New Sensitive One-Step Real-Time Duplex PCR Method for Group A and B HIV-2 RNA Load

Véronique Avettand-Fenoel, a, b Florence Damond, c, d, e Marie Gueudin, f, g Sophie Matheron, c, d, h Adeline Mélard, b Gilles Collin, c, d, e Diane Descamps, c, d, e Marie-Laure Chaix, c Christine Rouzioux, a, b Jean-Christophe Plantier, f, g for the ANRS-COS HIV-2 and the ANRS-AC11 Quantification Working Group

Laboratoire de Virologie, AP-HP, Hôpital Necker Enfants Malades, Paris, France; EA7327, Université Paris-Descartes, Sorbonne Paris Cité, Faculté de Médecine, Paris, France; INSERM, IAME, UMR 1137, Paris, France; Université Paris Diderot, Sorbonne Paris Cité, Paris, France; AP-HP, Hôpital Bichat-Claude Bernard, Laboratoire de Virologie, Paris, France; Laboratoire de Virologie Associé au Centre National de Référence du VIH, Hôpital Charles Nicolle, CHU de Rouen, Rouen, France; GRAM, Equipe d’Accueil 2656, Faculté de Médecine-Pharmacie, Institut de Recherche et d’Innovation en Biomédecine, Université de Rouen, Rouen, France; AP-HP, Hôpital Bichat-Claude Bernard, Service de Maladies Infectieuses et Tropicales, Paris, France; Laboratoire de Virologie, AP-HP, Hôpital Saint-Louis, Paris, France

The Agence Nationale de Recherche sur le Sida et les hépatites virales (ANRS) previously developed a widely used method for HIV-1 RNA quantification (Biocentric). Here, we report the development of a new specific and sensitive method for HIV-2 RNA quantification, based on an adaptation of the existing HIV-1 protocol. The new test is based on TaqMan one-step reverse transcription-quantitative PCR (qRT-PCR) targeting two conserved consensus regions of HIV-2 (long terminal repeat [LTR] and gag). Analytic performances were determined in three laboratories. Clinical performances were evaluated on 100 plasma samples from HIV-2-infected patients (groups A, B, and H) by comparison with the assay currently used for the ANRS HIV-2 cohort. The specificity was 100%. Sensitivity was 50 copies/ml (cp/ml) and was optimized to 10 cp/ml. The within-run coefficients of variation varied from 2.28% to 6.43%. Of the 39 clinical samples below 2 log10 in the current assay, the new test improved the detection or quantification of 17 samples, including eight group B samples. For quantifiable samples, similar loads were obtained with the two assays for group A samples. The median difference between the two assays for group B samples was +0.18 but with greater heterogeneity than for group A. The HIV-2 group H sample had similar results with the two assays. This new assay is highly sensitive and accurately quantifies the most prevalent HIV-2 groups. This test will be useful for monitoring low viral loads in HIV-2-infected patients.

HIV-2 is characterized by less efficient transmission through the sexual and vertical routes than HIV-1 (1, 2) and by a slower natural clinical course (2–4); nevertheless, HIV-2 infection eventually leads to AIDS. HIV-2 infection must be distinguished from HIV-1 infection, as HIV-2 is naturally resistant to non-nucleoside reverse transcriptase inhibitors, T20, and some protease inhibitors, and as patient follow-up differs from that of HIV-1 infection (5, 6).

Compared to HIV-1, HIV-2 is characterized by lower viral replication (4, 7–10). In the French National HIV-2 Cohort (974 patients in June 2013), 61% of untreated patients had plasma viral loads below 250 copies/ml (cp/ml). Likewise, in a British study, only 8% of patients with CD4 of >500 cells/mm³ and 62% of patients with CD4 of <300 cells/mm³ had detectable viral loads (11), implying that 38% of patients had undetectable viral loads in an assay with a quantification limit of 100 copies/ml.

