Antiretroviral drug susceptibility tests facilitate therapeutic management of HIV-1-infected patients. Although genotyping systems are affordable, inaccuracy in the interpretation of complex mutational patterns may limit their usefulness. Currently available HIV-1 phenotypic assays are based on the generation of recombinant viruses in which the specific viral gene of interest, derived from a patient plasma sample, is cloned into a susceptible genetic viral backbone prior to in vitro drug susceptibility evaluation. Nevertheless, in the case of protease inhibitors, not only are mutations in the HIV-1 protease-coding region involved in resistance, but the role of Gag in drug susceptibility has also recently been reported. In order to avoid the inherent limitations resulting from partial cloning of the viral genome, we designed and evaluated a new experimental strategy to test the in vitro susceptibility of primary viral isolates to protease inhibitors. Our protocol, which is based on a two-round infection protocol using the reporter TZM-bl cell line, showed a good correlation with genotypic resistance prediction and with the Antivirogram phenotypic assay, in both protease-recombinant viruses and primary viral isolates. The protocol is suitable for any HIV-1 subtype and enables rapid in-house measurement of protease inhibitor susceptibility, thus making it possible to evaluate the concomitant effects of both patient-derived gag and protease-coding regions.

Several methods have been developed for the assessment and quantification of HIV-1 drug resistance (14). Genotypic resistance tests are based on the detection of resistance mutations in the target viral genes. Drug susceptibility can be inferred from the additive effect of these HIV-1 mutations in reducing drug susceptibility, as previously described (10). Alternatively, a "virtual phenotype" can be obtained using mathematical models based on information from large databases which contain pairs of genotypes or phenotypes (23). Viral genome sequencing is rapid, and associated costs are relatively low. In addition, some genotypic interpretation algorithms are available online (Stanford HIVdb, Los Alamos Resistance DB, Geno2Pheno, and RegaDB), making genotyping the preferred technique for clinical drug resistance testing (51). However, the difficulties in recognizing viral mixtures and interpreting complex combinations of mutations may limit the usefulness of genotyping (17, 50), which should, therefore, be complemented by direct methods of testing drug susceptibility.

Phenotypic drug susceptibility assays are based on in vitro testing of the capacity of the virus to replicate in the presence of a drug, mostly in a cell culture. Standard phenotypic tests, such as Antivirogram (Janssen Diagnostics BVBA) and PhenoSense (Monogram Biosciences, Inc.), are based on the generation of recombinant viruses in which the gene of interest (derived from the plasma sample) is cloned into a susceptible HIV-1 backbone (16, 42). In both assays, the resulting recombinant virus contains patient-derived reverse transcriptase (RT), protease (PR), the 3′ half of the gag gene (including the p7/p1/p6 cleavage sites), and the connection domain (Antivirogram assay). To evaluate viral susceptibility to protease inhibitors (PIs), other noninfectious phenotypic methods have been developed to measure the in vitro activity of recombinant patient-derived HIV-1 proteases, either in cell-based settings or in cell-free experimental settings (3, 18, 19, 27, 28). However, the recently reported additional effect of Gag on restoration of viral fitness and on susceptibility to PIs is brought about mainly by mutations located at the p7/p1 and p1/p6 cleavage sites (8, 9, 21, 26, 46, 55), as well as in other residues along the Gag polyprotein sequence (12, 37, 40, 41, 47). Of note, preexistent polymorphisms at p2/p7 have been associated with virological failure in a first-line PI monotherapy regimen, despite the absence of major PI resistance mutations in the protease gene (13). Therefore, phenotyping methods which include only the 3′ half of the patient-derived gag sequences or PR alone might underestimate drug susceptibility. For this reason, several groups have high-
lighted the importance of including the full-length patient-derived gag sequences in viral recombinants used for evaluation of susceptibility to PIs (15, 45).

In order to bypass the inherent limitations resulting from partial cloning of the viral genome, we designed a new method to test the in vitro PI susceptibility of primary viral isolates and validated its performance and correlation with standard genotyping and phenotyping methods. This protocol enables the evaluation of susceptibility to PIs regardless of the viral tropism or subtype. Thus, it might be a useful tool for the study of PI resistance mechanisms in non-B subtypes, which are not as well characterized, as no subtype-matched cloning vectors are available for their evaluation by standard methods.

