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Rapid and Sensitive Method for Detection of Hepatitis C Virus RNA by Using Silica Particles

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We describe a rapid, sensitive, and economic method for detection of hepatitis C virus (HCV) RNA. This method uses silica particles for purification of nucleic acid and then a modified reverse transcription-PCR that minimizes the risk of contamination and reduces the amount of reagents used. We found purification by silica particles to be at least as sensitive and in certain circumstances more sensitive than that by traditional phenol-chloroform extraction. This improved sensitivity may be due to more efficient recovery of HCV RNA by silica particles. HCV RNA appears to bind to silica particles in a saturable fashion, and the addition of extraneous nucleic acids (salmon sperm DNA or tRNA) decreases the binding in a dose-related fashion. The reverse transcription-PCR is performed by using a modified single tube method which further simplifies and reduces the cost of this assay. Finally, this method may be applied to clinical specimens such as liver tissue.

Hepatitis C virus (HCV) is a newly isolated, flavivirus-like, positive-strand RNA virus approximately 9,500 bp in length (see reference 8 for a review). HCV is recognized as the agent responsible for more than 90% of the cases of posttransfusion hepatitis and at least 50% of the cases of sporadic, community-acquired non-A, non-B hepatitis (8). Unlike the situation with hepatitis B virus, the viral load of HCV in serum is generally quite low (7). Currently, the only means to detect viremia is by performing reverse transcription-PCR (RT-PCR) on extracted nucleic acid. The conventional method for purification of nucleic acid from clinical specimens for PCR involves the use of chaotropic reagents such as guanidinium isothiocyanate to lyse cells, followed by phenol-chloroform, and then chloroform extraction. Finally, the nucleic acid is precipitated and washed. The PCR is usually carried out twice by using two different sets of primers (nested PCR) in order to yield sufficient amplified products that can be visualized on a gel. After the first PCR, part of the PCR product is transferred to a second tube containing the inner primers for the second round of PCR (6, 9, 12, 15). These protocols involve manipulating the specimen multiple times during nucleic acid extraction and subsequent PCRs and hence are prone to contamination. Furthermore, phenol is toxic and needs to be disposed of in a glass receptacle. The process of extraction and subsequent PCR is tedious, making it difficult to run a large number of samples. As a result, PCR for detection of viral nucleic acid remains mostly a research tool.

Silica has been shown to bind nucleic acid in the presence of chaotropic agents and has been used mainly to purify DNA, such as hepatitis B virus DNA from serum (2). Chungue et al. (5) recently applied this method to extract dengue virus RNA from serum. DNA or RNA extracted by such methods was suitable for PCR or RT-PCR, respectively. DNA extracted by silica also appears to be suitable for most laboratory procedures, including sequencing, restriction enzyme digestion, and transfection (4). In this report, we describe a simplified rapid method that uses silica for extraction of HCV RNA from serum for PCR. The method is based on a modification of that described by Boom et al. (3) and is followed by a modified nested PCR technique that further simplifies the process and reduces the risk of contamination.

Sera used were usually prepared and frozen within 6 h after collection. Although repeat freezing and thawing was avoided by dividing samples into small aliquots, some samples were frozen and thawed several times. Sera derived from a single aliquot were used to compare the simplified rapid silica extraction method with traditional phenol-chloroform extraction, thus ensuring that the sera were exposed to identical conditions prior to RNA extraction. Normal sera were obtained from uninfected individuals without any evidence of liver disease and repeatedly tested negative for HCV antibodies by enzyme immunoassay (EIA II; Abbott Laboratories).

Size-fractionated silica (SC) was prepared as described by Boom et al. (3). Briefly, 60 g of amorphous silica (silicon dioxide, SiO_2; Sigma S5631) was suspended in 500 ml of demineralized water, and the suspension was allowed to settle for 24 h. The settled particles were recovered and resuspended in demineralized water for an additional 5 h, and 440 ml of the supernatant was removed. Finally, 600 μl of concentrated HCl was added to adjust the pH of the suspension to 2, prior to autoclaving. The final silica preparation was stored in aliquots in the dark. The lysis and washing buffers were also prepared as described by Boom et al. (3). Lysis buffer consisted of 120 g of guanidine thiocyanate (GuSCN; Fluka Chemical) dissolved in 100 ml of 0.1 M Tris-HCl (pH 6.4)–22 ml of 0.2 M EDTA (pH 8.0)–2.6 g of Triton X-100. Washing buffer was made by dissolving 120 g of GuSCN in 100 ml of 0.1 M Tris-HCl (pH 6.4). In contrast, washing buffer used by Chungue et al. (5) consisted of 50% ethanol in TNE buffer (10 mM Tris-HCl [pH 7.4], 50 mM NaCl, 1 mM EDTA).

