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Quantitative detection of human immunodeficiency virus type 1 (HIV-1) viral load by SYBR green real-time RT-PCR technique in HIV-1 seropositive patients

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Abstract

HIV-1 viral load represents a basic marker for evaluation of the rate and severity of HIV-1 related disease and to monitor the effectiveness of treatment. An SYBR green-based real-time RT-PCR (SYBR green real-time RT-PCR) revealed by Light Cycler technology was evaluated for quantitation of HIV-1 RNA viral load in plasma of HIV-1 seropositive patients. The performance of the SYBR green real-time PCR was assessed on 56 HIV-1 seropositive patients under highly active retroviral therapy (HAART) and 25 blood donors. The results demonstrated that this technique detected 50 HIV-1 RNA copies per millilitre of plasma. Moreover, we compared real-time RT-PCR with the b-DNA technique considered widely a reference technique for HIV-1 RNA viral load measurement. The parallel quantitative analysis of HIV-1 positive samples showed a high correlation ($r = 0.908$) between the two methods. Although b-DNA and the real-time-based method gave similar sensitivity, the assay determined quantitatively HIV-1 RNA copies in 4 out of 16 samples shown as undetectable by b-DNA. The SYBR green real-time RT-PCR represents a good alternative to b-DNA assay in HIV-1 viral load determination especially during the monitoring of HAART treatment.

Keywords: HIV-1 viral load; SYBR green; Real-time RT-PCR

1. Introduction

HIV-1 laboratory diagnosis is committed to the application of molecular biology techniques that, together with classical serological methods, represent a useful approach to the correct diagnosis and monitoring of HIV-1 disease (Clementi et al., 1996; Yilmaz, 2001; Schmitt, 2001). Recently, the assessment of the level of viral replication with quantitative assays of HIV-1 RNA load in plasma changed radically the use of laboratory markers of HIV-1 disease. The quantitative analysis of HIV-1 viral load yields information that predicts the rate and severity of HIV-1 disease more effectively than other markers such as CD4+ cell counts (Coste et al., 1996; Mellors et al., 1996; Perelson et al., 1996; Murphy et al., 1999). Accurate determination of viral load is a basic tool for monitoring HAART therapy in HIV-1 infected patients as the decrease of plasma viral load and improved clinical outcome are linked closely (Bratt et al., 1998; Raboud et al., 1998). This viral load quantitative decrease during therapy also reflects a parallel decrease of viral replication in lymphoid tissue (Wong et al., 1997). Sensitive quantitative assays have been developed to achieve flexible and reliable tests for general use in diagnostic laboratories. Available technology for quantitative evaluation of HIV-1 viral load includes RT-PCR, branched DNA (b-DNA) and nucleic acid sequence-based amplification (NASBA) (O'Shea et al., 2000). These methods were applied in several standard commercial kits employed for HIV-1 infection monitoring. Recently, real-time PCR indicated a new diagnostic route for the sensitive, specific and quantitative management of viral and bacterial infection (Morrison et al., 1998; Desire et al., 2001; Aldea et al., 2002).

A real-time quantitative RT-PCR method using SYBR green as a fluorescence dye and LightCycler technology as a detection system is described for quantifying HIV-1 RNA viral load in plasma. This quantitative method was applied on a group of HIV-1 seropositive patients' plasma samples

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and the data were compared with results obtained with a reference technique represented by b-DNA.

2. Materials and methods

2.1. Patients

Fifty-six HIV-1 seropositive patients and twenty-five HIV-1 seronegative blood donors were enrolled after informed consent following the Helsinki declaration. HIV-1 positive and healthy blood donor plasma samples were obtained from patients living in Italy. Sequence analysis indicated that all HIV-1 positive patients were infected by HIV-1 subtype B. All tested HIV-1 positive patients followed an antiretroviral therapy characterized by multiple NRTIs (nucleoside reverse transcriptase inhibitors) plus one NNRTI (non-nucleoside reverse transcriptase inhibitors) treatment for at least 1 year. CD4+ cell numbers of all samples was determined by a flow cytometric procedure, as described previously (Perfetto and McCrary, 1999).