MATERIALS AND METHODS

Clinical samples. In order to validate our assay, we used 11 plasma samples from HIV-1 B subtype-infected patients whose PI-containing regimens had failed. Blood samples were collected during routine patient monitoring. Plasma samples were processed and stored at −80°C until use. The study was approved by the Germans Trias i Pujol Hospital Ethics Committee, and informed consent was obtained from the study participants.

Cell lines and antiretroviral drugs. The NIH AIDS Research and Reference Reagent Program (Division of AIDS, National Institute of Allergy and Infectious Diseases [NIAID]), NIH) provided the cell lines MT-4 and TZM-bl, which contain the luciferase and β-galactosidase genes under the control of the HIV-1 promoter (53), and the PIs used in this study: tipranavir (TPV), darunavir (DRV), atazanavir (ATV), lopinavir (LPV), amprenavir (APV), and saquinavir (SQV).

Generation of GPR recombinant viruses. Patient-derived HIV-1 genome segments containing gag p7–p1–p6 and the full-length PR (termed GPR) were cloned into the pJM11ΔGPR vector (30), which was derived from the p83-2 HIV-1 hemiplasmid. The plasma samples had been collected after failure of a PI-containing regimen and before the initiation of new regimens. Briefly, viral RNA from plasma samples was purified (QIAamp Viral RNA Minikit), and reverse transcription-PCR (RT-PCR) was performed with the primers 1811U25 (5′-CTACTAGAAGAAGATGTA CAGC-3′) and 3482L31 (5′-TGGTCTATAACATCTCCATGACC GG TTCT-3′) using a SuperScript III One-Step Kit (Invitrogen). Nested PCR was performed using Platinum Taq DNA Polymerase High Fidelity polymerase (Invitrogen) and cloned into the pJM11ΔGPR vector using T4 DNA Ligase (New England BioLabs) by standard techniques. GPR recombinant plasmids were used to transform competent One Shot OmniMAX 2-T1 cells (Invitrogen). Plasmid DNA was purified from an overnight culture of the whole transformed bacterial culture (instead of selecting a limited number of bacterial clones) to maintain the quasispecies diversity of the original viral population. Infectious viral stocks were produced by cotransfection of 5 × 10⁶ MT-4 cells by electroporation (at 250V and 960 μF) with 2.5 μg of both the corresponding GPR-pJM11 recombinant plasmid and the hemiplasmid p83-10, which was previously digested with EcoRl. Seven days after transfection, culture supernatants were harvested, and the protease gene of the viral stocks was PCR amplified and sequenced to assess genetic similarity with the original plasma viral RNA sample of each patient (BigDye Terminator, version 3.1, Cycle Sequencing Kit; Applied Biosystems).

In vitro expansion of primary viral isolates. Four plasma samples, collected at different time points over a 2-year period from an HIV-1-infected patient who experienced successive viral failure to diverse PI-containing regimens (viral load range 10⁴ to 10⁶ RNA copies/ml), were selected to obtain viral isolates using anti-CD44 beads (Miltenyi Biotec) as previously described (43). Briefly, CD8⁺-depleted peripheral blood mononuclear cells (PBMCs) from three HIV-1-seronegative donors were pooled and stimulated under three different conditions (three-by-three method; Miltenyi Biotec) with the following low-dose phytohemagglutinin (PHA; 0.5 μg/ml), high-dose PHA (5 μg/ml), and plate-bound anti-CD3 monoclonal antibody (OKT3). After 3 days, cells were mixed to a final concentration of 10⁶ cells/ml in RPMI medium supplemented with 10% fetal bovine serum (both from Invitrogen) and 100 U/ml of interleukin-2 (IL-2; Roche), and 200 μl of the extracted virus was then added to the culture. Cultures were fed weekly with 10⁶ cells/ml of fresh cells stimulated according to the three-by-three method. Viral growth was monitored using a p24 enzyme-linked immunosorbent assay (Innogenetics) until at least 100 ng/ml was reached, typically after 1 to 3 weeks, depending on the sample viral load. Culture supernatants were then harvested, and viral RNA was sequenced.

