JOURNAL OF CLINICAL MICROBIOLOGY, Feb. 1997, p. 520–522 Vol. 35, No. 2
0095-1137/97/$04.00+0

Comparison of QIAamp HCV Kit Spin Columns, Silica Beads, and Phenol-Chloroform for Recovering Human Immunodeficiency Virus Type 1 RNA from Plasma

ROBERT W. SHAFER,[1]* DARCY J. LEVEE,[1] MARK A. WINTERS,[1] KATHRYN L. RICHMOND,[1] DIANA HUANG,[2] AND THOMAS C. MERIGAN[1]

Division of Infectious Diseases, Stanford University Medical Center, Stanford, California,[1] and Virology Quality Assurance Laboratory, Department of Immunology/Microbiology, Rush Presbyterian—St. Luke's Medical College, Chicago, Illinois[2]

Received 27 August 1996/Returned for modification 23 October 1996/Accepted 1 November 1996

Human immunodeficiency virus type 1 (HIV-1) *pol* mutations are responsible for HIV-1 resistance to current antiretroviral drugs. HIV-1 RNA extraction with QIAamp HCV kit spin columns (Qiagen, Chatsworth, Calif.) followed by reverse transcription-PCR successfully recovered a 1,008-bp *pol* fragment from the plasma of 31 of 34 HIV-1-infected patients that was suitable for sequencing and recombinant-virus studies. The minimum HIV-1 RNA concentration required for gene recovery was 30 to 40 copies/ml, which was similar to the minimal HIV-1 RNA concentration required when phenol-chloroform or silica beads are used for RNA extraction.

Sensitive reverse transcription (RT)-PCR protocols that amplify short (100- to 200-bp) regions of human immunodeficiency virus type 1 (HIV-1) have been developed for detection and quantitation of HIV-1 RNA in plasma (4, 5, 8). However, the sensitivity and yield of extraction of HIV-1 RNA from plasma and RT-PCR for amplifying larger HIV-1 gene fragments for genotypic and phenotypic analyses have not been examined. Sequencing of HIV-1 protease and reverse transcriptase genes is commonly used to study the genetic mechanism of resistance to antiretroviral drugs. We describe a method for extracting HIV-1 RNA from plasma with the QIAamp HCV kit (Qiagen, Chatsworth, Calif.), and we compare this method to two previously described methods of RNA extraction with phenol-chloroform or silica beads.

Test samples. (i) Plasma. Plasma samples from HIV-1-seropositive patients were collected into tubes containing acid-citrate-dextrose anticoagulant. Plasma was separated from whole blood by centrifugation at 400 × *g* to 800 × *g* for 10 to 20 min. Separated plasma was recentrifuged at 800 × *g* for 10 to 20 min to sediment platelets. Clarified plasma was frozen at −70°C in 1-ml aliquots. The plasma HIV-1 RNA concentration was determined by a quantitative RT-PCR assay, the Amplicor HIV Monitor test (Roche Diagnostics Systems, Branchburg, N.J.) (4, 5).

(ii) HIV-1-seronegative plasma spiked with HIV-1 from a virus stock prepared at the Virology Quality Assurance Laboratory, Rush Medical College (Chicago, Ill.). The HIV-1 stock concentration was assessed by electron microscopy (described in reference 3), the Amplicor HIV Monitor test, and the Quantiplex branched-DNA signal amplification assay (Chiron, Inc., Emeryville, Calif.). Multiple 200-μl plasma aliquots containing 10^5, 10^4, 10^3, 10^2, and 10^1 virions/200 μl were then created.

Nucleic acid extraction. Three methods were used for RNA extraction: one involving QIAamp HCV kit spin columns, one involving silica beads, and phenol-chloroform phase separation purification. The latter two methods have been previously described (1, 7, 8); extraction with QIAamp HCV kit spin columns is described here. Thawed plasma aliquots were dispensed into 1.5-ml microcentrifuge tubes and centrifuged for 10 min at 125,000 × *g* at 10°C to concentrate the virus. The nonvisible virus pellet was resuspended in 140 μl of diethyl pyrocarbonate (DEPC)-treated H_2O. The manufacturer's instructions for the QIAamp HCV kit were then followed. (i) A total of 560 μl of virus lysis buffer-carrier RNA was added to 140 μl of concentrated virus from plasma, and the mixture was vortexed and kept at 20°C for 10 min. (ii) A total of 560 μl of 100% ethanol was added, and the mixture was vortexed. (iii) The new mixture was added to the spin column, which was centrifuged at 6,000 × *g* for 1 min in a microcentrifuge. (iv) The spin column was washed twice with 500 μl of wash buffer (6,000 × *g* for 1 min). (v) To elute the bound nucleic acid, 50 μl of 80°C DEPC-treated H_2O was added to the column, which was again centrifuged at 6,000 × *g* for 1 min.

