ABSTRACT

The detection of hepatitis A virus (HAV) negative-strand RNA, which is synthesized during replication of the positive-strand RNA genome, proved to be difficult. We developed a method for the specific detection of HAV negative-strand RNA by RNA–DNA hybridization and luminescence detection using an anti-RNA:DNA hybrid antibody. This method, which is also applicable for the specific detection of positive-strand RNA, offers a simple, yet relatively rapid and certain means of detecting low amounts of RNA such as HAV negative-strand RNA. By using appropriate hybridization DNA probes, the method should be applicable for the detection of single-stranded RNA species of different viruses in general.

The replication of the positive-sense genomic RNA of hepatitis A virus (HAV), a picornavirus, proceeds via a negative-strand RNA intermediate (1,2). Therefore, the detection of viral negative-strand RNA is indicative for replication of HAV. Several laboratories attempted to detect HAV negative-sense RNA by hybridization techniques (2–6) or by applying a PCR approach (7). Their findings that HAV negative-strand RNA was either detectable only for a brief period or not at all revealed that HAV negative-sense RNA is present only in very low amounts. As these methods proved to be relatively uncertain and/or complicated by the presence of the positive strands, there is still a need for a technically simple, rapid and reproducible procedure for the specific detection of HAV negative-strand RNA. We describe an assay which is easy to handle and provides the reliable, sensitive and specific detection of negative-strand RNA of HAV. The principle of this hybrid detection assay (HDA) is a solution hybridization (8,9) of the HAV minus-strand RNA with a specific 5′-biotinylated single-stranded DNA probe. The resultant RNA:DNA hybrids are captured through biotin onto the surface of streptavidin-coated microwells. Immobilized hybrids are then incubated with an anti-hybrid antibody conjugated to alkaline phosphatase (Digene Diagnostics, Silver Spring) and detected with a chemiluminescent substrate.

The HAV negative-strand RNA specific single-stranded DNA probe was synthesized by run-off polymerization with Taq DNA polymerase (10). The polymerization was carried out with 200 pmol of negative-strand specific 5′-biotinylated primer, 5′-AAGCAA-CTACTGCTCCTTGT-3′ (nucleotides 1921–1940 of HAV positive-sense sequence), 200 µM each of dATP, dGTP, dTTP and dCTP, and 0.5 pmol template DNA [HAV nucleotides 633–2374, which were obtained from the plasmid pHAV/7 (11) by digestion with BamHI] in 100 µl of a solution of PCR buffer (Boehringer Mannheim). Taq DNA polymerase (4.2 U, Boehringer) was added after 2 min at 94°C and the reaction was performed as follows: 1 min at 94°C, 1 min at 65°C and 3 min at 72°C for 40 cycles. Finally the extension step was continued for 7 min at 72°C. The size of the polymerization product (454 bases) was verified by gel electrophoresis, and the demonstration that the reaction product was not cleavable with restriction enzymes confirmed its single-stranded nature.

In order to determine the sensitivity and strand specificity of the HDA using the single-stranded DNA probe in vitro transcribed negative-strand and positive-strand RNA from nucleotide positions 745–2993 of the HAV genome was used. The HDA was performed as follows: 250 fmol in vitro transcribed HAV RNA and 50 fmol single-stranded DNA probe in 50 µl hybridization buffer (50% formamide, 1× Denhardt’s, 5× SSCPE) were incubated for 5 min at 95°C and hybridized for 30 min at 57°C. The hybridization mixture was transferred to a well of the streptavidin-coated plate (Digene Diagnostics, Silver Spring). The covered plate was incubated at room temperature for 30 min on a shaker at 1200 r.p.m. The supernatant was removed, 50 µl of the monoclonal anti-hybrid antibody conjugated to alkaline phosphatase (Digene Diagnostics, Silver Spring) was added and incubated for a further 30 min at room temperature. After removal of the antibody solution, the well was washed five times with wash buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.05% Tween 20) and once with water. Fifty µl of substrate solution (Troplex CDP-Star with Emeralds enhancer) were added, the plate was incubated at room temperature for 30 min in the dark and...
analyzed with the luminometer function of the Micro Beta Trilux counter (Berthold-Wallac, Bad Wildbad). With negative-stranded HAV RNA 56 000 luminescence counts/s (LCPS/100) were detected, whereas positive-stranded RNA only resulted in 100 LCPS/100 (Table 1). The presence of a 40-fold excess of positive-strand RNA (10 pmol) over negative strands (250 fmol), which simulates the ratios of negative- and positive-stranded RNA found in cells infected with poliovirus (12), another picornavirus, did not affect the result (58 000 LCPS/100). In order to determine the sensitivity, the HDA was performed with different amounts of negative-strand RNA. The lower limit for detection of negative-strand RNA was 100 amol. These results show that the HDA is a specific and sensitive method for the detection of HAV negative-strand RNA.