Fifty microliters of serum was added to an Eppendorf tube containing 20 μl of SC in 900 μl of lysis buffer. The sample was mixed with an Eppendorf 5432 mixer for 10 min at room temperature. The supernatant was aspirated after a quick spin (12,000 × *g* for 1 min), and the silica-RNA pellet was washed twice with 450 μl of washing buffer and then twice with 1 ml of 70% (vol/vol) ethanol. After a final wash with 1 ml of acetone,

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the pellet was dried on a heat block (Fisher Scientific) at 56°C for 10 min. The RNA was eluted by resuspending the pellet in 20 to 50 μl of diethyl pyrocarbonate-treated water at 56°C for 10 min. The supernatant recovered after pelleting the silica particles (12,000 × *g* for 3 min) contained the RNA and was used for RT-PCR. For comparison, HCV RNA was also extracted from sera by using phenol-chloroform as described in detail previously (6, 9, 12, 15). Briefly, 50 μl of serum was extracted in GuSCN buffer (4.2 M GuSCN, 25 mM Tris-HCl [pH 8], 0.5% sodium Sarkosyl, 0.7% 2-mercaptoethanol), followed by phenol-chloroform extraction and chloroform extraction. RNA was precipitated with isopropanol at −70°C overnight or on dry ice for a minimum of 2 h. Precipitates were washed in 70% ethanol (vol/vol), precipitated as described above, pelleted at 14,000 × *g* in the cold room, dried in a Speed Vac (Savant), and resuspended in 10 to 50 μl of diethyl pyrocarbonate-treated water.

The RT-PCR was performed as described previously (9, 15) by using primers from the highly conserved 5′ noncoding region. Outer primers were 5′-GGCGACACTCCACCATA GAT-3′ and 5′-CATGGTGCACGGGTCTACGAGA-3′. Inner primers were 5′-GGAACTACTGTCTTCACGGGAG-3′ and 5′-TCGCAAGCACCCTATCAGGCA-3′. The reaction mixture consisted of the nucleic acid extract (corresponding to 5 μl of serum or its dilution), 2 pmol of each primer, 10 mM deoxynucleoside triphosphates, 0.5 U of *Taq* DNA polymerase (Boehringer Mannheim), 2 U of avian myeloblastosis virus reverse transcriptase (Seikagaku America, Inc.), and *Taq* buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$, and 0.01% gelatin) in a final reaction volume of 20 μl. The thermocycler (Perkin-Elmer Cetus) was programmed to incubate the sample at 43°C for 40 min for the initial RT step, followed by 35 cycles consisting of 94°C for 1 min, 50°C for 1 min, and 72°C for 1.5 min. For the second PCR, 40 μl of solution containing 20 pmol of each of the inner primers, 10 mM deoxynucleoside triphosphates, 0.4 U of *Taq* DNA polymerase, and *Taq* buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$, and 0.01% gelatin) was added to the product of the first PCR and centrifuged for 1 min to allow mixing. PCR was carried out using the identical conditions (as described above) for another 35 cycles. PCR products were analyzed by gel electrophoresis on a 3% agarose gel stained with ethidium bromide. Visualization of a 259-bp band that corresponded to the expected nested PCR product was considered a positive result. All necessary precautions were taken to prevent contamination (10), and positive and negative controls were included at the purification and amplification steps.

To determine the optimal quantity of silica particles required for binding, serum was extracted in the presence of 5, 10, 20, and 40 μl of silica particles. RT-PCR was performed on serial 10-fold dilutions of the eluted nucleic acids to determine the amount of HCV RNA recovered by various quantities of silica particles. Twenty microliters of SC was determined to be the optimal amount for recovering HCV RNA from 50 μl of serum, as indicated by semiquantitative RT-PCR on serial 10-fold dilutions of eluted nucleic acid (data not shown). There was no difference when 20 or 40 μl of SC was used for extraction, but there was a 10-fold decrease in recovery when only 10 or 5 μl of SC was used. This result suggests that a minimum amount of silica particles is required for binding viral nucleic acid, but an excessive amount will not enhance recovery. Therefore, 20 μl of SC was used for all subsequent experiments, unless specified otherwise. In addition, a lower rate of recovery of RNA was observed if the washing step with the washing buffer was omitted or if washing was done with the buffer described by Chungue et al. (5) (data not shown).