2.2. Branched DNA HIV-1 RNA viral load determination

All seropositive plasma were extracted and quantified by the Quantiplex HIV-1 RNA 3.0 assay branched DNA kit (Chiron, East Walpole, MA) using Chiron Diagnostics Quantiplex bDNA System 340 (Chiron) following the manufacturers' instructions. The amount of HIV-1 RNA is expressed as the number of copies per millilitre of plasma. The lower detection limit is determined at 50 copies/ml.

2.3. HIV-1 RNA extraction and purification from plasma for HIV-1 RNA quantitation by SYBR green RT-PCR

Whole blood samples were collected from HIV-1 seropositive and blood donor patients by venepuncture in EDTA-containing tubes. The samples were centrifuged at 2500 rpm for 20 min and the plasma stored at −80 °C until use. In order to extract and purify HIV-1 RNA genome, 1 ml of plasma obtained from HIV-1-infected patients or healthy blood donors was processed by QIAamp Ultrasens Virus kit (Qiagen, Hilden, Germany) following the manufacturers' instructions. Purified RNA was eluted in 40 μl of kit elution buffer and stored at −80 °C. In parallel, scalar plasma dilutions of standard reference curve were treated by the same procedure.

2.4. Assessment of standards for HIV-1 RNA quantitation by SYBR green RT-PCR

The standards for HIV-1 RNA quantitation were assessed by scalar dilution of HIV-1 positive control plasma used for the b-DNA technique shown above (Chiron). The stock virus concentration contained 6.5×10^5 RNA copies/ml. Prior to the RNA purification procedure the HIV-1 stock was diluted in HIV-1-negative plasma to achieve several scalar dilutions (from 5×10^5 to 5 copies/ml).

2.5. PCR primers selection

To avoid major mismatches due to HIV-1 variability, two specific oligonucleotides that recognize specific and highly conserved sequences on the *gag* region of HIV-1 different subtypes were chosen as described by Christopherson et al. (1997). The sequence of HIV-1 *gag* primers is: 5′ TGCTATGTCAGTTCCCCTTGGTTCTCT 3′ and 5′ AGTTGGAGGACATCAAGCAGCCATGCAAAT 3′. The amplification with this pair of oligonucleotides yielded a 142 bp *gag* fragment.

2.6. Determination of HIV-1 RNA viral load by SYBR green real-time PCR

SYBR green real-time PCR assay was carried out in 20 μl PCR mixture volume consisting of 10 μl of 2X Quantitect SYBR green RT-PCR Master Mix (Qiagen), containing HotStarTaq DNA polymerase, 0.5 μl of 500 nM of each oligonucleotide primer, 0.2 μl of 100X QuantiTect RT Mix (containing Omniscript and Sensiscript reverse transcriptases) and 8 μl of RNA extracted from plasma samples or from scalar dilution of plasma standard virus stock (from 5×10^5 to 5 copies/ml). In some experiments, additional dilutions of stock virus standard solution (25 and 10 copies/ml) were also analyzed.

HIV-1 *gag* gene amplification was carried out as follows: reverse transcription at 50 °C for 20 min; initial activation of HotStar Taq DNA Polymerase at 95 °C for 15 min; 45 cycles in four steps: 94 °C for 10 s, 60 °C for 30 s, 72 °C for 45 s and 78 °C for 3 s. At the end of amplification cycles, melting temperature analysis was carried out by a slow increase in temperature (0.1 °C/s) up to 95 °C. Amplification, data acquisition and analysis were carried out by LightCycler instrument (Roche, Mannheim, Germany) using LightCycler 5.3.2 software (Roche). This software, coupled to the LightCycler instrument, determines the threshold cycle (Ct) that represents the number of cycles in which the fluorescence intensity is significantly above the background fluorescence. Ct is proportional directly to the $\log_{10}$ of the copy number of the input templates with respect to a standard curve generated in parallel. SYBR green molecules bind all double stranded DNA molecules emitting a fluorescent signal, on binding, proportional to the amplicon synthesis during the PCR reaction. This property elicited an accurate analysis of the melting temperature curve of the amplified fragments generated by real-time PCR to determine the detection and quantitation of specific products. Real-time target amplification profile demonstrated a specific main peak with a melting temperature (Tm) at 81 °C and, sometimes, a second weak peak at 75 °C representing non-specific products (i.e. dimer primers). For this reason, the quantitative single fluorescence analysis was carried out at 78 °C for all the experiments

to rule out the possible interference of these non-specific products in the quantitative analysis carried out by LightCycler 5.3.2 software program thus achieving a maximal degree of assay specificity and a more accurate evaluation of HIV-1 RNA copy number as described by Morrison et al. (1999).

