Fully Automated Detection of Hepatitis C Virus RNA in Serum and Whole-Blood Samples

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In this study, we established a fully automated molecular assay for qualitative detection of hepatitis C virus (HCV) in serum and whole-blood samples and compared it with conventional molecular assays, including manual HCV RNA extraction protocols. Whole-blood samples were collected from patients with and without chronic HCV infection in EDTA tubes and nucleic acid stabilization tubes (NASTs). Prior to HCV RNA extraction, the HCV Internal Control (IC), derived from the COBAS AMPLICOR HCV test, version 2.0 (Roche Molecular Diagnostics), was added. The new assay was based on an automated extraction protocol on the MagNA Pure LC instrument (Roche Applied Science), followed by automated reverse transcription, amplification, hybridization, and detection on the Cobas Ampliprep analyzer (Roche Molecular Diagnostics). The detection limit of the new assay was found to be similar to those of conventional molecular assays. In clinical samples, 100% agreement between the new assay and conventional methods was observed. The introduced amount of IC was detected in 45 of 45 serum samples, 41 of 45 EDTA tube whole-blood samples, and 43 of 45 NAST whole-blood samples. Retesting led to more frequent IC detection. The fully automated molecular assay was found to be suitable for detection of HCV RNA in different kinds of sample materials. It may be recommended for use in the high-throughput routine molecular diagnostic laboratory.

Molecular techniques have been shown to be effective tools for direct detection of hepatitis C virus (HCV). Such assays for detection of pathogens basically consist of several steps: extraction of HCV RNA (also called sample preparation), reverse transcription (RT), amplification of cDNA, hybridization of amplified products, and detection of nucleic acid hybrids. Molecular techniques are labor-intensive and time-consuming when manual home brew methods are used. To meet the needs of the routine diagnostic laboratory, PCR amplification and detection of amplified products have recently been automated with the COBAS AMPLICOR (Roche Molecular Diagnostics, Pleasanton, Cal.) analyzer (1, 4, 5, 14).

Sample preparation is currently considered the major weakness in molecular detection of HCV RNA. Conventional sample preparation protocols are usually time-consuming, labor-intensive, and susceptible to contamination. It has been demonstrated that the probability of obtaining false-positive results because of contamination increases in relation to the number of manipulations involved in sample processing (3, 11). To save time and labor, more rapid and automated nucleic acid extraction protocols with fewer manipulation steps have largely replaced conventional protocols. Several ready-to-use sample preparation kits, available either separately or as part of the entire molecular kit, have been brought to market and found suitable for inclusion in molecular assays for detection of RNA viruses (6, 9, 15). Recently, a new automated specimen preparation instrument, the MagNA Pure LC (Roche Applied Science, Mannheim, Germany), was developed to automate sample preparation (7).

Previous studies have shown that HCV can infect peripheral blood mononuclear cells (PBMCs) in patients with chronic hepatitis C (2, 17, 19). The presence of HCV in PBMCs may have implications for the response to antiviral therapy (10, 13, 16). It has been demonstrated recently that detection of HCV RNA in PBMCs may be an additional tool to demonstrate the persistence of HCV RNA. Reappearance of HCV RNA is detected earlier in EDTA tube whole-blood samples than in sera (6). Therefore, recovery of total HCV RNA (i.e., intracellular RNA as well as plasma RNA) appears to be of major importance to detect low-level viremia. Until recently, the sustained virological response rate to anti-HCV therapy was rather low. Introduction of novel drugs, such as, e.g., pegylated interferon α-2b, has been shown to significantly increase the rate of patients with nondetectable serum HCV RNA (12, 18). These new therapeutic approaches have led to a strong increase in the number of EDTA tube whole-blood samples to be tested with HCV RNA. The aims of the present study were to establish a fully automated molecular assay for qualitative detection of HCV RNA in serum and whole-blood samples, which included automated RNA extraction with the MagNA Pure LC instrument, and to compare it with conventional molecular assays, which include manual HCV RNA extraction protocols.

Blood samples were collected from patients with chronic hepatitis C (nonresponders and those with sustained virological response) and from healthy blood donors. For qualitative detection of serum HCV RNA, blood was collected in 9-ml
Within 2 h of blood being drawn, 9-ml tubes were centrifuged at 1,500 x g for 20 min at room temperature. After centrifugation, serum aliquots were prepared, which were immediately frozen at −70°C until testing. Whole-blood collection tubes were frozen at −70°C without prior preparation.

In our ISO 9002-certified laboratory, serum samples were tested for the presence of HCV RNA with the COBAS AMPLICOR HCV MONITOR test, version 2.0 (Roche Molecular Diagnostics), according to the manufacturer's instructions. Whole-blood samples were analyzed with a combination of the High Pure Viral Nucleic Acid kit, version 3 (Roche Applied Science), and the COBAS AMPLICOR HCV MONITOR test, version 2.0, as described previously (8).

