Impact of HIV-1 Genetic Diversity on Plasma HIV-1 RNA Quantification

Usefulness of the Agence Nationale de Recherches sur le SIDA Second-Generation Long Terminal Repeat–Based Real-Time Reverse Transcriptase Polymerase Chain Reaction Test

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Summary: The high genetic diversity of HIV-1 has a major impact on the quantification of plasma HIV-1 RNA, representing an increasingly difficult challenge. A total of 898 plasma specimens positive for HIV-1 RNA by commercial assays (Amplicor v1.5; Roche Diagnostic Systems, Alameda, CA or Versant v3.0; Bayer Diagnostics, Emeryville, CA) were tested using the Agence Nationale de Recherches sur le SIDA second-generation (G2) real-time reverse transcriptase polymerase chain reaction (RT-PCR) test: 518 samples containing HIV-1 of known subtype, including 88 from 2 subtype panels and 430 harboring B (n = 266) and non-B (n = 164) group M HIV-1 subtypes from patients followed up in 2002 through 2005 at Necker Hospital (Paris, France), and 380 samples from 10 different countries (Argentina, Cambodia, Cameroon, Central African Republic, France, Ivory Coast, Madagascar, Morocco, Thailand, and Zimbabwe). HIV-1 RNA values obtained by G2 real-time PCR were highly correlated with those obtained by the Amplicor v1.5 for B and non-B subtypes (R^2 = 0.892 and 0.892, respectively) and for samples from diverse countries (R^2 = 0.867 and 0.893 for real-time PCR vs. Amplicor v1.5 and real-time PCR vs. Versant v3.0, respectively). Approximately 30% of specimens harboring non-B subtypes were underquantified by at least -0.51 $\log_{10}$ in Amplicor v1.5 versus 5% underquantified in G2 real-time PCR. Discrepant results were also obtained with subtype B samples (14% underquantified by Amplicor v1.5 vs. 7% by G2 real-time PCR). Similar percentages were observed when comparing results obtained with the G2 real-time PCR assay with those obtained using the Versant assay. Addressing HIV-1 diversity, continual monitoring of HIV-1 RNA assays, together with molecular epidemiology studies, is required to improve the accuracy of all HIV RNA assays.

Key Words: HIV-1 diversity, HIV-1 RNA viral load, real-time polymerase chain reaction

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One of the major characteristics of HIV-1 is its extreme genetic variability attributable to high rates of mutation,[1] recombination,[2] and viral turnover.[3] At present, HIV-1 has been subdivided into 3 phylogenetically distinct groups: M for major (or main), O for outlier, and N for non-M/non-O (or new).[4–6] Within group M, which is responsible for the pandemic, 9 nonrecombinant subtypes (A–D, F–H, J, and K) and at least 19 circulating recombinant forms (CRFs) with epidemic significance are currently recognized.[7,8] In addition,

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unique recombinant forms (URFs) are frequently found in areas where multiple variants circulate concomitantly.[9] The dynamic geographic spread of these multiple groups/subtypes/CRFs/URFs varies.[10] In Europe and the United States, subtype B is the predominant genetic form, but the prevalence of non-B subtypes is regularly growing.[11] In Africa, subtypes A, C, CRF02_AG, and CRF06_cpx are the most frequent strains, but all HIV-1 variants are concomitantly present.[12] In Southeast Asia, CRF01_AE predominates.[13] In South America, subtype B predominates but subtypes F, C, and CRF12_BF are increasing.[14]

Consequently, a key factor in assessing the sensitivity of a molecular-based assay measuring HIV-1 RNA levels in plasma is its ability to quantify all relevant HIV-1 genetic forms accurately. Commercial HIV-1 RNA quantification assays were initially developed and regularly evaluated with samples harboring B subtypes, which predominate in industrialized countries.[15] Even though the latest generations of these kits have been improved for the quantification of non-B group M variants,[16–18] different reports showed underquantification with some of these kits.[19–24] In comparison to commercial assays using end-point amplification methods, the kinetic real-time fluorescence-based technologies combine the amplification and detection steps into 1 homogeneous assay.[25,26] Because of their simplicity, rapidity, sensitivity, specificity, and reproducibility, together with the ongoing introduction of new chemistries and less expensive instrumentation, they are extensively used in virology.[27,28] This includes the field of plasma HIV-1 RNA detection/quantification, using commercial kits[29–31] or low-cost in-house techniques.[32–38] Recently, under the auspices of the French Agence Nationale de Recherches sur le SIDA (ANRS), an in-house TaqMan real-time reverse transcriptase polymerase chain reaction (RT-PCR) assay targeting consensus sequences in the long terminal repeat (LTR) region of HIV-1 and using a FAM/TAMRA-labeled probe has been developed for plasma HIV-1 RNA quantification. This first-generation (G1) test has been successfully evaluated, especially in Ivory Coast,[39] where CRF02_AG strains are largely predominant.[40] Some HIV-1 strains from Cameroon and Thailand were not detected by the G1 test, however. Thus, further optimization was required to evaluate the impact of HIV-1 diversity fully on the reliability of the assay.

In this study, a second generation (G2) of this LTR-based real-time RT-PCR test has been developed in the Virology Laboratory at Necker Hospital (Paris, France), using a reduced length minor groove binder (MGB) probe. A large collection of plasma samples harboring most clinically relevant B and non-B HIV-1 subtypes from panels and patients followed at Necker Hospital and plasma specimens from HIV-1–infected subjects living in several distinct countries were assessed using this modified test, and results were compared with those obtained with commercially available assays.

PATIENTS AND METHODS

Sample Collection

Samples With Defined HIV-1 Subtypes

As summarized in Table 1, a total of 518 plasma samples harboring well-defined HIV-1 subtypes were assessed for HIV-1 RNA level using the ANRS G2 real-time RT-PCR assay. Approximately 48% of these specimens harbored non-B group M variants. All subtypes, with the exception of subtype K and 8 CRFs, were represented.