Viral stock titration. Chemiluminescence titration assays in TZM-bl cells, with some modification of the original protocol (33), were used to determine the mean 50% tissue culture infective dose (TCID₅₀) of each viral stock. To avoid hypersusceptibility effects or experiment failures due to the fitness cost associated with resistance mutations in the protease gene, we normalized viral input in the drug susceptibility assay in order to have similar infection rates for all viral stocks tested in the absence of drug. Thus, we modified the titration protocol so that it also consisted of two infection cycles. Briefly, titration plates were prepared as in the standard protocol, and, after 3 days of culture, 100 μl of supernatant from each well was transferred to new TZM-bl cultures, keeping the same culture conditions and plate distribution. After 3 days, HIV-1 infection in this second round was monitored by measuring luciferase expression (Bright-Glo Luciferase Assay; Promega).

Two-round infection PI susceptibility assay. The protocol for the PI susceptibility assay is based on a modification of the luciferase reporter gene assay for titrating HIV-neutralizing antibodies described by Montefiori (34). As the inhibitory effect of PIs cannot be detected in a single-cycle infection in the target cells, we designed a two-round infection assay in TZM-bl cells in order to evaluate PI susceptibility (Fig. 1). In the first round of infection, 50 TCID₅₀ of each viral stock were used to infect 10,000 TZM-bl cells (multiplicity of infection, 0.02) in quadruplicate in 96-well plates containing 9.35 μg/ml of DEAE-dextran and 4-fold serial dilutions of each PI tested. After 3 days of culture, 100 μl of supernatant containing de novo produced viral particles from this first infection was transferred to new TZM-bl cultures (10,000 cells per well) in opaque-walled 96-well plates (Nunc). HIV-1 infection in this second round was monitored by measuring luciferase expression in infected target cells 3 days after infection using a Bright-Glo Luciferase Assay (Promega). The percentage of inhibition was determined by calculating the difference in relative light units (RLUs) between test wells and negative-control (non-infected) wells, dividing this result by the difference in the number of RLUs between positive-control wells (without drug) and negative-control wells, subtracting from one, and multiplying by 100. The effective drug concentrations required to inhibit virus replication by 50% (EC₅₀) were calculated by fitting data to a sigmoid dose-response curve with a variable slope using GraphPad Prism, version 5.0. The fold change (FC) in drug susceptibility was determined by dividing the EC₅₀ of every sample virus by the EC₅₀ of the drug-susceptible HIV-1 (HIVdb).

Determination of genotypic resistance. Viral stocks were sequenced (BigDye Terminator, version 3.1, Cycle Sequencing Kit; Applied Biosystems), and PI resistance mutations were identified according to the HIV Drug Resistance Database of Stanford University. Also from the HIVdb systems), and PI resistance mutations were identified according to the HIV Drug Resistance Database of Stanford University. Also from the HIVdb program, genotypic resistance interpretation algorithm and the total mutation score were used to obtain qualitative and quantitative inferred levels of PI susceptibility, respectively (HIVdb program, version 6.1.1; Stanford University Drug Resistance Data Base [http://sierra2.stanford.edu/sierra/servlet/Sierra]). Correlation was evaluated using a Spearman...
correlation coefficient \((r)\) and the associated two-tailed \(P\) value (GraphPad Prism, version 5.0).

RESULTS

Performance of the two-round infection PI susceptibility assay.

As PIs affect viral replication by inhibiting protease activity in recently produced immature virions (24), PI susceptibility cannot be evaluated in a single-cycle infection assay. For this reason, standard PI susceptibility assays are based on the generation of multiple viral stocks in the presence of increasing concentrations of drug. Here, we designed a rapid two-round infection assay in which TZM-bl cells are used as virion-producing cells in the first infection round and as reporter cells in the second round (Fig. 1). Thus, the phenotype of viral stocks previously produced in the absence of drug (in cell lines or in primary cultures) might be evaluated by using this protocol.