RT-PCR and nested PCR. The conditions for RT-PCR were the same for each of the three methods compared. A 25-μl master mixture containing ≥200 U of murine leukemia virus reverse transcriptase (Pharmacia Biotech), 25 mM deoxynucleoside triphosphate, primer RT21 (250 ng; 5′-CTGTATT TCTGCTATTAAGTCTTTTGATGGG-3′), 2.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris HCl (pH 8.3), 10% Nonidet P-40, 0.1 M dithiothreitol, and 40 U of RNasin (Promega, Madison, Wis.) was added to 25 μl of the eluted nucleic acid. The reaction mixture was incubated for 30 min at 42°C and then for 5 min at 95°C.

The 50-μl reverse-transcribed sample served as a template for the nested PCR, which was performed by a modification of a previously described protocol (6). The first PCR used primers RT18 (250 ng; 5′-GGAAACCAAAAATGATAGGGGG AATTGGAGG-3′) and RT21 (added for the RT step), 2.5 U of *Taq*, 0.25 mM deoxynucleoside triphosphates, and 2.5 mM $MgCl_2$, in a final volume of 100 μl. Thirty-five cycles of 94°C for 60 s, 55°C for 60 s, and 72°C for 120 s were performed in a thermocycler. Ten microliters of the product from the first PCR served as a template for the second PCR, performed with primers RT19 (250 ng; 5′-GGACATAAAGCTATAGGTAC AG-3′) and RT20 (250 ng; 5′-CTGCCAGTTCTAGCTCTGC TTC-3′) and under the same conditions as the first PCR.

* Corresponding author. Phone: (415) 725-2946. Fax: (415) 725-2395.

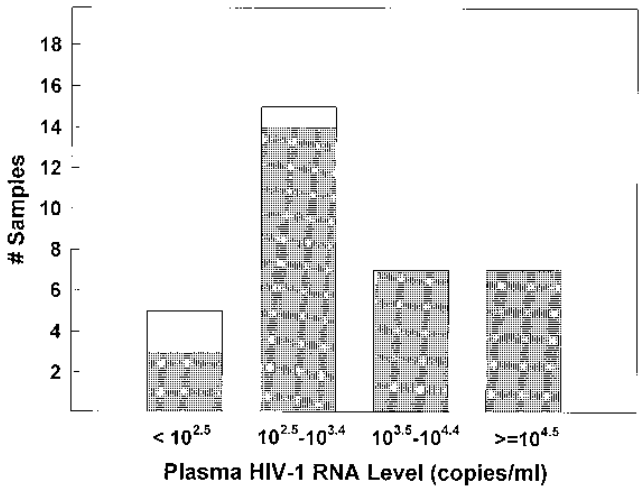

FIG. 1. Sensitivity of the QIAamp HCV kit and RT-PCR for extraction of RNA from 34 plasma samples from HIV-1-seropositive patients according to plasma HIV-1 RNA concentration. Shaded rectangles represent assays yielding an ≈1-kb HIV-1 *pol* gene. Empty rectangles represent negative assays.

Detection of amplified HIV-1 *pol* cDNA. Ten microliters of the product from the second PCR was analyzed by agarose gel electrophoresis and ethidium bromide staining. If an ≈1-kb fragment was present, the PCR product was purified. The genotype and phenotype of the *pol* gene were studied by DNA sequencing and/or the creation of infectious HIV-1 by homologous recombination with a proviral HIV-1 DNA clone containing a 780-bp *pol* gene deletion (Δ2-262, kindly provided by Wilco Keulen, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands) (2).

Sensitivity of QIAamp HCV kit spin columns and PCR for extraction of HIV-1 RNA from clinical plasma samples. A 1,008-bp HIV-1 *pol* fragment was amplified from the plasma of 31 of 34 (91%) HIV-1-infected persons (Fig. 1). The HIV-1 RNA concentrations in these samples were determined by quantitative PCR (Amplicor HIV Monitor test). The three plasma samples which did not yield a 1-kb fragment had relatively low HIV-1 RNA concentrations ($<10^{2.0}$ RNA copies/ml, $<10^{2.0}$ RNA copies/ml, and $10^{3.3}$ RNA copies/ml [Fig. 1]). In each of the 31 cases, amplified DNA was confirmed to be HIV-1 *pol* by either DNA sequencing or the creation of infectious recombinant HIV-1.