TABLE 1. Group/Subtype Distribution of Collected Samples: Necker Hospital, Paris, France

Genetic Group/Subtype	Panels		Clinical Samples	Total (%)
	WHO	ANRS AC11		
Group M				
B subtype	1	0	266	267 (51.5)
Non-B subtypes				
Subtype A	1	11	30	42 (8.1)
Subtype C	1	15	7	23 (4.4)
Subtype D	1	8	10	19 (3.7)
Subtype F	1	8	8	17 (3.3)
Subtype G	1*	9	9	19 (3.7)
Subtype H	1	7	4	12 (2.3)
Subtype J	0	2	2	4 (0.8)
CRF01_AE	1	1	4	6 (1.2)
CRF02_AG	0	3	60	63 (12.2)
CRF05_D/F	0	0	1	1 (0.2)
CRF06_cpx	0	7	8	15 (2.9)
CRF09_cpx	0	0	1	1 (0.2)
CRF11_cpx	0	4	2	6 (1.2)
CRF12_BF	0	2	0	2 (0.4)
CRF14_BG	0	1	0	1 (0.2)
Unclassified	0	0	18	18 (3.5)
Total	7	78	164	249 (48.1)
Group O	1	0	0	1 (0.2)
Group N	1	0	0	1 (0.2)
Total	10	78	430	518

*AG-GH (RU570) strain.

These specimens comprised 2 distinct HIV-1 RNA subtype panels:

1. A World Health Organization (WHO) HIV-1 RNA genotype reference panel (National Institute for Biological Standards and Control [NIBSC] no. 01/466, Hertfordshire, United Kingdom) consisting of 10 samples: 8 specimens belonging to group M with 1 A strain (92UG037, Uganda), 1 B strain (92TH014, Thailand), 1 C strain (98TZ017, Tanzania), 1 D strain (94UG114, Uganda), 1 F strain (93BR020, Brazil), 1 H strain (V1525, Gabon), 1 AG-GH strain (RU570, Russia), and 1 CRF01_AE strain (92TH001, Thailand); 1 group O strain (MVP5180, Cameroon); and 1 group N strain (YBF030, Cameroon).
2. A non-B group M subtype HIV-1 RNA panel recently established by the ANRS Working Group for Viral Quantification (coordinated action AC11) consisting of 78 well-characterized variants from 75 patients (11 A, 15 C, 8 D, 8 F, 9 G, 7 H, 2 J, 1 CRF01_AE, 3 CRF02_AG, 7 CRF06_cpx, 4 CRF11_cpx, 2 CRF12_BF, and 1 CRF14_BG). All the ANRS AC11 panel members were HIV-1 RNA-positive (except 1 subtype J sample) when quantified by the Cobas Amplicor HIV-1 Monitor v1.5 test (ultrasensitive protocol; Roche Diagnostic Systems, Alameda, CA).

Four hundred thirty independent clinical plasma samples (stored at −80°C), selected from patients infected with a known HIV-1 subtype and followed routinely at Necker Hospital from January 2002 to July 2005, were also tested using the ANRS G2 real-time PCR assay (see Table 1). All these HIV-1 RNA-positive specimens were quantified for HIV-1 RNA by the Cobas Amplicor HIV-1 Monitor v1.5 test (standard protocol). They consisted of 266 samples harboring B subtypes and 164 non-B subtypes (30 A, 7 C, 10 D, 8 F, 9 G, 4 H, 2 J, 4 CRF01_AE, 60 CRF02_AG, 1 CRF05_DF, 8 CRF06_cpx, 1 CRF09_cpx, 2 CRF11_cpx, and 18 unclassified). The patients originated from France and diverse sub-Saharan African countries (including Ivory Coast, Senegal, Mali, Burkina Faso, and Ghana in West Africa; Cameroon, Central African Republic [CAR], Democratic Republic of Congo, and Equatorial Guinea in Central Africa; and Burundi and Zambia in East Africa).

Samples From Different Countries

A total of 380 samples (−80°C ethylenediaminetetraacetic acid [EDTA] plasma) were also analyzed by G2 real-time PCR from HIV-1–infected individuals living in 10 distinct countries as follows: France (n = 88), Ivory Coast (n = 69), Zimbabwe (n = 52), Morocco (n = 50), Argentina (n = 30), CAR (n = 25), Cambodia (n = 24), Madagascar (n = 22), Cameroon (n = 10), and Thailand (n = 10). The HIV-1 subtype was characterized for samples from Ivory Coast (57 CRF02_AG, 8 A, and 4 CRF06_cpx samples), Argentina (10 subtype B and 20 CRF12_BF samples), and Madagascar (8 A, 6 C, 4 CRF02_AG, 2 CRF06_cpx, 1 B, and 1 CRF01_AE samples). All these HIV-1 RNA-positive specimens were quantified for HIV-1 RNA using commercial assays: samples from France, Zimbabwe, Morocco, CAR, Madagascar, Cambodia, and Thailand were tested by the Cobas Amplicor HIV-1 Monitor v1.5 RT-PCR test (standard protocol), whereas those from Ivory Coast, Cameroon, and Argentina were assessed by the Versant HIV-1 RNA v3.0 bDNA assay (Bayer Diagnostics, Emeryville, CA). The 20 samples from Cameroon and Thailand were previously found to be undetectable (<2.5 $\log_{10}$/mL) for HIV-1 RNA by the G1 real-time PCR test.

