulting fragment was transcribed in vitro with T7 RNA polymerase (T7 MEGAshortscript high yield transcription kit; Ambion) to yield high quantities of HAV RNA transcripts. RNA concentration was 2 × 10¹¹ RNA copies/ml. Serial 10-fold dilutions of HAV RNA were prepared in Tris-EDTA containing 1.0 U of RNasin plus RNase inhibitor/µl (Promega, Madison, WI) and 100 ng/µl of yeast tRNA (Ambion, Austin, TX) from the starting concentration of 10⁸ copies/ml and assayed by the TaqMan assay.

Prototype isolates representing HAV genotypes IA, IB, IIA, IIB, and IIIA were detected with the real-time TaqMan assay as shown in Fig. 2. RNA extracted from each genotype preparation was assayed as described above. HAV was detected in all preparations by ≤25 cycles as shown on the X axis. Samples of genotype IIA and IIB (or VII) showed a lower plateau than the other genotypes, which is likely explained by the two mismatches in the probe for this genotype (Fig. 1). However, the cycle number of detection was very low, 17 and 19 cycles, respectively, indicating good sensitivity (Fig. 2). Amplification using our primers in the presence of syber green (which measures double-stranded DNA) revealed that equivalent amplification was obtained for all the human genotypes (data not shown). Comparison of the one sequence available (genotype V) of the simian genotypes demonstrated two mismatches in the reverse primer and six mismatches in the probe, indicating that the assay is very unlikely to detect this virus (Fig. 1). Other simian virus sequences are not available for comparison and therefore may not be excluded from detection at this time. The primers and probe did not react with human hepatitis E virus (HEV), swine HEV, or several human enteroviruses in laboratory trials.

Quantitative evaluation of the assay for detection of the HM-175 stock and synthetic RNA is shown in Fig. 3. Detection is linear over five log₁₀ dilutions, and the most dilute sample (with an intercept at 36 cycles) represents detection of 0.5 PFU 24A HM175 HAV (Fig. 3A). A comparison of threshold cycle versus log₁₀ copy number of synthetic HAV RNA is shown in Fig. 3B. The estimated starting copy number was 2 × 10⁸ molecules/ml as measured by optical density at A₂₆₀/A₂₈₀ nm, and the sensitivity of the assay was at least 40 HAV copies/reaction (2 × 10⁴ copies/ml). Four copies per reaction yielded a negative test.

	Forward Primer (+) (392-410)	Probe (+) (413-441)	Reverse primer (-) (480-461)
	GGTAGGCTACGGGTGAAAC	CTTAGGCTAATACTTCTATGAAGAGATGC	GCGGATATTGGTGAGTTGTT
1A-x75215	GGTAGGCTACGGGTGAAAC	CtTTAGGCTAATACTTCTATGAAGAGATGc	ttttggatagggttaacagcgGCGGATATTGGTGAGTTGTT
1B-m14707c.....
IIA-m63025a.....g.....
IIB-ay032861a.....g.....
III-AY644337a.....c.....
V-d00924at.....c.....g.....atc.....a.....t.....cc.....

FIG. 1. Sequence alignment of HAV genotypes in the amplified region. The actual sequence of the reverse primer is 5'ACAACCTCACCA ATATCCGC3'. GenBank sequence numbers are indicated.