All samples from patients were run in duplicate and the average value of the copy number was employed to quantify HIV-1 viral load. For intra-assay validation, four replicates were done for each scalar dilution whereas for inter-assay analysis, four experiments were carried out in triplicate. Plasma standard virus stock dilutions were employed as reference curve when the clinical samples were assayed. HIV-1 RNA viral load final quantitative data were expressed as number of copies per plasma millilitre.

2.7. Statistical analysis

Linear regression and correlation were used to determine the relationship between b-DNA and SYBR green real-time RT-PCR technique. Statistical analysis was also carried out using two-tailed Student's *t*-test.

3. Results

3.1. Sensitivity and specificity evaluation of SYBR green real-time RT-PCR for HIV-1 viral load detection in plasma

The first set of experiments was carried out to determine the sensitivity and specificity of SYBR green based quantitative real-time RT-PCR for HIV-1 viral load detection in human plasma. Scalar (from 5×10^5 to 5×10^0 ml^{-1} HIV-1 RNA copies) dilutions of reference HIV-1 positive serum were extracted and then amplified by a *gag* gene specific oligonucleotide pair, which determines the synthesis of 142 bp HIV-1 *gag* specific product. The results shown in Table 1 demonstrate that repeated testing (four replicate each run of all scalar dilution tested) of our assay reveals 50 copies/ml in 100% of runs while no positive fluorescence signal was detected at 5 copies/ml. To determine the end point of sensitivity, other standard reference serum dilutions at HIV-1 RNA concentration below the 50 copies/ml were analyzed. The results showed that a positive signal was not always detected at 25 copies/ml (25% of replicates) whereas at 10 copies/ml no signal was detected in any replicates. Hence, the detection limit of our technique was set at 50 copies/ml. The assay encompasses at least five orders of magnitude with a high linear relationship ($r > 0.99$) between the Ct values and the standard serum input copies. The specificity of method was 100% since no healthy HIV-1 negative blood donors' plasma showed any detectable fluorescent signal.

The reproducibility of the technique both intra and inter-assay using scalar dilutions of standard HIV-1 positive serum was also tested. In particular, intra-assay reproducibility was evaluated using four replicates of each point of virus stock dilutions between 5×10^5 and 50 HIV-1 RNA copies/ml. The coefficient of variation (CV) of Ct was <3.2% for all standard scalar dilutions tested (Table 1). The CV of copy number was always <25% for all standard dilutions. The inter-assay reproducibility was obtained employing four different experiments performed in triplicate, indicating a Ct CV < 3.6% for all standard scalar dilutions (Table 2). The CV of copy number was always <30% for all scalar standard dilutions.

3.2. Analysis and comparison of plasma viral load quantitation by SYBR green real-time RT-PCR and b-DNA techniques in HAART-treated HIV-1 seropositive patients

To test SYBR green-based quantitative real-time PCR assay on patients' samples, viral RNA was extracted from 1 ml of plasma taken from 56 HIV-1 seropositive patients under HAART treatment and from 25 healthy blood donors to measure HIV-1 viral load. As reference curve, scalar dilutions (5×10^5 to 5×10^0 RNA copies/ml) of reference HIV-1 positive plasma were employed. The results demonstrated that SYBR green real-time RT-PCR quantitative assay quantified 43 HIV-1 seropositive patients' plasma (range from 79 to 3.2×10^5 copies/ml) whereas 13 samples showed undetectable values and were classified as samples

Table 1
Comparison of Ct of standard curves and intra-assay analysis

Reference serum dilutions (copies/ml)	Ct mean values[a]	S.D.	CV
5×10^5	23.76	0.6	2.52
5×10^4	27.19	0.6	2.20
5×10^3	30.56	0.8	2.61
5×10^2	34.02	1.0	2.94
5×10^1	37.73	1.2	3.18
2.5×10^1	ND	–	–
1×10^1	ND	–	–
5×10^0	ND	–	–

S.D.: standard deviation; CV: coefficient of variation; ND: not detected.
[a] For each sample, the Ct value is the average of results from four replicates.