Plasma HIV-1 RNA Quantification

Real-Time Reverse Transcriptase Polymerase Chain Reaction Test

The protocol used was established by the ANRS Working Group for HIV Quantification (AC11). HIV RNA was extracted from 0.2 mL of plasma using the QIAamp Viral RNA Mini kit (QIAGEN, Courtaboeuf, France). The primers and probe were selected from a region of LTR displaying low variability between subtypes, as determined by comparison of Los Alamos Genbank HIV-1 sequences. Further, because we observed underestimation of some samples from Cameroon and Thailand with the G1 test,[39] we analyzed their LTR sequences and made the decision to shorten the probe and the forward primer sequences. These modifications permitted us to obtain the expected quantification for those specific samples. Thus, these sequences were definitively used in the G2 test. The sequence of the internal HIV-1 TaqMan probe (LTR MLC-1) was 5′-AAGTRGTGTGTGCCC-3′. This probe carried a 5′ FAM reporter and a 3′ MGB-nonfluorescent quencher (Applied Biosystems, Foster City, CA). The sequence of the forward primer (NEC005) was 5′-GCC TCAATAAAGCTTGCC-3′, whereas that of the reverse primer (NEC131) was not changed (5′-GGCGCCACTGC TAGAGATTTT-3′). All reactions were performed in a 50-μL volume containing RNA extract (20 μL), primers (200 nM of each), probe (200 nM), 1 × PCR buffer, and 1 × RT mix (SuperScript III Platinum, One-Step qRT-PCR kit; Invitrogen Corporation, Carlsbad, CA). The external standard was a culture supernatant of an HIV-1 subtype B strain previously quantified using the Versant bDNA v3.0 assay. For each experiment, 1 aliquot of this standard was extracted along with the clinical samples and serially diluted (5-fold dilutions) to the following concentrations: from 4,980,000 (6.7 $\log_{10}$) to 300 (2.5 $\log_{10}$) copies/mL. Using 0.2 mL of plasma, the threshold of the assay was set at 2.5 $\log_{10}$ copies/mL.

Thermocycling conditions were as follows: 30 minutes at 48°C and 10 minutes at 95°C, followed by 50 cycles at 95°C for 15 seconds and 60°C for 1 minute. Amplification and data acquisition were carried out using the ABI Prism 7000 Sequence Detection System (Applied Biosystems).

Commercial HIV-1 RNA Viral Load Assays

Two commercial HIV-1 RNA assays were used. The Cobas Amplicor HIV-1 Monitor v1.5 test, which targets the *gag* p24 region of HIV-1, was performed according to the standard procedure (0.2 mL of plasma, with a dynamic range of 400 [2.6 $\log_{10}$]–750,000 [5.9 $\log_{10}$] copies/mL) or the ultrasensitive protocol (0.5 mL of plasma, working range of 50 [1.7 $\log_{10}$]–75,000 [4.9 $\log_{10}$/mL] copies/mL). The Versant HIV-1 RNA 3.0 assay, which targets the HIV-1 *pol* region, was performed according to the manufacturer's instructions (1.0 mL of plasma, dynamic range of 50 [1.7 $\log_{10}$]–500,000 [5.7 $\log_{10}$] copies/mL).

HIV-1 Phylogenetic Analyses

For the specimens of the ANRS panel and the clinical samples, RT sequences were performed on plasma viral RNA using the consensus sequencing technique of the ANRS Resistance Study Group, as previously reported elsewhere.[41] Phylogenetic analysis was done by estimating the relations among RT sequences and reference sequences of HIV-1 genetic subtypes and CRFs obtained from the Los Alamos Database (http://hiv-web.lanl.gov). After alignment using CLUSTAL W program version 1.7, the genetic distance between sequences was determined using the Kimura 2-parameter substitution model and phylogenetic analysis was performed using the neighbor-joining method with 500 bootstrapped data sets.[42]

Statistical Analyses

Plasma HIV-1 RNA values (copies/mL) were $\log_{10}$-transformed before statistical analysis. For samples from the ANRS AC11 panel with HIV-1 RNA values ranging between 50 and 300 copies/mL, as determined by the

Amplicor v1.5 ultrasensitive protocol, the G2 real-time PCR assay results distributed in this range were also considered to avoid biases in comparing the 2 techniques. If a sample was undetectable using one assay, the threshold of the assay was subtracted from the value obtained using the other test to determine the difference (δ). For the clinical samples, the performance of the 3 assays (G2 real-time PCR, Amplicor v1.5, and/or Versant v3.0) was evaluated by comparing the mean δ HIV-1 RNA values. Individual values obtained using different assays were compared by linear regression analysis. The data were also analyzed by the method of Bland and Altman.[43]

RESULTS

Comparative Quantification of HIV-1 Subtypes

Results From Panels

The group M and N members from the WHO panel exhibited similar concentrations by G2 real-time PCR and Amplicor v1.5 assays (Table 2). Like commercial techniques (except for the LCx assay, Abbott Laboratories, Chicago, IL), the G2 real-time PCR test failed to detect the group O isolate from the WHO panel.

The differences in $\log_{10}$ copies/mL (δ) between the G2 real-time RT-PCR HIV-1 RNA measurements and the Amplicor v1.5 results for the 85 non-B group M members are summarized in Figure 1. The samples harboring subtypes C, F, G, H, and CRFs were overall better quantified by the ANRS G2 real-time PCR assay than by the Amplicor v1.5 assay (mean δ at 0.46, 0.44, 0.53, 0.42, and 0.37 $\log_{10}$/mL, respectively). HIV-1 RNA results derived from the 2 assays were more evenly distributed for subtype A and D samples (mean δ at 0.09 and −0.07 $\log_{10}$/mL, respectively). Quantitatively, 33 (38.8%) of 85 samples (8 C, 5 G, 4 F, 4 CRF06_cpx, 3 A, 4 H, 2 CRF02_AG, 1 J, 1 CRF11_cpx, and 1 CRF14_BG) were quantified by the G2 real-time PCR assay at concentrations greater than 0.51 $\log_{10}$ differences from results with Amplicor v1.5 test. In contrast, 7 (8.2%) specimens (2 A, 2 D, 1 C, 1 F, and 1 G) were underquantified by at least −0.51 $\log_{10}$ in the G2 real-time PCR assay compared with the results with the Amplicor v1.5 test.

