Improved V3 genotyping with duplicate PCR amplification for determining HIV-1 tropism

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Objectives: To determine whether genotyping of HIV-1 by duplicate PCR amplification of the region encoding the V3 loop is more sensitive than single PCR for detecting CXCR4-using viruses.

Patients and methods: The V3 genotypes of the HIV-1 infecting 152 patients enrolled in the multicentre Geno-Tropism ANRS study were determined by all the participating laboratories using a single PCR and V3 bulk sequencing. In parallel, one laboratory determined the V3 genotype using duplicate PCR and bulk sequencing of pooled amplicons. HIV tropism was predicted with the geno2pheno10 algorithm. The phenotypes of all samples were determined with the Trofile assay and the Toulouse tropism test (TTT) recombinant virus assay.

Results: Geno2pheno10 was 56.8% sensitive and 75.9% specific when compared with the Trofile assay for detecting CXCR4-using viruses after a single PCR. Duplicate amplification and bulk sequencing of the pooled PCR amplicons increased the sensitivity to 68.2% and specificity to 79.6%. Geno2pheno10 was 64.1% sensitive and 77.0% specific when compared with the TTT assay for detecting CXCR4-using viruses after a single PCR. Duplicate amplification and sequencing of the pooled PCR amplicons increased sensitivity to 76.9% and specificity to 80.5%.

Conclusions: The genotypic determination of HIV-1 tropism can be improved by duplicate amplifications and sequencing the pooled PCR products. This is a good compromise between improved sensitivity and reasonable cost for the genotype-based determination of tropism.

Keywords: HIV-1 tropism, phenotype, CCR5 receptor, V3 genotype, bulk sequencing

Introduction

HIV type 1 (HIV-1) enters CD4-expressing cells using one or both of the chemokine receptors CCR5 and CXCR4. CCR5-using viruses are classified as R5 variants, CXCR4-using viruses as X4 variants and viruses using both co-receptors as R5X4 dual-tropic variants.1 HIV-1 co-receptor use must be determined before using a CCR5 antagonist in clinical practice because these drugs act only against R5 viruses. HIV-1 tropism can be assessed with phenotypic assays.2,3 However, these assays are rather complex and costly for routine use. HIV-1 tropism can be determined by genotyping the V3 env region that contains the main genetic determinants of HIV-1 co-receptor usage.4,5 But studies on the algorithms for interpreting V3 sequencing have found suboptimal concordance between genotypic and phenotypic approaches.6,7

We previously evaluated the genotypic prediction of HIV-1 tropism using single amplification in the multicentre ANRS Geno-Tropism study.2 We have now compared the performances of duplicate and single PCR amplification for V3 genotyping with reference to the Trofile and Toulouse tropism test (TTT) phenotypic assays.

Materials and methods

We studied 152 patients (140 infected with HIV-1 subtype B (92%), 4 with subtype CRF02-AG, 3 with subtype G, 3 with subtype D and 2 with...
subtype F1) included in the multicentre ANRS GenoTropism study who had undergone tropism determination by V3 genotyping by both single and duplicate PCR and by two phenotypic assays. Plasma HIV RNA was measured using standardized real-time PCR assays. The 18 participating laboratories performed single V3 amplifications on plasma samples and determined the bulk V3 sequences using a standardized protocol validated in each laboratory. The PCR primers and conditions, and sequencing primers are described in the ANRS consensus techniques (http://www.hivfrenchresistance.org). Duplicate amplification of the V3 region was performed at the Virology Laboratory of Toulouse University Hospital, France, using HIV-1 RNA isolated from 500 μL of plasma collected on the screening date. Two separate RT-PCR (RNA input 15 μL) and nested PCR (DNA input 2 μL) were performed for each sample to amplify a fragment encompassing gp120 and the ectodomain of gp41. The resulting products were pooled and sequenced in both directions by the dyeoxy chain termination method (BigDye Terminator; Applied Biosystems, Foster City, CA, USA).

Results were analysed in each laboratory, with the operator blinded to the phenotype. Minor species were detected when the automated sequencer electropherogram showed a second base peak. All possible combinations of V3 amino acid sequences present as a result of nucleotide mixtures were considered for predicting the genotype. The V3 sequences were interpreted using the Geno2pheno tool with a false-positive rate of 10% (http://coreceptor.bioinf.mpi-hbi.de/cgi-bin/coreceptor.pl) because this genotype algorithm provided the best prediction in the GenoTropism study. The sequences reported here have been given GenBank accession numbers HQ891668–HQ891817.

The phenotypes of all samples were determined with two versions of the Trofile assay (Monogram Biosciences, South San Francisco, CA, USA): the original Trofile assay (OTA) was used for 129 samples and the enhanced-sensitivity Trofile assay (ESTA) was used for 23 samples. The detection limit of X4 variants was 10% for OTA and 0.3% for ESTA. HIV-1 tropism was also determined using the TTT based on the pooled env PCR products obtained after duplicate amplification, as previously described. Minor X4 variants were detected when present at ≥0.5%.