Table 2
Inter-assay analysis of Ct mean values

Reference serum dilutions (copies/ml)	Ct mean values[a]	S.D.	CV
5×10^5	24.16	0.8	3.31
5×10^4	27.85	0.7	2.51
5×10^3	30.82	0.9	2.92
5×10^2	34.29	1.1	3.20
5×10^1	38.04	1.4	3.68
2.5×10^1	ND	–	–
1×10^1	ND	–	–
5×10^0	ND	–	–

S.D.: standard deviation; CV: coefficient of variation; ND: not detected.
[a] For each sample, the Ct value is the average of results from four different experiments performed in triplicate.

Table 3
Cross sectional data of viral load and CD4+ cell count of each HIV-1 seropositive patient

Patients	SYBR green real-time RT-PCR	Branched-DNA	CD4 cell number/mm^3
1	3.2×10^5	1.8×10^5	350
2	9.0×10^4	1.6×10^4	344
3	8.0×10^4	3.6×10^4	345
4	6.1×10^4	1.1×10^5	352
5	6.0×10^4	6.8×10^4	210
6	4.9×10^4	3.5×10^4	325
7	2.0×10^4	4.0×10^4	156
8	1.1×10^4	2.6×10^3	351
9	1.1×10^4	1.7×10^4	321
10	6.8×10^3	3.7×10^3	739
11	5.0×10^3	3.1×10^3	245
12	4.8×10^3	1.2×10^3	451
13	4.0×10^3	9.6×10^3	539
14	3.2×10^3	1.0×10^4	253
15	2.9×10^3	6.5×10^3	491
16	2.9×10^3	2.0×10^3	447
17	2.5×10^3	1.4×10^4	286
18	1.6×10^3	1.3×10^3	267
19	1.5×10^3	2.5×10^3	618
20	950	700	745
21	650	2.6×10^3	840
22	630	2.4×10^3	375
23	590	1.4×10^3	425
24	590	270	318
25	560	440	553
26	510	560	599
27	460	140	175
28	400	850	424
29	390	81	418
30	320	76	406
31	310	74	410
32	230	1.0×10^3	390
33	190	72	433
34	190	68	430
35	170	72	286
36	125	<50	1294
37	110	100	249
38	96	<50	701
39	94	<50	1314
40	91	<50	751
41	91	74	718
42	88	89	106
43	79	<50	521
44	<50	<50	1025
45	<50	<50	859
46	<50	<50	829
47	<50	<50	772
48	<50	<50	653
49	<50	<50	608
50	<50	<50	599
51	<50	<50	488
52	<50	<50	484
53	<50	<50	445
54	<50	<50	437
55	<50	<50	431
56	<50	86	214
	13.317 ± 46.216 Median 395	10.187 ± 29.686 Median 255	496 ± 253 Median 432

carrying <50 copies/ml. HIV-1 RNA copy mean was 13.317 ± 46.216 copies/ml with a median of 395 copies/ml (Table 3). As expected, none of 25 HIV-1 negative samples showed any positive fluorescent signal.

The b-DNA technique represents a reference technique for quantitative determination of HIV-1 RNA viral load in plasma. To correlate this reference technique to SYBR green real-time RT-PCR assay, we tested in parallel the same 56 selected samples using HIV-1 RNA 3.0 branched DNA assay, a commercial b-DNA kit, that shows cut-off sensitivity at 50 copies/ml like our technique. The results demonstrated that 40 samples were detectable (range from 68 to 1.8×10^5 copies/ml) whereas 16 were classified as samples with <50 copies/ml. HIV-1 RNA copy mean was 10.187 ± 29.686 copies/ml with a median of 255 copies (Table 3). None of the 25 HIV-1 negative samples showed any positive fluorescent signal by HIV-1 RNA 3.0 assay. The analysis of quantitative viral load data achieved by both methods demonstrated a high correlation ($r = 0.908$; Fig. 1). Interestingly, although both assays showed the same absolute limit of sensitivity, our SYBR green real-time RT-PCR quantified 43 out of 56 HIV-1 seropositive patients' plasma (76.9%) whereas the b-DNA assay determined viral load in only 40 samples (71.4%). Four of the 16 plasma samples (25%) that contained <50 copies/ml by b-DNA, were detected quantitatively by the real-time technique (mean 101 ± 16 copies/ml; median 95) whereas 12 samples were undetectable by both assays (Table 4). In contrast, b-DNA technology showed a detectable viral load for one sample (1/13; 7.6%) that was undetectable with real-time RT-PCR.