Results on Clinical Samples

Of 430 specimens quantified by the Amplicor v1.5 test, 427 were also quantifiable by the G2 real-time PCR assay. The overall sensitivity of the G2 real-time PCR test was thus 99.3% (427 of 430 samples, 95% confidence interval [CI]: 98.0 to 99.9). The 3 specimens that were positive using the Roche assay but negative using the G2 real-time PCR test contained subtype B viruses. Two of them had an HIV-1 RNA level, as quantified by the Amplicor v1.5 test, of ≤3.0 $\log_{10}$ copies/mL, whereas 1 had an HIV-1 RNA level of 5.6 $\log_{10}$ copies/mL.

For the 263 B subtype samples that were quantifiable using both assays, plasma HIV-1 RNA levels were highly correlated (R^2 = 0.8920, $P < 0.001$; Fig. 2A). The overall mean difference (δ) between the values obtained using the G2 real-time PCR assay and Amplicor v1.5 test was 0.10 $\log_{10}$/mL. Ten (3.8%) samples showed HIV-1 RNA levels more than ±2 SDs from the mean δ, with RNA levels for 5 samples being higher using the G2 real-time PCR assay than by using the Amplicor v1.5 assay and 5 samples with higher levels using the Amplicor v1.5 assay (see Fig. 2B). Thus, 96.2% (253 of 263) of the HIV-1 RNA values were homogeneously distributed within the 95% CI of the mean difference. HIV-1 RNA viral load determinations differed between the 2 assays by at least 0.51 $\log_{10}$ for 56 (21.3%) samples. Thirty-seven (14.1%) had higher levels and 19 (7.2%) lower levels in the G2 real-time PCR assay than in the Amplicor v1.5 assay (χ^2 test, P = 0.011).

Regarding the correlation and distribution obtained for the 164 non-B subtype specimens (see Figs. 2C, D), plasma HIV-1 RNA levels obtained using the G2 real-time PCR and the Amplicor v1.5 assays were highly correlated (R^2 = 0.8922, $P < 0.001$). The overall δ between the G2 real-time PCR and Amplicor v1.5 results was 0.23 $\log_{10}$/mL, with the following distribution: 0.22, 0.06, 0.05, −0.01, 0.20, 0.88, 0.43, 0.26, 0.06, and 0.36 $\log_{10}$/mL for A, C, D, F, G, H, CRF01_AE, CRF02_AG, CRF06_cpx, and unclassified strains, respectively. For 7 (4.3%) samples, the difference between the assays exceeded ±2 SDs, including 3 specimens (1 subtype H, 1 CRF02_AG, and 1 CRF06_cpx) that were better quantified by real-time PCR and 4 samples (1 subtype F and 3 CRF02_AG) that were better quantified by the Amplicor v1.5 test. HIV-1 RNA concentrations differed between the 2 assays by at least

TABLE 2. HIV-1 RNA Results Obtained on 10 Specimens From the WHO HIV-1 RNA Genotype Reference Panel Using the ANRS G2 Real-Time PCR Assay

Subtype/Group	A	B	C	D	AE	F	AG-GH	H	N	O
Strain	92UG037	92TH014	98TZ017	94UG114	92TH001	93BR020	RU570	V1525	YBF30	MVP5180
Origin	Uganda	Thailand	Tanzania	Uganda	Thailand	Brazil	Russia	Gabon	Cameroon	Cameroon
HIV-1 RNA results* ($\log_{10}$/mL)										
Monitor v1.5	3.38	3.15	3.43	3.50	3.63	3.67	3.76	4.11	3.61	Und
Nuclisens	3.55	3.53	3.59	3.81	3.53	2.33	Und	2.18	Und	Und
Versant v3.0	3.06	3.20	3.07	3.49	3.33	3.47	3.25	3.58	2.62	Und
LCx	3.57	3.40	3.44	3.71	3.64	3.53	3.34	3.62	2.48	2.18
ANRS G2 real-time PCR assay	3.01	3.44	3.45	4.08	3.72	3.72	3.40	4.46	4.01	Und

*For each panel sample, HIV-1 RNA values obtained by the 4 commercial HIV-1 RNA assays were included in the manufacturer's notice.
Und indicates undetectable.