Reproducibility of the assay was evaluated using a panel of six PIs (TPV, DRV, ATV, LPV, APV, and SQV). The means and standard deviations of EC_{50} for each PI were calculated based on data obtained from nine independent experiments testing the susceptible HIV-1_{NL4-3} viral strain (Fig. 2), with the following results: TPV, \(178.5 \pm 100.4 \text{ nM}\); DRV, \(0.93 \pm 0.45 \text{ nM}\); ATV, \(3.48 \pm 2.07 \text{ nM}\); LPV, \(2.53 \pm 1.53 \text{ nM}\); APV, \(5.92 \pm 4.17 \text{ nM}\); and SQV \(4.51 \pm 1.84 \text{ nM}\). All of these values are in the expected EC_{50} range for drug-susceptible viruses, according to previously reported phenotypic data. Despite these rather consistent results, HIV-1_{NL4-3} was eventually included as an internal reference control in each independent phenotypic determination to minimize interassay variability.

Correlation with viral genotype. To evaluate the performance of the assay with PR-resistant HIV-1 specimens, we used a panel of...
11 patient-derived viral stocks. Seven of these were GPR recombinant viruses, which contained the patient-derived 3′ half of gag (p7-p1-p6) and the full-length PR cloned in an HIV-1NL4-3 backbone. Samples were collected from these patients after failure of PI-containing treatment and before the initiation of new regimens. Furthermore, to explore the possibility of using this phenotypic assay with full-length patient-derived viral isolates, four plasma samples were collected at different time points from an HIV-1-infected patient who experienced successive virologic failures to antiretroviral therapy. All viral stocks underwent population-based sequencing, and PI resistance mutations in the PR coding region were identified based on the Stanford University HIV Drug Resistance Database (Table 1). The two-round infection PI susceptibility assays were then performed, and the FC in resistance of each viral sample to TPV, DRV, ATV, LPV, APV, and SQV was calculated. A sample phenotypic drug susceptibility profile for a representative primary viral isolate is shown in Fig. 3.

The genotypic resistance interpretation algorithm (HIVbd program) was used to obtain qualitative inferred levels of PI susceptibility for each viral sample based on its protease-coding region sequence (23). This algorithm classified each virus sample as susceptible, potential or low-level resistant, intermediate, or high-level resistant. When FC data from all our phenotypic assays were categorized according to these inferred genotypic resistance levels (Fig. 4A), increasing FC values were observed in the groups with potential/low-level resistance (median FC, 10.7), intermediate resistance (median FC, 31.6), and high-level resistance (median FC, 285.9).

### Table 1 Major and minor PI resistance mutations in the protease-coding region of the viral variants used for the evaluation of the novel two-round infection susceptibility assay

<table>
<thead>
<tr>
<th>Virus</th>
<th>HIV PR residue at the indicated position&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>NL4-3</td>
<td>L V L V L E K M G I F I Q A G T V I N L L</td>
</tr>
<tr>
<td>GPR-1</td>
<td>I · · · · · · · · · · · · · · · · · · ·</td>
</tr>
<tr>
<td>GPR-2</td>
<td>I · · · I F · · · · · · · · · · · · ·</td>
</tr>
<tr>
<td>GPR-3</td>
<td>F · · · · · · · · · · · · · · · · · · ·</td>
</tr>
<tr>
<td>GPR-4</td>
<td>I · · · · · · · · · · · · · · · · · · ·</td>
</tr>
<tr>
<td>GPR-5</td>
<td>I · · · · · · · · · · · · · · · · · · ·</td>
</tr>
<tr>
<td>GPR-6</td>
<td>· · · · · · · · · · · · · · · · · · ·</td>
</tr>
<tr>
<td>GPR-7</td>
<td>I · · · · · · · · · · · · · · · · · · ·</td>
</tr>
<tr>
<td>PVI-1</td>
<td>I · · · · · · · · · · · · · · · · · · ·</td>
</tr>
<tr>
<td>PVI-2</td>
<td>I · · · · · · · · · · · · · · · · · · ·</td>
</tr>
<tr>
<td>PVI-3</td>
<td>I · · · · · · · · · · · · · · · · · · ·</td>
</tr>
<tr>
<td>PVI-4</td>
<td>I · · · · · · · · · · · · · · · · · · ·</td>
</tr>
</tbody>
</table>

<sup>a</sup> GPR-1 to GPR-7 are recombinant viruses derived from samples collected after failure of a PI-containing regimen and before the initiation of new regimens. PVI-1 to PVI-4 are primary viral isolates (in chronological order) derived from samples collected at different time points from an HIV-1 patient who experienced successive virologic failures to antiretroviral therapy.