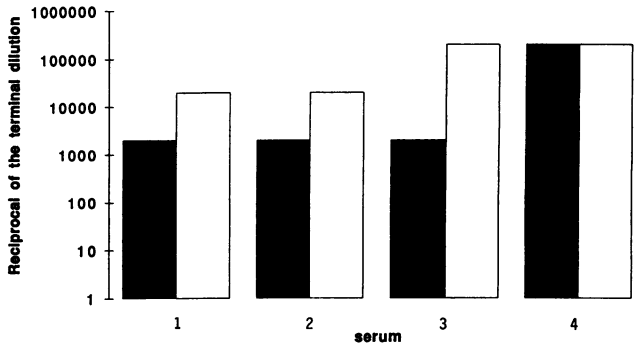

FIG. 1. Comparison of end point dilution of purified RNA by phenol-chloroform (■) and silica (□) methods. HCV RNA was purified from 50 μl of HCV antibody-positive sera by either the phenol-chloroform or silica method. RT-PCR was performed on serial 10-fold dilutions of extracted nucleic acid, starting with nucleic acid from 5 μl of serum. Terminal dilution was defined as the last dilution that continued to be PCR positive, i.e., the sample became PCR negative at the next 10-fold dilution. This is expressed as the reciprocal of the terminal dilution positive by PCR or PCR unit per ml.

To compare the efficiency of purification by each method and the suitability of the extracted nucleic acid for subsequent RT-PCR, serial RT-PCRs were performed. End point dilution of sera was estimated by performing HCV RNA RT-PCR on serial 10-fold dilutions of nucleic acid purified from 50 μl of serum from four chronically infected HCV (EIA II) antibody-positive patients. This semiquantitative assay is an estimate of the HCV RNA recovered by the purification process. Using phenol-chloroform extraction, an end point dilution that ranged from 10^3 to 10^5 PCR units per ml was achieved (Fig. 1). The titer achieved was either the same (one sample) or higher (three samples), using the silica extraction protocol. The end point dilution titer was 10-fold higher in two of the three samples and 100-fold higher in one sample (Fig. 1). Of note, the silica extraction method appeared to recover more HCV RNA from the three samples with the lowest titers as determined by the phenol-chloroform method.

The sensitivity of the assay is a function of two parameters: the amount of viral nucleic acid that is recovered during purification and the presence of extraneous product(s) that may be coextracted with the RNA of interest. The latter includes nonviral nucleic acid that may interfere with or decrease the sensitivity of the RT-PCR. To estimate the sensitivity of the assay as applicable to clinical specimens, positive serum was serially diluted 10-fold in normal seronegative serum prior to extraction. Similar to the findings above, extraction by silica particles resulted in a 10- to 1,000-fold increase in sensitivity (Fig. 2). In addition, most of the higher-titer samples in this experiment also had higher titers in the earlier experiment, suggesting the increased sensitivity of the silica purification method is, in part, a direct consequence of the improved recovery of HCV RNA during the extraction step. In contrast to the results of the earlier experiment when both HCV RNA and extraneous nucleic acid in serum were serially diluted, all samples in this experiment contained a constant amount of extraneous nucleic acid or other (if any) inhibitor(s). The findings of this experiment thus would be a better indicator of the sensitivity of this assay when applied to clinical samples which differ mainly in the level of viremia.

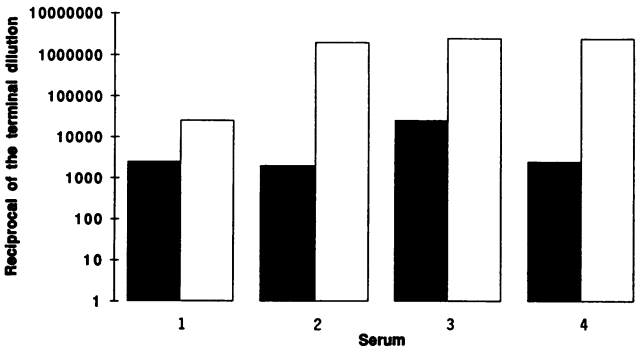

FIG. 2. Comparison of sensitivity of phenol-chloroform (■) and silica (□) purification methods. HCV RNA was purified from 50 μl of HCV antibody-positive serum serially diluted 10-fold in normal serum by either the phenol-chloroform or the silica method. RT-PCR was performed on nucleic acid extracted from 5 μl of sample. Terminal dilution was defined and expressed as defined in the legend to Fig. 1. The format of these experiments was similar to that described for Fig. 1. The only difference was that HCV RNA purified from 50 μl of HCV antibody-positive sera was first serially diluted 10-fold in normal serum, using either the phenol-chloroform extraction or the silica method.