Table 1. Strand specificity of the HDA

<table>
<thead>
<tr>
<th>Sample</th>
<th>LCPS/100</th>
<th>(\chi^2) n–1</th>
</tr>
</thead>
<tbody>
<tr>
<td>(–)-strand RNA (250 fmol)</td>
<td>56106.15</td>
<td>267.6</td>
</tr>
<tr>
<td>(+)-strand RNA (250 fmol)</td>
<td>108.6</td>
<td>30.4</td>
</tr>
<tr>
<td>(–)-strand RNA (250 fmol) + 40-fold amount of (+)-strand RNA</td>
<td>58218.75</td>
<td>3209.2</td>
</tr>
</tbody>
</table>

Each data point is an average value obtained from two separate experiments and is adjusted by deduction of the value of the negative control. \(\chi^2\) n–1 indicates the standard deviation of the mean.

In order to investigate whether the HDA is applicable for the proof of HAV replication by detection of negative-strand RNA intermediates, replication kinetics were performed. Figure 1 shows the results of a representative experiment. FRhK-4 cells grown on 6 cm cell culture plates were infected with tissue culture-adapted HM175 strain of HAV (11) at a multiplicity of infection (m.o.i.) of 1. The inoculum was washed out after an incubation time of 2 h. and cytoplasmic extracts were prepared after removal of the inoculum and at 8, 16, 24, 36 and 48 h and 3, 4, 6 and 8 days post infection (p.i.), and total cell RNA was extracted with phenol-chloroform (13). RNA extracted from mock infected cells were used as negative controls. The HDA was performed with the HAV negative-strand specific DNA probe using 5 out of 50 µl of the extracted RNA as described above. In order to determine temporal differences in the appearance of negative-strand and positive-strand HAV RNA and to estimate the relative ratios of the two RNA forms, the HDA was also performed with a HAV positive-strand specific single-stranded DNA probe. The 5’-biotinylated positive-strand specific DNA probe, containing nucleotides 633–1090 of HAV negative sequence, was prepared using an antisense primer from nucleotide position 1071–1090, biotin-5’-CCTTGAACAGCAAACCT-GTCT-3’, by run-off synthesis as described above. Strand specificity of this probe was ascertained as described for the negative-strand specific probe.

HAV negative-strand RNA was after its first detection at 8 h p.i. detectable in increasing amounts until 36 h p.i. (Fig. 1A). Between 36 h and 6 days p.i. the amount was constant, followed by an increase until day 8 p.i. (Fig. 1A). Positive-strand RNA was detectable for the first time 16 h p.i. (Fig. 1B), with increasing amounts until day 8 p.i.. Between 36 and 48 h p.i., the amount of positive strands exceeded the amount of negative strands significantly (Fig. 1A). The positive strands also exhibited a phasic course, with steady state levels between 16 and 36 h p.i. (Fig. 1B) and 48 h and 6 days p.i. (Fig. 1A). The luminescence signals for each time point of two parallel infections were almost identical. The relative ratios of the two RNA species and the times at which the RNAs were detectable for the first time were similar in three independently performed kinetic studies. The results obtained by tail-PCR for the detection of HAV negative strands, which was performed as described in the literature (7), were similar with these obtained by HDA. These results show that the HDA is reproducible and as sensitive as the PCR approach. Moreover, changes in the amount of RNA during the time course of the infection are detectable with the HDA, which is not possible with the PCR method.

Applying a technique based on solution hybridization with a DNA probe, selective capture of the hybrids and specific immunological detection via a sensitive chemiluminescence reaction, we were able to detect low numbers of single-stranded RNA. The length of the single-stranded DNA probe should be at least 300 nucleotides to enable the monoclonal anti-DNA:RNA-hybrid antibody to bind to the hybrids. The capture step and the
specificity of the anti-hybrid antibody guarantee the specificity of the HDA.

This sensitive method enables kinetic studies without *in vivo* labeling methods, allows with its reproducibility the investigation of single samples and should be readily adaptable to detection of low amounts of single-stranded RNA of other viruses such as positive or negative-strand RNA of hepatitis C virus. As the HDA is easy to handle, performed in only 3 h and as many samples can be investigated simultaneously, the method is suitable for use in clinical laboratories.

**ACKNOWLEDGEMENTS**

We thank Digene Diagnostics, Silver Spring, for providing us with the anti-hybrid antibody DR1 as well as streptavidin-coated plates and helpful discussion in the course of this work. This work was supported by the Tönjes-Vagt-Stiftung, Bremen.

**REFERENCES**