Clinical management of HIV-2 infection is hampered by the lack of validated commercial RNA viral load assays. In-house assays are therefore widely used (12–18). The ACHIEV2E international collaboration on HIV-2 infection showed that plasma HIV-2 RNA values vary considerably between laboratories (19, 20). The high genetic diversity of HIV-2, with 9 groups designated A to I, of which only groups A and B are epidemic, also represents an obstacle to accurate viral load quantification (21–25). Previous studies showed that group B viruses are particularly difficult to quantify (13, 19, 20).

As most cases of HIV-2 infection occur in resource-limited settings, an affordable test is urgently needed, particularly to identify HIV-1/HIV-2 coinfection. The Agence Nationale de Recherche sur le Sida et les hépatites virales (ANRS) has previously promoted the development of an HIV-1 RNA assay, which is now marketed by Biocentric (Bandol, France) and is widely used in resource-limited settings (26, 27). This test is easy to perform and affordable, and it has been proven to be useful for patient monitoring.

The aim of the present work was to develop a new sensitive method for quantifying HIV-2 RNA, particularly that of epidemic groups A and B. It is based on a one-step real-time duplex PCR method using the same amplification protocol as the Biocentric generic HIV-1 charge virale assay. The new test was compared with the HIV-2 assay currently used for the French HIV-2 cohort and was validated by three laboratories belonging to the ANRS AC11 quantification working group.
MATERIALS AND METHODS

The new HIV-2 RNA assay. The new test is based on a one-step duplex TaqMan PCR approach targeting a conserved consensus region in the long terminal repeat (LTR) region and the gag region. The forward and reverse primers for the LTR region are 5′-TCTTATAGCAAGGAGCGT GG-3′ and 5′-AGCAGGTAGCCGGTTTGT-3′, respectively (28), with a new internal probe (5′-FAM-CTTGGGCCGYYRCTGGCCAGABHQ1-3′ [FAM, carboxyfluorescin; BHQ1, black hole quencher 1]) to optimize efficiency for HIV-2 group B. The forward and reverse primers for the gag region are F3 (5′-GGCCGAAAATCCGGTCTTG-3′) and R1 (5′-TTCGGTGGACCAATATGTT-3′), respectively (13), and the internal HIV-2 TaqMan gag probe is S65GAG2 (5′-FAM-TAGGTATCAGG CCAGGGGAAAAA-BHQ1-3′ [Eurogentec, Seraing, Belgium] (13).

RNA was extracted from 200 μl of plasma by using the QIAamp viral RNA minikit (Qiagen, Courtaboeuf, France), as in the Biocentric generic HIV-1 charge virale assay, in labs A and B (Necker Hospital, Paris, and Charles Nicolle Hospital, Rouen) or 1 ml with the total nucleic acid (NA) large-volume MagNA Pure kit (Roche Automated System, Meylan, France) in lab C (Bichat Claude Bernard Hospital, Paris).

The reaction mix consists of a 20-μl volume containing the RNA extract (10 μl), primers (500 nM each), probes (250 nM each), and 1× PCR buffer (4× one-step mix; Invitrogen, Cergy-Pontoise, France).

The thermocycling conditions are those used for the Biocentric HIV-1 assay (10 min at 50°C and 5 min at 95°C, followed by 50 cycles of 95°C for 15 s and 60°C for 1 min). Amplification and data acquisition were carried out with the TaqMan ABI real-time PCR system (Applied Biosystems, Courtaboeuf, France). The log_{10} number of targets initially present is proportional to the cycle threshold (C_{T}) and is determined from the standard curve.

A BIOQ HIV-2 RNA group A quantification panel (P0182; Rijswijk, the Netherlands) was used as the external standard. The standard, evaluated on July 2, 2017 by guest http://jcm.asm.org/ Downloaded from

...analytical sensitivity was determined by dilution in RPMI of the BIOQ external standard in 200 μl of plasma. The sample was centrifuged at 17,000 rpm and the pellet was resuspended in 1 ml of RPMI medium prior to manual extraction as previously described, with elution in 60 μl. This yielded 90% sensitivity at 10 cp/ml (1 log_{10} cp/ml).