3.3. Comparative analysis of SYBR green real-time RT-PCR and CD4+ lymphocyte count

We also investigated the statistical correlation between HIV-1 viral load determined by our SYBR green real-time RT-RCR assay or b-DNA and classical infection parameters such as CD4 cell count. As demonstrated previously by several researchers, statistical analysis of HIV-1 plasma viral load of all patients disclosed a significant inverse correlation between HIV-1 viral load and CD4+ cell count using both techniques. In the patients with CD4+ cell

Table 4
SYBR green real-time RT-PCR and b-DNA assay analysis of HIV-1 seropositive plasma samples

	b-DNA positive samples	b-DNA negative samples	Total
SYBR green real-time RT-PCR positive samples	39/56	4/56	43/56
SYBR green real-time RT-PCR negative samples	1/56	12/56	13/56
Total	40/56	16/56	56/56

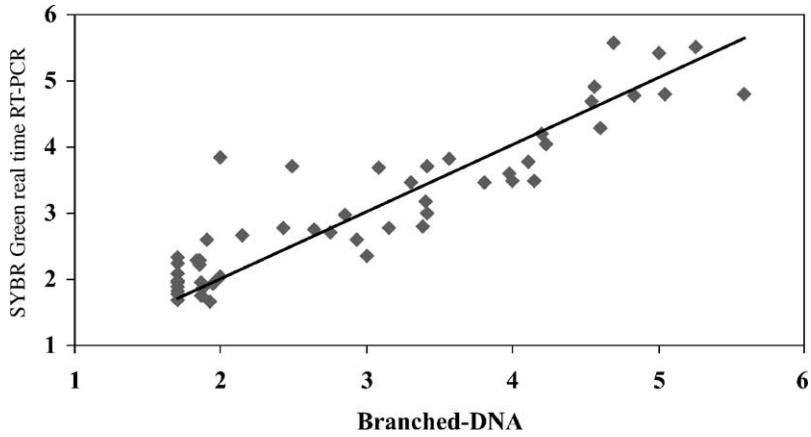

Fig. 1. Correlation between logarithmic viral load values detected by SYBR green real-time RT-PCR and b-DNA ($r = 0.908$). Points clustered at the bottom indicate undetectable HIV-1 viremia (limit of detection 50 copies/ml) revealed by single or both methods.

Table 5
Relationship between CD4+ cell count and HIV-1 viral load

Patients (n)	CD4 count cell (amount/mm^3)	SYBR green real-time RT-PCR RNA viral load (copies/ml) mean	b-DNA RNA viral load (copies/ml) mean
Group 1 ($n = 35$)	(>400)	832 ± 1562	952 ± 2018
Group 2 ($n = 21$)	(<400)	34.125 ± 71.712	25.578 ± 44.928

counts $<400\ \mu l^{-1}$, HIV-1 mean viral load, calculated by SYBR green real-time RT-PCR, was significantly higher ($34\,125 \pm 71\,712$) than in the patients with CD4+ cell count $>400\ \mu l^{-1}$ (832 ± 1562) ($P < 0.01$; Table 5). This statistical significance was also observed when b-DNA viral load data were compared with CD4+ cell count (25.578 ± 44.928 versus 952 ± 2018; $P < 0.01$).