To determine the minimum plasma HIV-1 RNA concentration required for HIV-1 *pol* gene recovery, we performed four limiting dilution analyses with clinical plasma samples (Fig. 2). The estimated HIV-1 RNA copy number for each dilution was based on the Amplicor HIV Monitor test results for these samples. HIV-1 *pol* was detected in eight of eight replicates of 1,000 RNA copies, eight of eight replicates of 250 RNA copies, three of eight replicates of 62.5 RNA copies, and three of eight replicates of 15.6 RNA copies. As determined by a linear logistic model, the estimated number of plasma HIV-1 RNA molecules required to yield the *pol* fragment in 50% of the assays was 41 RNA copies/ml (95% confidence interval [CI], 15 to 108 copies/ml).

To assess the frequency of contaminating viral or proviral DNA, a non-reverse-transcribed nucleic acid control was also amplified in 22 of 34 of the plasma samples by nested PCR. For these samples, the column eluate was split into 25 μl for RT-PCR and 25 μl for nested PCR without a preceding RT step. Only 1 of the 22 non-reverse-transcribed controls yielded an ≈1-kb PCR product reflecting the presence of contaminating viral or proviral DNA (9).

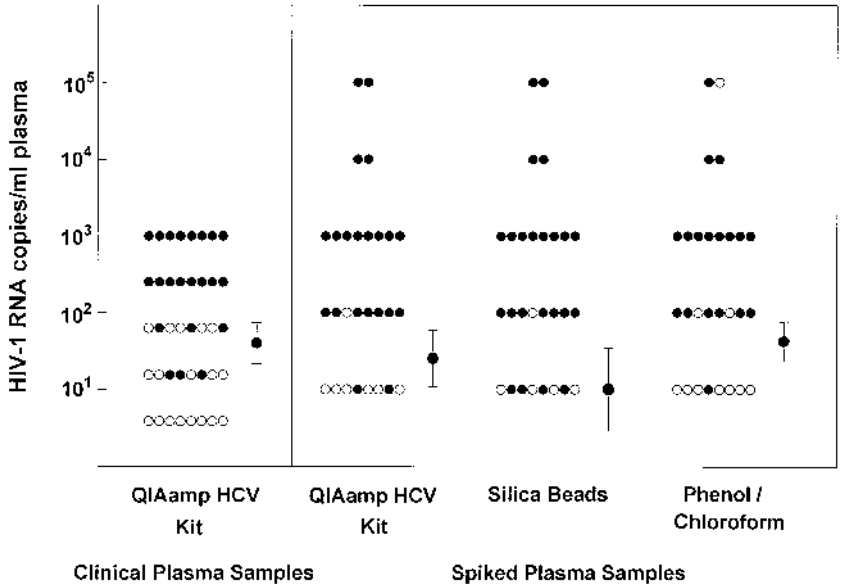

FIG. 2. Limiting-dilution analysis experiments to assess the sensitivity of the QIAamp HCV kit and RT-PCR for extraction of HIV-1 RNA from clinical plasma samples and of the QIAamp HCV kit, silica beads, and phenol-chloroform for extraction of HIV-1 RNA from spiked plasma samples. Solid circles indicate detection of a 1-kb fragment following RT-PCR. Open circles indicate a failure to detect a 1-kb fragment following RT-PCR. The plasma HIV-1 RNA concentration in clinical samples (leftmost titration) was determined by the Amplicor HIV Monitor test. The spiked-plasma HIV-1 RNA concentration was determined by electron microscopy (described in reference 3), the Amplicor HIV Monitor test, and the Chiron Quantiplex branched-DNA signal amplification assay. Error bars show the standard errors associated with the estimates of the number of plasma HIV-1 RNA molecules required to yield the *pol* fragment in 50% of the assays. The negative result at 10^5 copies for phenol-chloroform was attributed to a pipetting error and was not included in the titration analysis.

Comparison of HIV-1 RNA extraction from spiked plasma samples with the QIAamp HCV kit, silica beads, and phenol-chloroform. The minimum plasma HIV-1 RNA concentration required for HIV-1 *pol* gene recovery by three different extraction methods was compared with aliquots of HIV-1-seronegative plasma spiked with serial 10-fold dilutions of a supernatant from an HIV-1 stock culture. The estimated 50% minimum plasma HIV-1 RNA concentrations were 26 copies/ml (95% CI, 7 to 100) for the QIAamp HCV kit, 43 copies/ml (95% CI, 16 to 112) for phenol-chloroform, and 11 copies/ml (95% CI, 0 to 107) for silica beads. There was no statistically significant difference between each of these results and those obtained with the QIAamp HCV kit and the clinical plasma samples.