For the fully automated molecular assay, isolation of HCV RNA was done on the MagNA Pure LC instrument with the MagNA Pure LC Total Nucleic Acid Isolation kit (Roche Applied Science). MagNA Pure LC software, version 2.1, was used. For extraction of HCV RNA from serum and EDTA tube whole blood, the protocol “total nucleic acid serum, plasma, blood-variable elution volume” was employed. The input sample volume was 200 μl. The appropriate amount of HCV Internal Control (IC) from the COBAS AMPLICOR HCV test, version 2.0, was added to the lysis reagent prior to the start of the extraction procedure. When NASTs were used, 200 μl of NAST whole blood was mixed with 300 μl of preincubation solution and 8 μl of a suspension, which contained the appropriate amount of IC. After incubation at room temperature for 15 min and vortexing, 500 μl was used as the input sample volume, and the protocol “total nucleic acid isolation-external lysis” was employed. The protocol was run without use of the lysis buffer included in the kit. In each run, an elution volume of 60 μl and a dilution volume of 0 μl were chosen. Other details, such as reagent volumes and the number of reaction tips needed for the run, were automatically calculated by the software. The MagNA Pure LC automatically performed all of remaining steps of the procedure with specially designed nucleic-acid-free, disposable reaction tips. These reaction tips not only transferred the samples, but also served as reaction vials for the procedure. Within the tips, nucleic acids were bound to magnetic beads, washed free of impurities, and finally eluted from the magnetic beads into a cooled sample cartridge. During the run, used reaction tips were automatically discarded into an attached, autoclavable waste bag.

After completion of HCV RNA isolation, the specially designed MagNA Pure LC Cooling Block for A rings was placed into the posteluton area. After the start of the posteluton protocol, which had been programmed prior to the start of the first run, the MagNA Pure LC automatically pipetted 50 μl of the master mix and 50 μl of the processed sample into each of the A ring tubes. RT, amplification, hybridization, and detection of amplification products were done on the COBAS AMPLICOR analyzer with the COBAS AMPLICOR HCV test, version 2.0.

In a first step, the optimal amount of IC copies per reaction to be introduced into the new molecular assay was determined. Serum and EDTA tube whole-blood samples were collected from five healthy donors. In the first run, the IC from the COBAS AMPLICOR HCV test, version 2.0, was added to the lysis reagent in an amount similar to that required for that assay. In the second run, the IC was added in double the amount, and in the third run, it was added in fourfold the amount. Three aliquots of each of the sample types were analyzed: i.e., 15 results per sample type per defined amount of IC were obtained. When the IC, taken from the COBAS AMPLICOR HCV test, version 2.0, was introduced in an amount similar to that required for the assay, it could be detected in 9 of 15 serum samples and in 5 of 15 EDTA tube whole-blood samples. When double the amount of IC was introduced, it was detected in all 15 sera and in 13 of 15 EDTA–whole-blood samples. With fourfold the amount (e.g., for 32 samples, 282.6 μl of IC was added to 10.6 ml of lysis reagent, equivalent to 8 μl of IC per 300 μl of lysis reagent used for each sample), the IC was detected in all 15 sera of each of the sample types.

In a second step, the detection limit of the fully automated molecular assay was determined. Serum and whole-blood (EDTA tubes and NASTs) samples were collected from another healthy donor. Samples were spiked with members no. 2 (HCV genotype 1) and 5 (HCV genotype 3) of the Second European Union Concerted Action (EUCA) HCV Pro

Panel (www.qcca.org.uk). Dilutions, which contained 200, 100, 50, 25, and 12.5 HCV RNA IU/ml, were prepared and analyzed three times. The results for HCV genotypes 1 and 3 were identical for all kinds of samples. Serum samples, which contained 50 or 25 HCV RNA IU/ml, consistently tested positive. When serum samples contained 12.5 HCV RNA IU/ml, the fully automated assay produced inconsistent (negative, borderline, and positive) results. All EDTA tube whole-blood samples, which contained 100 HCV RNA IU/ml, tested positive. When EDTA tube whole-blood samples contained 50 HCV RNA IU/ml, inconsistent results were obtained. All NAST whole-blood samples, which contained 50 HCV RNA IU/ml, tested positive. When NAST whole-blood samples contained 25 HCV RNA IU/ml, the assay produced inconsistent results.