<sup>b</sup> Mutations are noted relative to the PR sequence of HIV-1NL4-3. A dot indicates that there was no mutation.

![Graph showing phenotypic drug susceptibility profiles from a representative patient-derived primary viral isolate.](http://jcm.asm.org/)
132.8), compared to the susceptible group (median FC, 3.9), although statistically significant differences were found only in the intermediate and high-level groups (P = 0.002 and P = 0.0004, respectively, Mann-Whitney test). Moreover, the genotype-based total mutation score (also from the HIVbd program) was used to predict quantitative resistance levels for each viral sample and drug. When our phenotypic data were compared with those scores, a good correlation was also observed for the overall data set (Spearman ρ = 0.69, P < 0.0001) (Fig. 4B). These results showed the consistent performance of the two-round infection PI susceptibility test with PR-resistant viruses.

Correlation with a standard HIV-1 phenotypic assay. To assess the reliability of the two-round infection PI susceptibility assay, the same viral stocks were evaluated using the in vitro phenotypic resistance test Antivirogram (Janssen Diagnostics BVBA), and correlation analysis (Spearman test) was used to compare both methods. A strong statistical correlation was observed between the FC data obtained with both assays for the GPR recombinant viruses (ρ = 0.79, P < 0.0001) and for the primary viral isolates (ρ = 0.84; P < 0.0001) (Fig. 5A and B). When all data were analyzed together, regardless of the origin of the virus and the PI used, we observed an overall Spearman correlation ρ value of 0.79 (P < 0.0001) (Fig. 5C). For individual PIs, given the low number of viruses analyzed and the irregular distribution of resistance levels among them, a significant correlation was found only in four out of the six of the drugs tested, namely, LPV (P = 0.04), TPV, DRV, LPV, ATV, SQV, and APV.

FIG 4 Comparison with genotype interpretation. A total of 11 viral stocks were tested for drug susceptibility using a panel of six PIs: TPV, DRV, LPV, ATV, SQV, and APV. (A) Data were classified according to the genotypic resistance level predicted by the Stanford HIVdb algorithm into four groups: susceptible (-), potential or low-level resistant (-/+), intermediate (+), and high-level resistant (+/+). The FC values obtained with the two-round infection PI susceptibility assay are plotted on the y axis; the median FC values, 25th to 75th percentiles (boxes), and 10th to 90th percentiles (whiskers) are shown for each group. The P values (Mann-Whitney test) after comparison of the median FC of the different genotypic resistance groups with the susceptible group are shown. (B) Correlation of the overall FC data obtained in the novel PI susceptibility protocol and the Stanford genotype score; drugs are color coded as shown. The nonparametric Spearman correlation coefficient (ρ) and the associated two-tailed P value were calculated (GraphPad Prism, version 5.0). No interpretation of resistance or genotype score was obtained for APV.

FIG 5 Correlation of the results from the two-round infection PI susceptibility method and the standard Antivirogram phenotypic assay. (A) Data from the GPR recombinant viruses. (B) Data from the primary viral isolates. (C) Overall correlation of data from the 11 viral stocks tested; drugs are color coded as shown. In all cases, the Spearman correlation coefficient (ρ) and the associated two-tailed P value were calculated (GraphPad Prism, version 5.0).
ATV ($P = 0.02$), APV ($P = 0.03$), and SQV ($P = 0.02$) (data not shown). In summary, our experimental approach demonstrates high reliability and an excellent correlation with standard commercially available methods for both recombinant viruses and primary viral isolates.