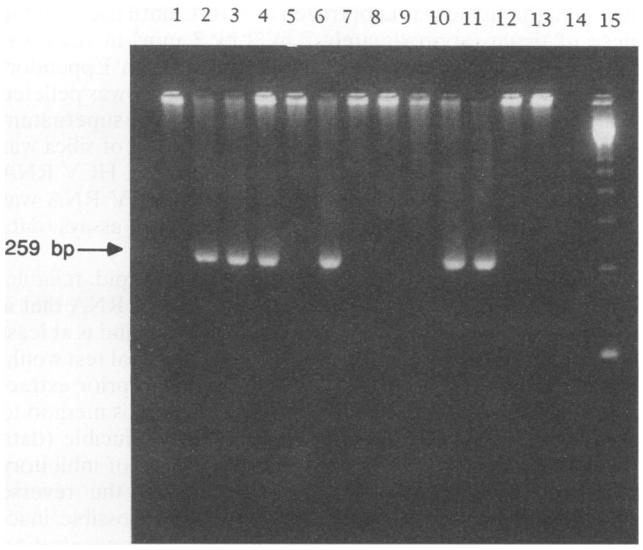

FIG. 3. Effect of yeast tRNA on recovery of HCV RNA from serum by silica purification. Ethidium bromide-stained 3% agarose gel of RT-PCR products is shown. A 259-bp band which corresponds to the expected nested PCR product was considered to be positive. Lanes: 1, negative serum; 2 to 5, serial 10-fold dilutions of positive serum purified by silica particles; 6 to 9, serial 10-fold dilutions of the same serum extracted in the presence of 100 μl of yeast tRNA (10 μg/ml); 10 to 13, serial 10-fold dilutions of the same serum extracted by silica in the presence of 10 μl of yeast tRNA (10 μg/ml); 14, RT-PCR product in the absence of nucleic acid; 15, 123-bp molecular size ladder.

The sensitivity of the two assays was further compared by testing 30 coded sera, 20 of which were HCV antibody positive. Coded sera from 10 patients undergoing interferon treatment for chronic hepatitis C (many were also alcoholics), 8 sera from patients with end-stage liver disease of various etiologies obtained prior to liver transplantation, and 12 miscellaneous samples including sera from one patient with cryoglobulinemia, an asymptomatic blood donor, a patient on hemodialysis, and other normal individuals were compared by both extraction methods. There was excellent correlation between the two extraction methods. Eighteen of 30 were positive by both methods, and 11 were negative by both methods. One serum sample, obtained during a relapse from a HCV antibody-positive patient who had been treated with interferon, was repeatedly negative by phenol-chloroform extraction but repeatedly positive by silica purification. Serum from this patient was positive for HCV RNA prior to interferon treatment. This single discordant specimen appeared to contain low levels of HCV RNA, since the sample became negative by the silica purification method when RT-PCR was performed after a 10-fold dilution.

Although it was not our aim to study the sensitivity and specificity of the HCV antibody serology assay, we found a good correlation between HCV antibody and HCV RNA results in the selected group of patients studied. Twenty of 30 sera were HCV antibody positive by EIA I or II (all EIA I-positive sera were also confirmed by RIBA II [Ortho Diagnostics]). The remainder were HCV antibody negative by EIA II. Discordant results were observed in only three samples (10%). Seventeen of 18 sera that were positive by both purification methods were HCV antibody positive. The only exception was serum from a post-cardiac transplant patient on immunosuppressive therapy who had received numerous transfusions and had marked elevations of transaminases with mild symptoms of liver disease. EIA II was repeatedly negative and RIBA II was indeterminate, but two different samples from the patient were positive for HCV RNA by both purification methods. Wright et al. (15) and others have shown that a significant percentage of posttransplant immunosuppressed patients are viremic but antibody negative. Two of the 11 patients that were negative for HCV RNA by both purification methods were HCV antibody positive. One patient was a liver transplantation candidate, the other was a volunteer blood donor with normal transaminases and an uncertain history of prior blood transfusion. The patients we studied had either an acute infection and were no longer viremic or, more likely, the level of viremia was below the detection limit of RT-PCR (7, 13).

In order to understand the mechanism(s) and specificity of binding to silica particles, HCV RNA was purified from sera in the presence of yeast tRNA (Bethesda Research Laboratories) and salmon sperm DNA (Sigma). Increasing volumes of salmon sperm DNA or tRNA (both at 10 μg/ml) at 10-fold increments were added to an HCV RNA-positive serum prior to purification by silica particles. After eluting the nucleic acids from silica particles, serial 10-fold dilution of the eluate was performed prior to RT-PCR. The addition of 10 or 100 μl of salmon sperm DNA resulted in a more than 1,000-fold decrease in the amount of HCV RNA recovered, whereas the addition of 1 μl resulted in a 100-fold decrease, and 0.1 μl of salmon sperm DNA had no effect. Similarly, 100 and 10 μl of tRNA decreased the titer by 100- and 10-fold, respectively (Fig. 3). In addition, the efficiency of recovery was decreased by the presence of other extraneous cellular material. For example, HCV RNA purified from serum in the presence of a cell pellet (approximately 2.5 million cells) resulted in a 100-fold decrease in the HCV RNA titer (data not shown).