Within-run reproducibility was evaluated in the three labs by using the BIOQ external standard with theoretical virus concentrations of 10,000 and 100 cp/ml (4 and 2 log_{10} cp/ml). For the 4 log_{10} cp/ml value, we obtained a mean of 3.91 log_{10} cp/ml at lab C, 4.1 log_{10} cp/ml at lab A, and 4.2 log_{10} cp/ml at lab B, with within-run coefficients of variation of 1.61%, 0.54%, and 1.10%, respectively. At the concentration of 2 log_{10} cp/ml, we obtained mean values of 2.03 log_{10} cp/ml at lab B, 2.07 log_{10} cp/ml at lab A, and 2.17 log_{10} cp/ml at lab C, with within-run coefficients of variation of 10.72%, 14.32%, and 7.24%, respectively.

In between-run assays, the positive control with a theoretical concentration between 10,000 (4 log_{10}) and 100,000 cp/ml (5 log_{10}) was evaluated at 4.61 log_{10} cp/ml in lab C, 4.70 log_{10} cp/ml in lab A, and 4.88 log_{10} cp/ml in lab B, with coefficients of variation of 2.28%, 6.43%, and 3.03%, respectively.

Clinical performances. The clinical performances of the new assay were evaluated in lab C. Clinical samples of 1 ml were extracted with the automated MagNA Pure method, and then the same eluate was used to perform the two assays in parallel with the ABI device for the new assay and the Light Cycler 1.5 device for the current assay. The results obtained with the new assay were categorized into four groups (Table 1), undetectable (<40 cp/ml), detectable but not quantifiable (0 to <40 cp/ml), quantifiable be-
between 40 and 100 cp/ml, and above the lower limit of quantification of the current assay (100 cp/ml).

Of the 39 samples below the quantification limit of 100 cp/ml in the current assay, 22 samples (56%) were also undetectable with the new assay (Table 1), while 10 samples (26%; 3 A, 3 B, and 4 nongenotypable) were detected at values between 0 and 40 cp/ml (range, 1 to 36 cp/ml). Three samples (7.7%; 1 B and 2 nongenotypable) were quantified between 40 and 100 cp/ml (range, 56 to 79 cp/ml), and four samples (10%; all B) were quantified above 100 cp/ml (range, 102 to 970 cp/ml). The latter corresponded to true false-negative samples, taking into account the 100 cp/ml cutoff the current assay. These results showed that the new test improved the detection or quantification of 17/39 samples (43.6%), including eight group B samples (Table 1).

All 61 plasma samples with values above 100 cp/ml in the current assay were detectable with the new test. One sample at 209 cp/ml (2.32 log10 cp/ml) in the current assay gave a value of 46 cp/ml (1.69 log10 cp/ml) in the new test (Table 1). A scatter plot was constructed with the values obtained for the 78 samples detectable or quantifiable with the new assay (Fig. 2). It showed a wider dispersion of values for quantifiable group B samples than for quantifiable group A samples, as well as better detection or quantification of group B and nongenotypable samples. This was confirmed by scatter equations specific for group A samples (n = 32; y = 0.9485x + 0.0294; r2 = 0.9619) and group B samples (n = 39; y = 0.7766x + 0.8087; r2 = 0.8184) and also by Bland-Altman representations (Fig. 3). Homogeneous quantification (±1.96 standard deviation [SD], range from −0.6 to 0.35) and similar values (median difference of −0.13) were obtained with the new and current assays for group A samples. The median difference between the two assays for group B samples was +0.18, but with greater heterogeneity (±1.96 SD, range −0.98 to 1.33).