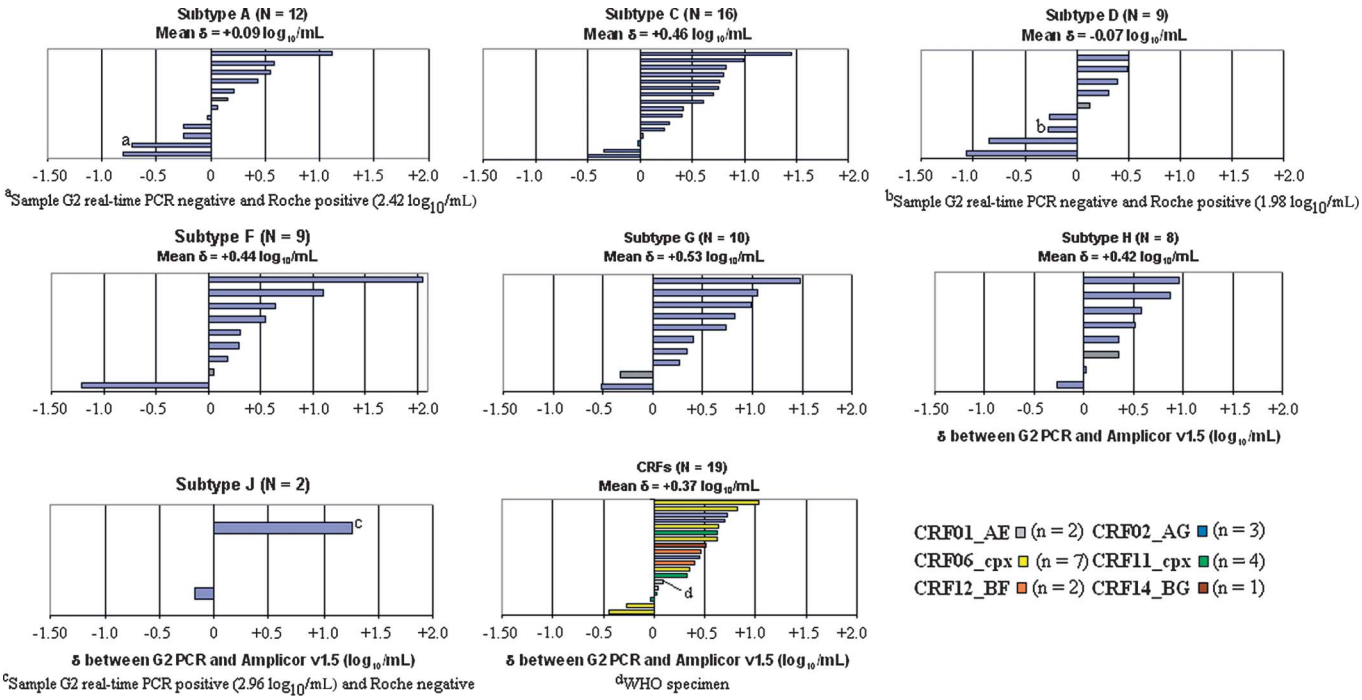

FIGURE 1. Comparison of HIV-1 RNA quantification of 85 non-B group M HIV-1 subtypes from the WHO and ANRS AC11 panels, using the ANRS G2 real-time PCR and Amplicor v1.5 assays. Subtypes A (n = 12), C (n = 16), D (n = 9), F (n = 9), G (n = 10), H (n = 8), J (n = 2), CRF 01_AE (n = 2), CRF02_AG (n = 3), CRF06_cpx (n = 7), CRF11_cpx (n = 4), CRF12_BF (n = 2), and CRF14_BG (n = 1) are included. For subtypes A to J, blue bars indicate the δ values (in $\log_{10}$ copies/mL) obtained for each ANRS AC11 panel sample and gray bars indicate those obtained for the WHO panel samples.

0.51 $\log_{10}$ for 49 (29.9%) samples. Forty-two samples (25.6%) (including 18 CRF02_AG, 7 A, 7 unclassified, 3 H, 2 G, 2 CRF06_cpx, 1 F, 1 J, and 1 CRF01_AE samples) had higher levels, and 7 (4.3%) samples (3 CRF02_AG, 1 A, 1 F, 1 CRF06_cpx, and 1 CRF11_cpx samples) had lower levels using the G2 real-time PCR assay than with the Amplicor v1.5 test (χ^2 test, $P < 0.001$).

Comparative Quantification of Samples Derived From Diverse Countries

Regardless of the geographic origin of the specimens, plasma HIV-1 RNA levels obtained by the ANRS G2 real-time PCR assay correlated significantly with those obtained by the Amplicor v1.5 (Fig. 3) or Versant v3.0 assay (Fig. 4). No specimen that tested positive by the Roche (or the Versant) assay tested negative by the G2 real-time PCR assay. The overall mean δ between the ANRS G2 real-time PCR and Amplicor v1.5 techniques was 0.21 $\log_{10}$/mL, ranging from 0.07 (Zimbabwe) to 0.41 (Morocco) $\log_{10}$/mL. The overall δ between the ANRS G2 real-time PCR and Versant techniques was 0.35 $\log_{10}$/mL. Overall, 71.2% (193 of 271 samples) and 69.7% (76 of 109 samples) were within 0.50 $\log_{10}$ copies/mL, comparing values generated by the ANRS G2 real-time PCR assay with those of the Amplicor v1.5 and Versant v3.0 assays, respectively.

DISCUSSION

As more HIV-1 variants intermix in different parts of the world, the probability of generating highly diverse recombinant strains of HIV-1 is continuously growing. This dynamic process has a major impact on the quantification of HIV-1 RNA in plasma, which already represents an increasingly difficult challenge. Worldwide, continuous and large-scale evaluations of the available HIV-1 RNA assays using large and genetically diverse panels are thus crucial.

In the present study, the ANRS G2 LTR-based real-time RT-PCR test was used to quantify plasma HIV-1 RNA in samples that were genomically and geographically diverse. Quantitative results obtained by the ANRS G2 real-time PCR test and 2 different commercial assays were highly correlated. We also found some significant differences in HIV-1 RNA concentrations by using these tests, however, probably because these different tests target different sequences of the virus (*gag*, *pol*, and LTR for the Amplicor v1.5, Versant v3.0, and ANRS G2 real-time PCR tests, respectively). For approximately 35% of specimens harboring non-B subtypes, values produced by the real-time PCR assay and the Amplicor v1.5 test differed by at least 0.51 $\log_{10}$ (30% were lower by the Amplicor v1.5 test, whereas 5% were lower by the G2 real-time PCR assay). Similar percentages were observed when comparing results obtained by the real-time PCR assay and the Versant v3.0 test. Discrepancy between assays was not associated with a particular subtype; varying levels of discrepancy were found within each subtype, suggesting "intrasubtype" genetic diversity in the entire HIV-1 genome. Discrepant results were also obtained among samples harboring B subtypes (14% had lower values by the Amplicor