**DISCUSSION**

In the last 10 years, drug resistance testing has become the standard of care in the management of HIV-1-infected patients whose antiretroviral treatment has failed. Genotypic resistance tests are the most widely used, given their fast turnaround time and affordable cost. Several algorithms are currently available for the interpretation of genotyping results; however, only mutations in the HIV-1 protease-coding region are taken into account for the inference of susceptibility to PIs. Other Gag mutations affect susceptibility to PIs, not only at cleavage sites (8, 21, 26) but also at noncleavage residues located at the matrix (p17) and capsid (p24) coding regions (40, 41). Even so, current standard phenotypic assays do not include the patient-derived full-length Gag region in the recombinant viruses used for PI susceptibility evaluation. Indeed, most commercially available and in-house methods for the determination of PI susceptibility are based on the use of recombinant pseudoviruses or replication-competent chimeric viruses, which harbor the patient-derived 3rd half of gag (containing the protein p7 C terminus, p1, and p6) and the whole PR-coding region (6, 11, 52, 54). In order to avoid the drawback of partial cloning of the viral genome, we designed and developed a new two-round experimental infection strategy to test the susceptibility of primary viral isolates to PIs.

Our phenotypic test is based on the use of the HIV-1 susceptible TZM-bl reporter cell line. These cells were first described and used for the evaluation of resistance to fusion inhibitors by a single-round infection protocol in 2002 (53). Since then, this methodology has been widely used to evaluate neutralizing antibodies (22, 31) and viral susceptibility to other entry inhibitors (1), nucleoside and nonnucleoside reverse transcriptase inhibitors (25), and integrase inhibitors (4). In previous studies using this system for PI susceptibility testing, multiple recombinant viral stocks were generated in the presence of different drug concentrations prior to infection of the reporter cells (35, 36) since the inhibitory effect of PIs cannot be detected in a single-cycle infection in the target cells (24). Our assay overcomes this restriction by using the TZM-bl cell line as a virion-producing and reporter cell line in a rapid two-round infection protocol. This cell line had been previously determined to be susceptible to infection with PBMC-grown primary isolates regardless of HIV-1 tropism or clade (2, 32); therefore, we tested this methodology with patient-derived primary viral isolates and GPR recombinant viruses harboring the X4-tropic HIV-1 LAV-1 backbone. Our results showed a good overall correlation with the Stanford HIVdb (similar to the correlation between the standard Antivirogram test and the HIVdb results; Spearman $\rho = 0.71$, $P < 0.0001$) (data not shown). Likewise, an excellent correlation was observed with the standard Antivirogram phenotypic test. However, before our test can be applied in diagnosis, additional analyses with a broader diversity of patient-derived susceptible and resistant viral samples should be performed to determine the biological cutoffs for each drug and the overall concordance with standard methods. Interestingly, discordant results—samples showing FC resistance values of >15 in our phenotypic test while remaining susceptible according to the Antivirogram results (FC of <2.5)—were found only in patient-derived primary viral isolates and not in GPR recombinant viruses (Fig. 5). These viral isolates were generated from the plasma samples of a patient who had experienced successive virological failures with PI-containing antiretroviral therapy. Further studies should be performed to determine whether mutations in the 5’ half region of Gag of these primary isolates (not included in the recombinant viral construction used in the Antivirogram assay) cause higher levels of resistance when full-length viruses are tested since the direct contribution of residues in HIV matrix to PI resistance and the coevolution of these residues with drug-resistant proteases have been reported (40, 41).