Discussion. HIV-1 RNA extraction with QIAamp HCV kit spin columns followed by RT-PCR is a new method for recovering plasma HIV-1 RNA for sequencing and other applications. The sensitivity of the method across a range of clinical samples was demonstrated by recovering HIV-1 *pol* genes from the plasma of 31 of 34 HIV-infected persons. Limiting dilution analysis of plasma from HIV-1-infected persons and of plasma spiked with known numbers of virions demonstrated that the minimum HIV-1 RNA concentration required for gene recovery is ≈30 to 40 copies/ml.

There were no statistically significant differences in the minimal plasma HIV-1 RNA concentration required for *pol* gene recovery when QIAamp HCV kit spin columns, silica beads, or phenol-chloroform was used for RNA extraction. Although ultracentrifugation is not included in the manufacturer's instructions for the QIAamp HCV kit, this step improves sensitivity by concentrating the plasma HIV-1 RNA before the spin columns are used. RNA extraction with silica beads is sensitive even in the absence of ultracentrifugation, but this method requires that complicated buffers be made consistently and also requires careful pipetting to remove the RNA solution from the silica beads (1). RNA extraction with phenol-chloroform is the most labor-intensive method and requires additional safety measures for handling and disposing of phenol.

Recovery of HIV-1 gene fragments from plasma samples may be reduced by several factors, including incomplete RNA recovery, incomplete protection from plasma RNases, and incomplete RT due to RNA secondary structure. RNA extraction with QIAamp HCV kit spin columns can be used to reproducibly recover HIV-1 RT cDNA directly from the plasma of HIV-1-infected individuals for DNA sequencing and drug susceptibility testing of recombinant viruses.

REFERENCES

1. **Boom, R., C. J. A. Sol, M. M. M. Salimans, C. L. Jansen, P. M. E. Wertheim-van Dillen, and J. van der Noordaa.** 1990. Rapid and simple method for purification of nucleic acids. J. Clin. Microbiol. **28:**495–503.
2. **Kellam, P., and B. A. Larder.** 1994. Recombinant virus assay: a rapid, phenotypic assay for assessment of drug susceptibility of human immunodeficiency virus type 1 isolates. Antimicrob. Agents Chemother. **38:**23–30.
3. **Layne, S. P., M. J. Merges, M. Dembo, J. L. Spouge, S. R. Conley, J. P. Moore, J. L. Raina, H. Renz, H. R. Gelderblom, and P. L. Nara.** 1992. Factors underlying spontaneous inactivation and susceptibility to neutralization of human immunodeficiency virus. Virology **189:**695–714.
4. **Lin, H. J., L. E. Myers, B. Yen-Lieberman, F. B. Hollinger, D. Henrard, C. J. Hooper, R. Kokka, S. Kwok, S. Rasheed, and M. Vahey.** 1994. Multicenter evaluation of quantification methods for plasma human immunodeficiency virus type 1 RNA. J. Infect. Dis. **170:**553–562.
5. **Mulder, J., N. McKinney, C. Christopherson, J. Sninsky, L. Greenfield, and S. Kwok.** 1994. Rapid and simple PCR assay for quantitation of human immunodeficiency virus type 1 RNA in plasma: application to acute retroviral infection. J. Clin. Microbiol. **32:**292–300.
6. **Nijhuis, M., C. A. B. Boucher, and R. Schuurman.** 1995. Sensitive procedure for the amplification of HIV-1 RNA using a combined reverse transcription and amplification reaction. BioTechniques **19:**178–182.
7. **Shafer, R. W., M. A. Winters, D. L. Mayers, A. J. Japour, D. R. Kuritzkes, O. S. Weislow, F. White, A. Erice, K. J. Sannerud, A. Iversen, F. Pena, D. Dimitrov, L. M. Frenkel, and P. S. Reichelderfer for the AIDS Clinical Trials Group Virology Committee Drug Resistance Working Group.** 1996. Interlaboratory comparison of sequence-specific PCR and ligase detection reaction to detect a human immunodeficiency virus type 1 drug resistance mutation. J. Clin. Microbiol. **34:**1849–1853.
8. **Winters, M. A., L. B. Tan, D. A. Katzenstein, and T. C. Merigan.** 1993. Biological variation and quality control of plasma human immunodeficiency virus type 1 RNA quantitation by reverse transcriptase polymerase chain reaction. J. Clin. Microbiol. **31:**2960–2966.
9. **Zhang, H., Y. Zhang, T. Spicer, D. Henrard, and B. J. Poiesz.** 1995. Nascent human immunodeficiency virus type 1 reverse transcription occurs within an enveloped particle. J. Virol. **69:**3675–3682.