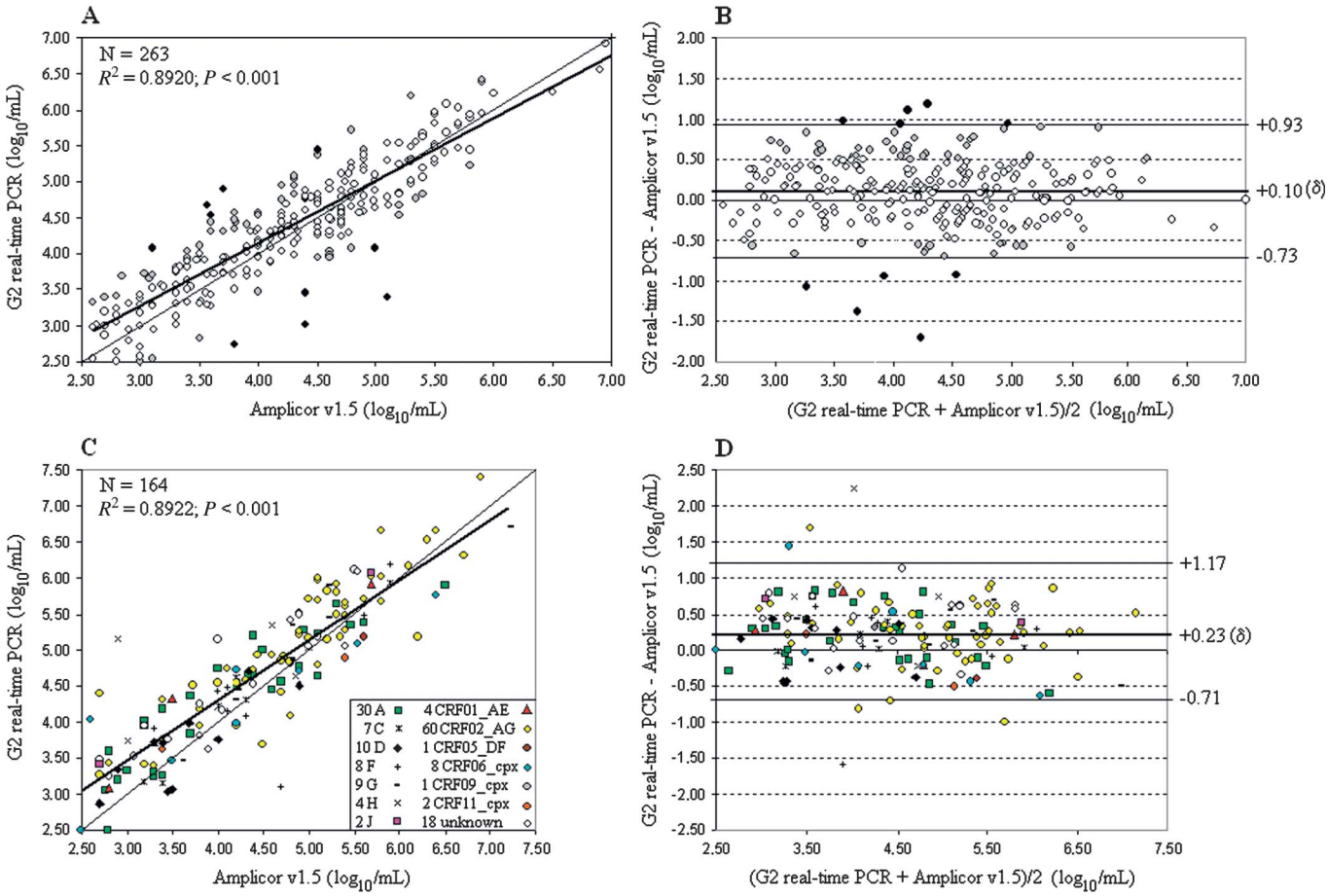

FIGURE 2. Correlation and difference (Bland-Altman representation) between HIV-1 RNA viral load measurements obtained with the ANRS G2 real-time PCR test and the Amplicor v1.5 assay, according to HIV-1 subtype/CRF distribution. Comparison of quantitative results obtained on 263 specimens containing B subtypes (A, B) and 164 harboring non-B subtypes (C, D). For the correlations, the fitted regressions are represented by solid lines. For the Bland-Altman representations, the mean differences and the 95% CIs are represented by solid lines. In Figures 2A and 2B, log_{10} differences are shown as follows: black circles, greater than the mean difference ±2 SDs; gray circles, 0.51 to 1 log_{10}; and open circles, within 0.5 log_{10}.

v1.5 test vs. 7% by the G2 real-time PCR assay). This also suggests increased genetic diversity within B subtypes resulting from strain recombination and indicates the necessity to characterize sub-subtypes within the subtype B further.

Thus, the genetic diversity of HIV continues to pose problems (underquantification or failure to detect some HIV-1 strains). Because of this singular characteristic, there is definitively no "perfect" HIV-1 RNA viral load assay. Ideally, each laboratory should initially compare different HIV-1 RNA tests and choose the assay that performs best based on the subtypes circulating in the country at that time. Given population migration and viral recombination, however, assay evaluation must be a continual process. This is particularly important when high HIV-1 genetic diversity is observed, as it is in Central Africa and Madagascar.[44] The accurate quantification of non-B subtypes is also becoming important in the French context. In France,[11] as in other European countries,[45] change in subtype distribution is being observed, with an increased proportion of patients of sub-Saharan African origin migrating to France (particularly young women), who are infected with non-B subtypes. The strong representation of CRF02-AG in our clinical samples from Necker Hospital reflects this epidemiologic trend in France.