![FIG 1 Standard curve of the HIV-2 RNA real-time viral load assay. The cycle threshold (Ct) is the number of cycles at which fluorescence passes a fixed limit (time to positivity). Median values and 25% and 75% interquartile ranges (box plot) of the Ct are indicated. The vertical lines show the ranges of the Ct.](http://jcm.asm.org/)

### TABLE 1 Clinical performance of the new assay using automated extraction (MagNA Pure plus ABI prism) compared to that of the current assay (MagNA Pure plus Light Cycler method)

<table>
<thead>
<tr>
<th>VL&lt;sup&gt;a&lt;/sup&gt; (cp/ml) with new version of HIV-2 assay</th>
<th>No. of samples tested by current version of HIV-2 assay per indicated group at a VL (cp/ml) of:</th>
<th>Group A (n = 9)</th>
<th>Group B (n = 19)</th>
<th>NA&lt;sup&gt;b&lt;/sup&gt; (n = 11)</th>
<th>Group A (n = 29)</th>
<th>Group B (n = 31)</th>
<th>Group H (n = 1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;100 cp/ml (2 log) (n = 39)</td>
<td>Group A Group B NA</td>
<td>6</td>
<td>11</td>
<td>5</td>
<td>6</td>
<td>28</td>
<td>31</td>
</tr>
<tr>
<td>&lt;40 cp/ml (1.7 log)</td>
<td>Group A Group B NA</td>
<td>3</td>
<td>3</td>
<td>4</td>
<td>4</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>0 to &lt;40 cp/ml</td>
<td>Group A Group B NA</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>40 ≤ VL &lt; 100 cp/ml</td>
<td>Group A Group B NA</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>1</td>
<td>28</td>
<td>31</td>
</tr>
<tr>
<td>≥100 cp/ml (2 log)</td>
<td>Group A Group B NA</td>
<td></td>
<td></td>
<td></td>
<td>28</td>
<td>31</td>
<td>1</td>
</tr>
</tbody>
</table>

<sup>a</sup> VL, viral load.  
<sup>b</sup> NA, nongenotypeable samples.
Among the samples that were quantifiable with both assays, 10 samples showed differences above 0.5 log_{10}, 9 of them belonging to group B. Five of these samples were better quantified with the new assay (differences of 0.53, 0.60, 0.68, 0.76, and 0.89 log_{10}/ml) and five with the current assay (differences of 0.51, 0.52, 0.55, 0.66, and 0.70 log_{10}/ml). These differences illustrate the difficulty of correctly assessing HIV-2 group B viral load.

The only HIV-2 group H sample gave very similar results with the two assays (4.33 log_{10} and 4.34 log_{10}).

**DISCUSSION**

HIV-2 infection differs markedly from HIV-1 infection, notably by its slower natural course, different therapeutic management, and genetic diversity. Specific molecular methods are therefore necessary for diagnosis and patient monitoring. Current assays, mainly consisting of in-house methods or unvalidated derivatives of commercial kits, suffer from major limitations in terms of their sensitivity, accuracy, and coverage of HIV-2 genetic diversity (19, 20).

The aim of this work was to develop a quantitative assay that takes into account both the low viral load seen in most HIV-2-infected patients and the broad genetic diversity of HIV-2, especially group B (13, 19, 20). In addition, as most cases of HIV-2 infection occur in West Africa, such a test must be easy to implement in developing countries, as previously achieved with the generic HIV-1 viral load assay marketed by Biocentric.

We chose to optimize the assay currently used to monitor the French HIV-2 cohort based on amplification of the HIV-2 gag region, which gave accurate results in the ACHIEV2E study (19, 20), but with a lower limit of quantification of 100 cp/ml (13) and not completely adapted to some group B strains. We also used the same operating conditions as those of the Biocentric HIV-1 assay kit, in order to facilitate its use either for HIV-2 alone or jointly for HIV-1 and HIV-2.

The new test exhibits good linearity (40 to 1,000,000 cp/ml) and within-run reproducibility (<15%). Its interlaboratory reproducibility was validated by evaluation at three different sites. Both manual and automated extraction methods were validated for compatibility with local practices in resource-limited countries.