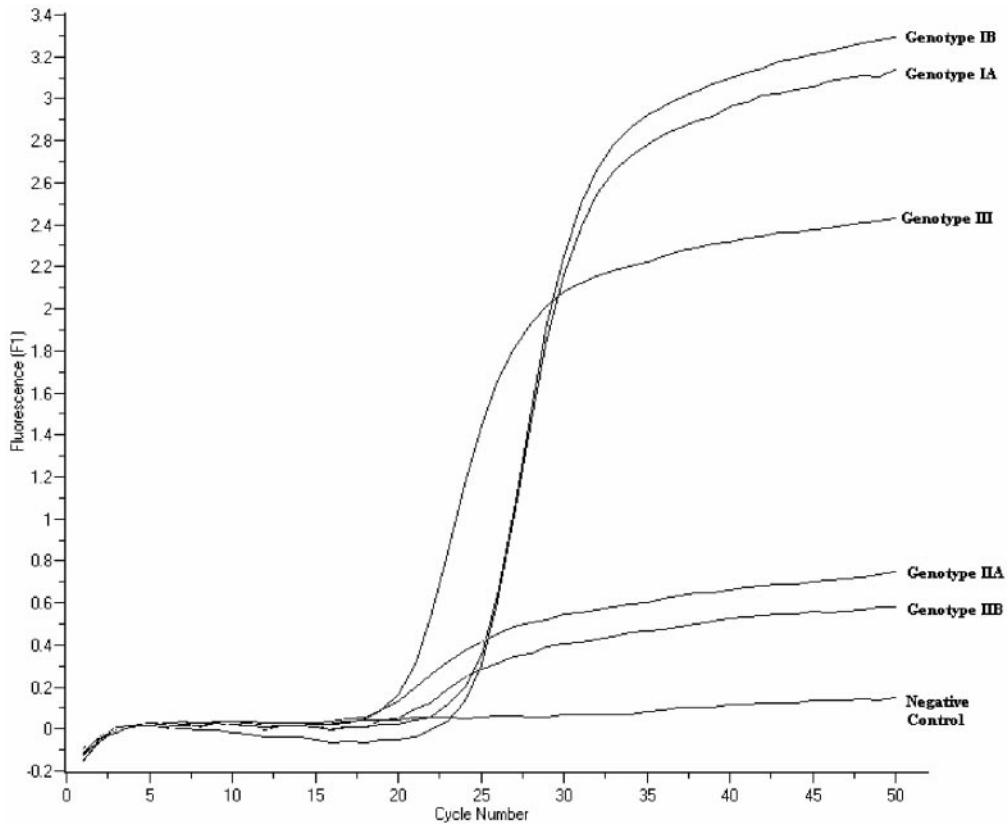


FIG. 2. Detection of human HAV genotypes with the 5'UTR TaqMan RT-PCR assay.

In addition to the assay of undiluted preparations of each genotype shown in Fig. 2, assay of a 10-fold dilution series of each genotype was performed with the real-time assay (Table 2). Only the genotype IB sample (assayed for infectivity in cell

culture) was assayed quantitatively for comparison in the real-time assay. Other isolates tested do not have any quantitative measurement, such as the plaque assay, for comparison with real-time detection in RT-PCR. The average results of two