In comparison with commercial assays, one significant advantage of our research PCR test is its important flexibility, allowing the improvement of protocols through easy modification of the nucleotide sequences of the primers and/or probe and their chemistries. In our study, the subtype reactivity was improved significantly by modifying the probe, forward 5′ primer, and amplification mix reagent. The use of a short MGB probe limits nucleotide mismatches at the probe binding sites and greatly enhances hybrid stability, which seems to be optimal for the amplification of sequences possessing a high degree of variability, such as HIV-1.[46]

The underquantification of HIV-1 RNA levels may have practical implications in the clinical management of HIV-1–infected subjects because it can lead to an inappropriate strategy for antiretroviral treatment. Thus, clinicians should interpret HIV-1 RNA viral load results cautiously: when a low (<4 log_{10}/mL) HIV-1 RNA viral load result using one HIV-1

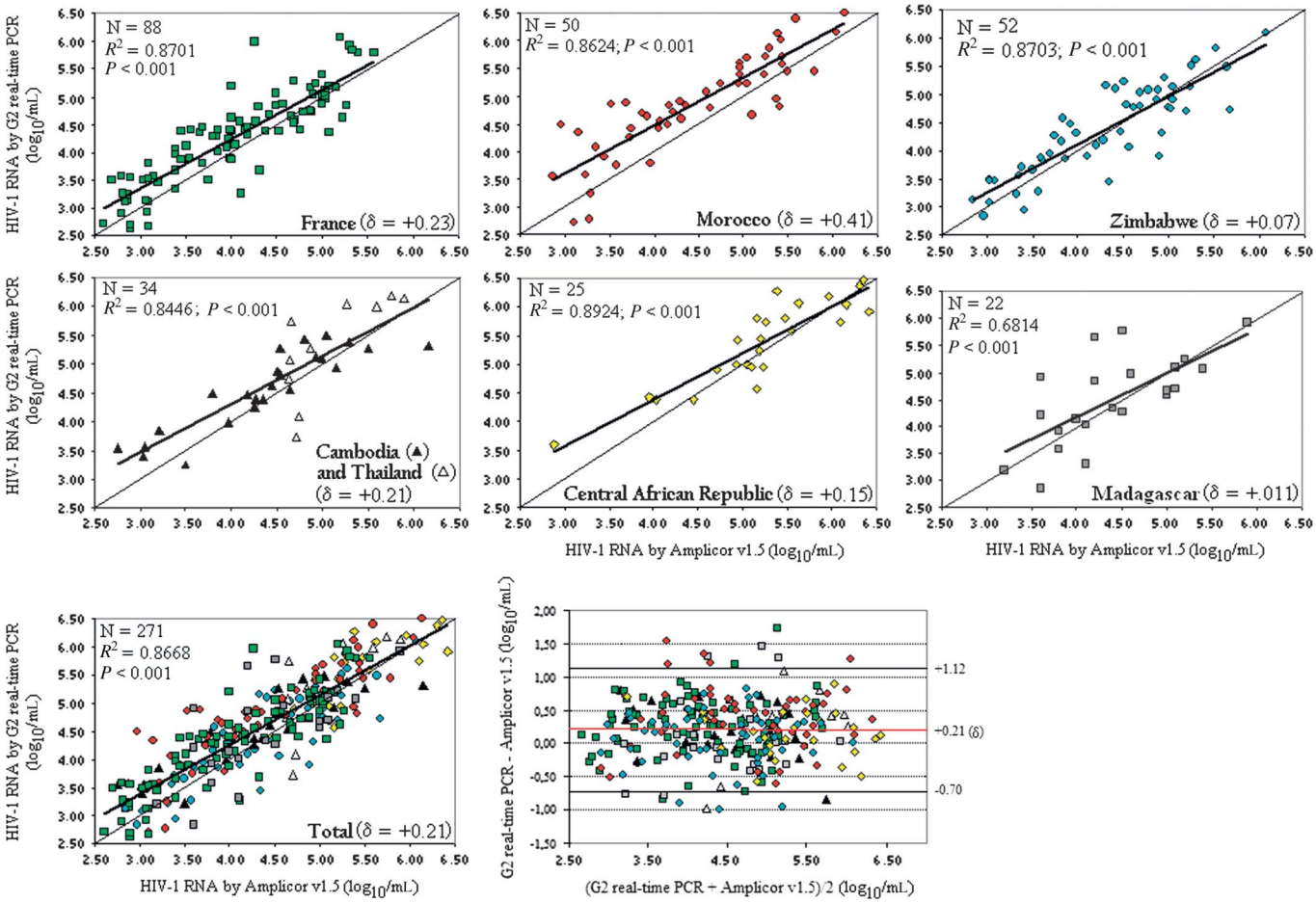

FIGURE 3. Correlation between HIV-1 RNA viral load levels obtained with the ANRS G2 real-time PCR test and Amplicor v1.5 test in 271 samples derived from 7 distinct countries: France (n = 88), Zimbabwe (n = 52), Morocco (n = 50), CAR (n = 25), Cambodia (n = 24), Thailand (n = 10), and Madagascar (n = 22). The Amplicor v1.5 test was performed in Necker Hospital on specimens from France, Zimbabwe, CAR, and Madagascar. Samples from other countries were tested locally.