Relative to the current assay, the new test has a significantly better analytical limit of quantification, reaching 50 cp/ml with manual extraction of 200 µl of plasma and 40 cp/ml with automated extraction of 1 ml. Assuming a probit rate of 90%, the detection limits with 1 ml of plasma would be 10 cp/ml and 20 cp/ml, respectively. This very good analytical sensitivity matches that of recently published in-house methods (12, 14, 18) and is compatible with virological monitoring of HIV-2 infection, as >60% of untreated patients have viral loads below 250 cp/ml. The new test detected and/or quantified more than one-third of samples that were undetectable with our current assay, which has a quantification limit of 100 cp/ml. This excellent sensitivity should prove useful both for pathophysiological studies and for treatment monitoring.
The most difficult issue facing the development of HIV-2 viral load assays is the genetic diversity of this virus (especially group B), with some variants being underquantified or escaping detection with current tests (13, 19, 20). Three teams recently reported improved sensitivity for HIV-2, but they mainly used supernatants (14) or a limited number of samples (12, 18) or validated detection but not quantification (18), leaving questions as to their clinical performance, especially for group B viruses. We evaluated our new assay on 100 clinical samples, 39% of which were undetectable with our current assay. We chose to develop a duplex method capable of simultaneously amplifying the LTR and gag regions, in order to retain the benefits of each previous test and to improve the detection of group B viruses by reducing the risk of mismatches. Our strategy, based on coupling of primers and probes in two distinct regions of the viral genome, has already been adopted by Roche in version 2.0 of its CAP CTM HIV-1 assay, resulting in better quantification of HIV-1 non-B subtypes (31). The new and current HIV-2 assay methods gave similar results for the single group H sample and for the group A samples (although 3 additional group A samples were detectable with the new test), whereas the new test developed by Delarue et al. gave values nearly 0.5 log_{10} lower than their reference test (14). Eight additional group B samples (42%) were detected or quantified with our new test, four samples having values of 102 to 970 cp/ml. This improvement is due to the addition of primers in the LTR region and to changes in the LTR probe (data not shown). However, the wider dispersion of values and the larger number of group B than group A samples with differences exceeding 0.5 log_{10} relative to the current assay illustrate the greater difficulty of group B quantification. In addition, six non-genotypable samples were better detected or quantified with our new assay.

We designed the new test for use under the same operating conditions as the generic HIV-1 RNA assay currently used with success in many resource-limited countries (26, 27), meaning it could be used on the same machine, with the same software program and even, if necessary, in the same amplification plate, as HIV-1 samples. This will reduce analytical costs by increasing the number of samples per run.

Although ours is an in-house assay, its performance is adequate for patient monitoring in the absence of a validated commercial test. In addition, the use of a standardized kit that includes an external standard will improve interlaboratory reliability, as shown by the 2nd International ACHV_{2E} study (19, 20).

The analytical performance of our new assay is similar to that of other newly developed tests (12, 14, 18). However, it is difficult to compare our method with the test described by Chang et al., adapted from the Abbott platform (Abbott Molecular, Chicago, IL), as the latter was evaluated on few group B samples and was not compared with other techniques (12). Styer et al. recently compared their method with this “Abbott” technique and observed a difference of $-0.35 \log_{10} \text{UI/ml}$ but they used a limited panel of uncharacterized samples, ruling out any evaluation of in terms of genetic diversity (18). Finally, Delarue et al. (14) and Styer et al. (18) used two-step methods, whereas our assay is performed in a single step.

In conclusion, we have developed and standardized an assay with better analytical sensitivity than the technique currently used to monitor HIV-2-infected patients in France. Our assay also has improved clinical sensitivity and has been validated on a broad, well-characterized sample panel, unlike other recently published tests (12, 14, 18). The analytical performance of this new assay, which is easy to perform, makes it suitable for use in resource-limited countries in which multiple HIV-2 variants circulate. In addition, our assay can be used on the same analytical platforms and in the same run as tests for HIV-1, thus improving its cost-efficiency for monitoring patients infected with HIV-1 and/or HIV-2. This possibility of simultaneous analysis will facilitate molecular diagnosis of mother-to-child transmission of HIV-1 and/or HIV-2, and also diagnosis and follow-up of dual HIV-1/HIV-2 infection in the same sample. Finally, use of this assay for virological monitoring will provide new insights into the natural history of HIV-2 infection at different clinical stages.

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