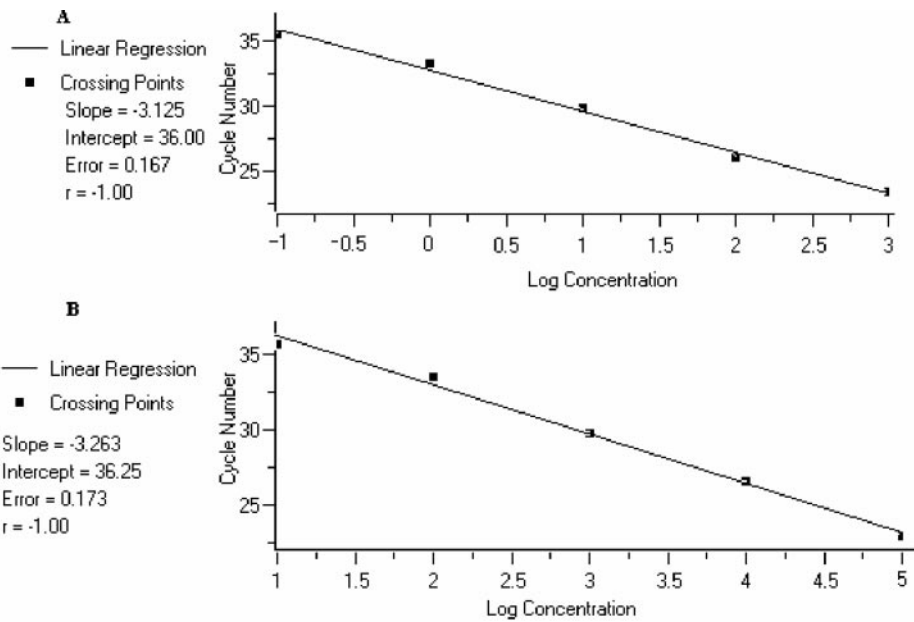


FIG. 3. Standard calibration curve for the HAV real-time RT-PCR assay. (A) The range of detection of Genotype IB (clone 24A HM-175). (B) Detection over a linear range of synthetic HAV RNA. The log of the initial template PFU (A) or copy number (B) is plotted horizontally, and the cycle number plotted at the crossing point is plotted vertically.

TABLE 2. Analysis of dilution series of each genotype of HAV^a

Genotype	Slope	Regression	Efficiency
1A	-3.131	0.99	2.09
1B	-3.125	1.00	2.09
IIA	-3.317	1.00	2.00
IIB	-3.526	0.98	1.92
IIIA	-3.448	1.00	1.95

^a Relative standard curves were derived from analysis of a 5 log₁₀ dilution series of virus of each genotype, except IIB (7 log₁₀ dilutions). PCR efficiency was calculated from the slope of the dilution curve using the formula $E = 10^{(-1/\text{slope})}$, where E refers to PCR efficiency.

separate experiments analyzing 10-fold dilution series for each genotype are shown in Table 2. Using the LightCycler software, the slope and regression values were calculated based on the data obtained for five to seven serial log₁₀ dilutions of each genotype sample from which RNA was extracted. The efficiency for each genotype was calculated with the formula described. The results of these experiments demonstrate good performance of the assay for detection of all tested genotypes over a significant range of concentrations (five to seven orders of magnitude).

The developed assay is highly reproducible over a wide range of dilutions for all human HAV genotypes. Detection of all human HAV genotypes is important in the evaluation of potential outbreaks or environmental contamination. The current practice of shipping food worldwide and international travel make it important to be able to rapidly detect all genotypes, including those that may not commonly occur in North America. There are several advantages of the TaqMan assay for detection of HAV in environmental or clinical samples including: (i) detection within 2 h in comparison to 1 or 2 days for confirmed standard RT-PCR detection; (ii) the real-time PCR assay is carried out in a closed tube format, substantially decreasing any opportunity for false positive results; (iii) specificity is equal to or greater than other hybridization assays or a second round of PCR, because the highly specific probe hybridizes in the central portion of the amplicon. The quantitative potential of the TaqMan assay is also important. The number of RNA copies in a specimen can be estimated by comparison with the standard curve and cycle numbers within the same reaction.

Based upon published reports for the ratio of physical particles to infectious particles for cytopathic HM-175 (58:1 to 79:1) (9, 15), the sensitivity of detection of HAV RNA (40 copies) is similar to the sensitivity of detection of infectious HM-175 (0.5 PFU). The sensitivity of the real-time assay was similar or better than standard RT-PCR followed by nested PCR or hybridization if volumes of RNA per reaction are considered (2). Furthermore, with the different real-time PCR platform machines that are now available, a larger volume may easily be evaluated to achieve even greater sensitivity.

HAV is very difficult, if not impossible, to culture from clinical and environmental samples, and therefore molecular detection is a very important analytical tool. Culture of wild-type virus requires a larger quantity of virus than is commonly found in environmental sample concentrates or requires many months of serial cell culture passage to grow the low quantity of virus present in these samples (7). Evaluation of the assay

for detection of HAV in spiked water and food samples is in progress. Use of the TaqMan assay will provide a rapid method for detection of HAV in food, water or clinical specimens.

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