RNA technique is inconsistent with clinical staging (eg, stage C) and/or low CD4 count (eg, <200 cells/mm^3), the HIV-1 RNA measurement should be repeated using another HIV-1 RNA assay to refute or confirm the former result. In addition, as recently reviewed by Calmy et al,[47] HIV-1 RNA viral load should increase in importance in resource-limited countries as a guide for clinical decisions on how to optimize the duration of the first-line antiretroviral treatment regimen and when to switch to second-line treatment. At present, no validated definition of immunologic treatment failure based on the CD4 cell count exists. Two recent studies suggested that neither clinical features nor CD4 cell counts alone can predict virologic failure.[48,49] Also, the HIV-1 RNA viral load is a useful tool for monitoring adherence to treatment, which may help to preserve long-term use of first-line regimens, performing sentinel surveillance, and diagnosing HIV-1 infection in children aged <18 months. Thus, the G2 ANRS real-time PCR assay could be an alternative option to commercial assays to ensure that assessment of the HIV-1 RNA viral load becomes more affordable (the unit price of the G2 test is ~7€), simpler, and easier to use in resource-limited settings.[50]

In conclusion, the ANRS G2 LTR-based real-time RT-PCR assay accurately quantified relevant HIV-1 subtypes and CRFs. This low-cost and easy-to-use assay is currently distributed by the manufacturer Biocentric (Generic HIV charge virale; Biocentric, Bandol, France). Targeting the functional LTR region, which is one of the most conserved regions among HIV-1 virus isolates and subtypes, seems to be an appropriate choice, as recently confirmed by Drosten et al.[38] Our assay could serve as an academic reference tool for regular evaluation of the impact of the HIV-1 genetic drift on the sensitivity of HIV-1 RNA quantification with commercial assays. For this purpose, the WHO panel is useful, but larger HIV-1 RNA B and non-B subtype panels are urgently needed, as initiated by the ANRS AC11 study group. Molecular epidemiology studies focusing on diversity in *gag*, *pol*, and LTR genes are also required to improve the accuracy of all HIV-1 RNA quantification assays and virologic monitoring of HIV-1–infected patients.

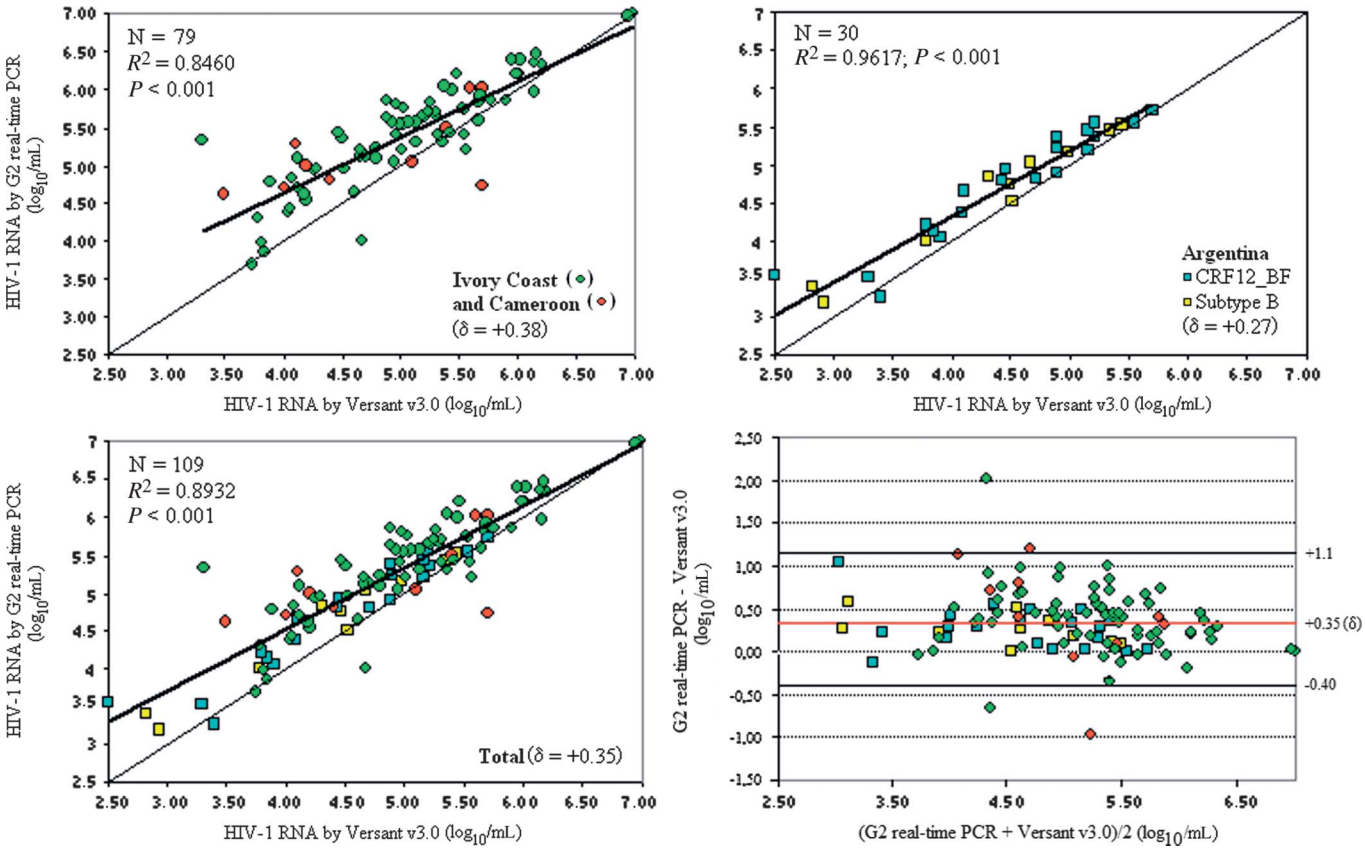

FIGURE 4. Correlation between HIV-1 RNA viral load levels obtained with the ANRS G2 real-time PCR test and the Versant v3.0 test in 109 samples from 3 different countries: Ivory Coast (n = 69), Argentina (n = 30), and Cameroon (n = 10). The Versant v3.0 assay was performed locally.

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