In a third step, the clinical performance of the fully automated molecular assay in the routine clinical laboratory was evaluated and compared with that of the routinely done assays. Blood samples were collected from 45 patients with chronic hepatitis C. Thirty-two patients were nonresponders (i.e., they had tested positive with the routinely performed assays), whereas the remaining 13 patients had a sustained virological response (i.e., no HCV RNA had been detectable in any of the samples). When 135 clinical samples were tested with the fully automated molecular assay, the results were in 100% agreement with those obtained by the routinely performed molecular assays. The IC was detected in all serum samples, but was missed in 4 of 45 (9%) EDTA tube whole-blood samples and in 2 of 45 (4%) NAST whole-blood samples. All samples with negative IC were from different patients; in two of the four EDTA tube whole-blood samples and in both of the NAST whole-blood samples, HCV RNA was not detected. When those samples were retested, the IC was detected in three of four EDTA tube whole-blood samples (in those without detectable HCV RNA and in one of those with detectable HCV
RNA and in both of the NAST whole-blood samples (Table 1).

The automated RNA extraction with the MagNA Pure LC was completed within 105 min for extraction of 32 samples. This included a 15-min set up of the MagNA Pure LC. The time required for the postelution protocol was 15 min. The manual extraction of 32 samples could be completed within 180 min for serum samples and within 150 min for EDTA tube whole-blood or NAST whole-blood samples.

The accepted criterion for assessment of efficacy in studies of anti-HCV therapy is sustained loss of HCV RNA in serum. However, it has been shown that HCV can infect PBMCs in patients with chronic hepatitis C (2, 17, 19). Hence, molecular assays for detection of serum HCV RNA only may be insufficient, and an additional qualitative molecular assay for detection of HCV RNA in PBMCs may be advisable. Because of improved therapeutic management of chronic hepatitis C, the number of patients with sustained loss of serum HCV RNA is continuously increasing (12, 18). Therefore, an increasing number of blood samples must be analyzed. To meet the requirements of a routine molecular diagnostic laboratory, a fully automated qualitative molecular assay for detection of HCV RNA in serum and whole-blood samples was established. This assay was compared to the conventional molecular assays, which include manual HCV RNA extraction protocols.

Amplification may fail because of interference from PCR inhibitors. It is therefore obligatory to incorporate an IC into every molecular assay. The qualitative COBAS AMPLICOR HCV test includes the HCV IC, an in vitro RNA transcript with primer binding regions identical to those of the HCV target sequence, a randomized internal sequence with a length and base composition similar to those of the HCV target sequence, a randomized internal sequence with a length and base composition similar to those of the HCV target sequence, and a unique probe-binding region that differentiates the IC amplification product from the HCV amplification product. The HCV IC is coamplified with the target RNA. When serum or EDTA tube whole blood was used, the appropriate amount of HCV IC was manually introduced into the lysis reagent prior to the start of the fully automated extraction procedure. When NASTs were used, several manual manipulations had to be done prior to automated extraction: the flakes had to be resuspended by gentle shaking, and the mixture consisting of NAST whole blood, HCV IC, and preincubation solution was prepared manually. Those steps, however, may be included in the automated protocol by a small modification (increase of sample input volume) of the protocol “total nucleic acid serum, plasma, blood-variable elution volume” in the future.

To obtain better standardization and reliability of nucleic acid extraction, semiautomated and automated platforms have been developed. The MagNA Pure LC employs magnetic beads for the isolation of DNA and total RNA. By using this instrument, the probability of false-positive results attributable to contamination by hands-on manipulation may be eliminated. On the other hand, the HCV IC, which has a length of only 244 bp, must be detected to exclude the presence of one or more inhibitors. In this study, the input volume of the HCV IC had to be increased to an amount fourfold that used in the conventional COBAS AMPLICOR HCV test to achieve a sufficient rate of recovery. In a single package of the COBAS AMPLICOR HCV test, there is a sufficient amount of IC included to do the same number of tests (100) with the new protocol. A further increase in the IC concentration, however, would not be useful because of the limited amount of IC.

With the fully automated assay, the detection limit for serum samples was found to be slightly better (25 HCV RNA IU/ml) than that of the COBAS AMPLICOR HCV test, version 2.0 (50 HCV RNA IU/ml). When the detection limit of whole-blood samples was determined, it was found to be equal to that of the COBAS AMPLICOR HCV test (NASTs) or slightly worse (100 HCV RNA IU/ml; EDTA tubes). No differences were observed when samples spiked with either HCV genotype 1 or 3 were analyzed.

In the clinical study, 100% agreement of results was found when the fully automated assay and the conventional molecular assays were compared. In a total of six samples (four EDTA tube whole-blood samples and two NAST whole-blood samples), however, the internal control tested negative. Upon repetition, five of the six samples were positive for the HCV IC, and one of the EDTA tube whole-blood samples was negative again for the HCV IC. It remains unclear whether the HCV IC was lost during extraction or amplification had failed because of interference from PCR inhibitors and because the new extracts were not inhibitory or at least were less inhibitory than the originals.

In summary, with the fully automated molecular assay described in this study, reliable results can be achieved for qualitative detection of HCV RNA in serum and whole-blood samples. Compared to the manual assay, the automated assay saved hands-on work and was easy to use. It brings full automation of molecular methods into the routine diagnostic laboratory and may help avoid human error.

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REFERENCES