4. Discussion

The quantitative evaluation of HIV-1 viral load in plasma is a pivotal marker for the diagnosis and prognosis of HIV-1 infection (Clementi et al., 1996; Yilmaz, 2001) since this parameter reflects directly viral replication status and disease evolution (Coste et al., 1996; Mellors et al., 1996; Murphy et al., 1999). In addition, the viral load yields basic information for therapy monitoring allowing a concrete analysis of treatment failure caused by emerging resistance to specific anti-retroviral compounds. For these reasons, studies on molecular procedures to determine viral load quantitatively have increased in recent years. Approaches such as b-DNA, NASBA and competitive RT-PCR are employed widely as reference techniques for diagnostic laboratories. Recently, the advent of real-time RT-PCR offers a diagnostic system able to quantify specific amplicons synthesis during PCR cycles (Gratzl et al., 1997; Morrison et al., 1999; Desire et al., 2001; Aldea et al., 2002).

In this paper, a SYBR green-based real-time RT-PCR evaluated by Light Cycler technology to measure HIV-1 viral load in the plasma of seropositive patients was developed. This approach presented a full specificity as all HIV-1 negative healthy donor samples failed to show any positive detection. Moreover, SYBR green real-time RT-PCR showed a high level of sensitivity as the detection limit of technique was assessed at 50 HIV-1 RNA copies/ml. b-DNA, commercially or in-house competitive quantitative RT-PCR assays did not show higher sensitivity (Venturi et al., 2000; Johanson et al., 2001). Other real-time quantitative studies carried out by different formats of real-time RT-PCR (i.e. Taqman or beacon formats) for HIV-1 or HIV-2 viral load detection showed a sensitivity lower than or similar to our technique (Schutten et al., 2000; Damond et al., 2002). Furthermore, SYBR green is less expensive than labelled probes that could also determine PCR artefacts beyond the 30th cycle during real-time RT-PCR. In addition, probe selected sequences may be prone to specific mutations (Espy et al., 2000; Aldea et al., 2002). Interestingly, the sensitivity of the assay also compares favourably with those of the SYBR green real-time PCR reported previously with other viral models such as Hepatitis C virus or Theiler's murine encephalomyelitis virus (Koumarian-Pradel et al., 2001; Trottier et al., 2002). According to our technique, the sensitivity of Light Cycler coupled with SYBR green detection was also shown at 10–100 RNA copies/reaction (Koumarian-Pradel et al., 2001; White et al., 2002). A direct clinical field evaluation of SYBR green RT-PCR was carried out testing a small group of 56 HAART-treated patients' plasma. In this cross-sectional analysis, the assay detected the viral load in 43 samples out of 56 (76.8%) also demonstrating a significant inverse correlation between CD4+ cell counts and HIV-1 RNA levels with a relative scattering

of values in agreement with other molecular assays (Zazzi et al., 1999; Damond et al., 2002). We also compared the SYBR green real-time RT-PCR with b-DNA reference standard technique for the viral load quantitative determination of HIV-1. This comparison clearly demonstrated a high correlation between the two methods ($r = 0.908$). Strikingly, the new method disclosed the viral load count in some samples assayed as undetectable with b-DNA: the b-DNA detected only 40 samples out of 56 (71.4%). Of the 16 HIV-1 seropositive patients' plasma, classified by b-DNA analysis as <50 copies/ml, four samples (25%) were positive with SYBR green real-time RT-PCR assay. In contrast, only one of 13 HIV-1 seropositive patients' plasma, classified by this technique as <50 copies/ml was detected by b-DNA assay. In spite of the same detection limit achieved by both techniques, this discrepancy may be explained either by two different RNA extraction and purification approaches used for two assays or by different algorithms employed by each assay to distinguish positive results from negative ones using a specific ratio of fluorescence between sample and background (Erice et al., 2000). SYBR green RT-PCR has some advantages over b-DNA. In particular, it is a more rapid assay (3 h versus 24) and is slightly more efficient in the quantitation of samples with a low viral load. However, b-DNA can analyze a larger number of samples in the same analytical run than our technique.

In conclusion, SYBR green real-time RT-PCR is a sensitive and reliable assay to ascertain HIV-1 viral load in plasma from HIV-1 seropositive patients. This method could be employed to monitor viral load variations in plasma in the course of HIV-1 infection and antiviral treatment thus representing a useful alternative to current HIV-1 viral load quantitative techniques.

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