To test whether this method is suitable for the purification of nucleic acids from tissue, HCV RNA was extracted from liver specimens of three HCV antibody-positive cirrhotic patients. Two were surgical specimens and one was an autopsy speci-

men, and the tissue was kept frozen at −70°C until use. A small piece of tissue (approximately 2 by 2 by 2 mm^3 in size) was minced with a razor blade and transferred to an Eppendorf tube containing the lysis buffer. Particulate matter was pelleted in a microcentrifuge (12,000 × *g* for 1 min). The supernatant was transferred to another Eppendorf tube, 20 μl of silica was added, and the mixture was incubated for 10 min. HCV RNA was extracted as described above for serum. HCV RNA was detected in all three liver specimens by using both assays (data not shown).

The major aim of this study was to develop a rapid, reliable, and simple method for routine detection of HCV RNA that is applicable to large numbers of clinical specimens and is at least as sensitive as the conventional techniques. An ideal test would directly amplify HCV RNA from serum without prior extraction. Although this is possible (1, 14), we found this method to yield highly variable results that were not reproducible (data not shown). This may be related to the presence of inhibitory factor(s) in serum that affect the activity of the reverse transcriptase and/or *Taq* polymerase or to the possible inaccessibility of the viral RNA in serum to RT. The concentration of these inhibitory factor(s) has to be balanced with the extremely low level of HCV RNA in serum. Others have noted that direct amplification is frequently more reliable when a smaller amount of serum is used (1).

We have shown extraction by silica to be as efficient as and, in some situations, even more efficient than extraction by phenol-chloroform. The efficiency of recovery of rRNA added to serum lysate and DNA by using silica particles has been estimated to be 60% (3) and 60 to 80% (4), respectively. In these studies, most of the DNA lost during extraction by silica occurred during the elution step in which approximately 30% of the DNA was not eluted. We also have found elution to be incomplete. We were able to detect residual HCV RNA bound to silica particles by a second elution after all the supernatant was removed (data not shown).

Silica has been used mainly for extraction of DNA from viruses such as hepatitis B virus (2). Chungue et al. (5) have recently used silica for the isolation of dengue virus RNA from serum followed by RT-PCR. They found the sensitivity of the silica RT-PCR method to be greater than or equal to that of other published methods, but these methods were not compared side by side. In addition, they used buffers different from that used in our experiments for RNA extraction and performed RT-PCR in the presence of silica. When we used the buffers they described, we found a lower sensitivity and multiple bands could occasionally be seen after RT-PCR (data not shown). In addition, the presence of more than a minute amount of silica particles in the purified sample appeared to interfere with the RT-PCR (data not shown).

The other major advantage of the silica extraction method is the savings in both time and cost. By scaling down the volume of the PCR mix and performing both PCRs in the same tube, we used less reverse transcriptase and *Taq* polymerase and minimized the risk of contamination. In prior studies, we noted that most of the contamination occurred during the transfer of reagents between the first- and second-stage PCR (unpublished data). The single tube method has been described to reduce the risk of contamination, but we found the concentration of primers in the reaction mixture previously described by Lin et al. (11) to be suboptimal to the concentrations we used (data not shown). Because of the short incubation periods of no more than 10 min, 8 to 10 specimens could be extracted in approximately 1 h. Up to 18 RT-PCR samples can be prepared in less than 2 h, making it possible to perform nested PCR and analysis of the products by gel electrophoresis within the same day. Furthermore, the protocol described in this report used only 40% of the reverse transcriptase and 80% of the *Taq* polymerase used in conventional methods we have described previously (9, 12, 15). The number of Eppendorf tubes used was reduced by one-third during the extraction steps and by one-half during the PCR steps. In addition, this method did not require dry ice or equipment such as a Speed Vac.

In summary, this report describes a fast, reliable, and economic method for the detection of HCV RNA that is potentially applicable to detecting viral nucleic acids in other clinical specimens. The method uses silica particles for extraction of HCV RNA, followed by a modified single tube method to perform nested PCR. This method is as least as sensitive as conventional methods and has the added advantages of requiring smaller quantities of reagents and supplies and less time to perform.

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