In the present work, a protocol based on the use of anti-CD44 beads was implemented to expand primary viral isolates from plasma samples (43). The protocol enables viral stocks to be generated from plasma samples containing >1,000 copies of viral RNA/ml in 1 to 2 weeks with 90% efficiency; stocks can be generated from samples containing 100 to 1,000 copies/ml in up to 3 weeks with 30% efficiency (7). Alternative protocols based on coculture of PBMCs from the patient and from healthy donors might also be used to expand primary viral isolates with 50% efficiency from low-level viremia samples (100 to 1,000 copies/ml) (7). Although the first standardized phenotypic drug susceptibility assays used primary viral isolates derived from patient samples cocultured with healthy-donor PBMCs (20), these protocols fell into disuse because they were time-consuming and not applicable for high-throughput processes. Consequently, they were replaced by recombinant virus-based protocols. As the number of antiretroviral drugs available for the treatment of HIV-1-infected individuals increased and so the number of viral target genes, the need to generate multiple recombinant viruses emerged. However, low viremia often limits the PCR amplification of multiple fragments, as well as the amplification of large single fragments. The enhanced sensitivity of the primary viral isolate expansion methodology, compared to other PCR-based standard assays (detection limit of 1,000 copies/ml), might help to characterize viruses from patients whose therapy fails with sustained viral loads below 1,000 RNA copies/ml (39, 48, 49). The need for extended culture time in biosafety level 2 (BSL-2) facilities to generate primary isolates is well compensated by the fact that any kind of phenotypic assay (including drug resistance and replication capacity assays in the presence or absence of inhibitors) might be performed using the same primary viral isolate, thus removing the higher expense of generating multiple recombinant viruses.

We observed that, especially in the case of highly resistant viruses, normalization of viral input is critical for optimal performance of the phenotypic assay. Therefore, we also designed a two-round infection titration protocol to ensure equivalent levels of target cell infection for all the viruses tested in the phenotype assay. Indeed, there have been reports of a highly significant fitness cost associated with PI resistance mutations (5, 29), which may lead to a hypersusceptibility effect in phenotypic assays or even failure in data recording. Nevertheless, viral input is not generally normalized in most phenotypic assays. Here, the use of the full-length patient-derived viral genome may help to recover viral fitness, as compensatory mutations along the Gag region restore otherwise compromised activity of viruses containing multiple amino acid substitutions associated with resistance to PIs (8, 9, 12, 26, 37, 40, 41, 46, 47, 55). Nevertheless, most of the samples in these studies were B subtype, and further research is needed to
better define PI resistance determinants and compensatory effects in non-B subtypes. Our assay could prove to be a useful tool for the characterization of both Gag and PR mutations in non-B subtypes. In addition, using either full-length Gag-containing recombinant viruses or primary viral isolates, this two-round infection protocol could also be applied to evaluate drug susceptibility to new antiviral agents such as bevirimat, a maturation inhibitor that specifically inhibits the p24/p2 cleavage event in the Gag precursor (38), a region which is not included in the main recombinant viruses used in standard phenotypic assays.

In conclusion, this novel two-round phenotypic infection assay is an affordable and rapid in-house method for the PI susceptibility evaluation of recombinant viruses and primary viral isolates. The synergic contribution of patient-derived gag and PR viral genes to PI resistance can be evaluated using this system. Thus, assays of this type might help to further characterize PI resistance mutations located outside the PR-coding region in not only HIV-1 B subtypes but also non-B subtypes.

ACKNOWLEDGMENTS

The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: MT-4 (Douglas Richman), TZM-bl (John C. Kappes, Xiaoyun Wu, and Tranzyme, Inc.). The AIDS Research and Reference Reagent Program also supplied the antiretrovirals used in the study (tipranavir, darunavir, atazanavir, lopinavir, and amprenavir).

P.V.D.E. is an employee of Janssen Diagnostics BVBA. We have no other conflicts of interest to declare.

Funding for this work was provided by grants from the Spanish AIDS network Red Temática Cooperativa de Investigación en SIDA (RD06/0006) and from the Fundación para la Investigación y Prevención del SIDA en España (36771/08). This study was also supported by the European Union’s Seventh Framework Programme (FP7/2007-2013) under the “Collaborative HIV and Anti-HIV Drug Resistance Network (CHAIN)” Project Grant agreement 223131 and grants CP09/00279 and P11/00249 by the ISCIII. J.G.P. holds a Miguel Servet Contract (MS09/00279) funded by FIS-ISCIII (Government of Spain).

REFERENCES

32. Montefiori DC. 2009. Optimization and validation of a neutralizing antibody assay for HIV-1 in TZM-bl cells using luciferase reporter gene

Downloaded from http://jcm.asm.org on August 29, 2017 by guest