### Results

The 152 samples screened included 108 identified by Trofile as R5 and 44 identified as R5X4 or X4. At the same time, the V3 genotype was determined in each participating laboratory by single PCR and bulk sequencing and in one laboratory by duplicate amplifications and bulk sequencing of the pooled products.

The concordance between genotypic and phenotypic approaches is shown in Table 1. Using Trofile as a reference, the duplicate PCR amplification identified five more X4 viruses than did the single PCR approach. The sensitivity of V3 genotyping for predicting CXCR4-using viruses therefore increased from 56.8% with single PCR to 68.2% with duplicate PCR (P = 0.13). The concordance between the single PCR approach and Trofile was 76% for HIV-1 subtype B and 83% for non-B HIV-1 subtypes.

The 152 samples screened included 113 identified by TTT as R5 and 39 identified as R5X4 or X4 (Table 1). Using the TTT result as a reference, the duplicate PCR system detected five more X4 viruses than did the single PCR approach. The sensitivity of V3 genotyping for predicting CXCR4-using viruses therefore increased from 64.1% with single PCR to 76.9% with duplicate PCR (P = 0.22). The five additional samples containing X4 viruses detected by the duplicate approach using TTT or Trofile as a reference were the same and phenotyped as R5X4/X4 by both phenotypic assays.

Thirty-three patients had a plasma virus load (VL) of <3000 copies/mL, while 115 had a VL of ≥3000 copies/mL (4 patients had unknown VLs). This cut-off was 3-fold greater than the usual threshold used for performing genotypic analyses in plasma samples (i.e. 1000 copies/mL). VLs ranging from 1000 to 3000 copies/mL are considered to be low VLs. We studied the influence of plasma HIV RNA load on the performance of V3 genotyping using TTT as a reference (Figure 1). The sensitivity for detecting CXCR4-using viruses increased from 50% with the single PCR to 63% with the duplicate PCR when the VL was ≥3000 copies/mL and from 68% to 81% when the VL was >3000 copies/mL. The plasma HIV RNA load had a similar influence on the sensitivity of V3 genotyping when using the Trofile assay as a reference (data not shown).

### Discussion

As the virological response to a treatment that includes a CCR5 antagonist is impaired by the presence of X4 viruses, these viruses must be detected by a tropism-determining assay. Population-based sequencing of the V3 env region and genotypic algorithms are useful for routine determination of HIV-1 tropism, but genotypic methods are often poorly sensitive for detecting

### Table 1. Determination of HIV-1 tropism by two genotype-based assays using the Trofile or TTT assays as a reference

<table>
<thead>
<tr>
<th>V3 genotype</th>
<th>Trofile phenotype</th>
<th>Performance of the V3 genotype using Trofile as a reference</th>
<th>TTT phenotype</th>
<th>Performance of the V3 genotype using TTT as a reference</th>
</tr>
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<tr>
<td></td>
<td></td>
<td>sensitivity&lt;sup&gt;a&lt;/sup&gt;</td>
<td>specificity&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Single PCR and Geno2pheno10</td>
<td>R5</td>
<td>82</td>
<td>56.8%</td>
<td>R5</td>
</tr>
<tr>
<td></td>
<td>X4</td>
<td>26</td>
<td>25</td>
<td>X4</td>
</tr>
<tr>
<td>Duplicate PCR and Geno2pheno10</td>
<td>R5</td>
<td>86</td>
<td>68.2%</td>
<td>R5</td>
</tr>
<tr>
<td></td>
<td>X4</td>
<td>22</td>
<td>30</td>
<td>X4</td>
</tr>
</tbody>
</table>

<sup>a</sup>Sensitivity, the capacity for detecting CXCR4-using viruses, was calculated by the number of concordant X4/R5X4 results divided by the number of viruses phenotyped as R5X4/X4.

<sup>b</sup>Specificity, the capacity for detecting exclusive CCR5-using viruses, was calculated by the number of concordant R5 results divided by the number of viruses phenotyped as R5.
with each new PCR run. We also separately analysed by both single PCR run, but the probability of it being amplified increases in the quasi species. A minor variant may not be amplified in a to reduced sampling bias and better detection of minor variants improved sensitivity obtained with replicate PCRs could be due from 64.1% to 76.9% with the TTT assay as a reference. This 11.4% gain was not determined with the geno2pheno10 algorithm.

Influence of plasma HIV-1 RNA load on the performance of V3 genotyping. There were two groups of patients with different plasma HIV RNA loads at the time of tropism testing; one (33 patients) had a VL of <3000 copies/mL and the other (115 patients) had a VL of ≥3000 copies/mL. The sensitivity of the V3 genotype for predicting X4 viruses was calculated for each group using both strategies of amplification and the TTT assay as a reference. The proportion of R5X4/ X4 viruses accurately predicted by the V3 genotype is indicated on the bars. NA, not applicable. *McNemar’s χ² test was used for comparing the sensitivities of the genotype-based assays using single and double amplification strategies.

CXCR4-using viruses. The highest sensitivity for detecting X4 viruses was 59.3% with geno2pheno10 in the multicentre ANRS GenoTropism study, while the sensitivity was 88% in a previous study with the same genotypic algorithm after duplicate amplification of the env region. We therefore compared single and duplicate PCR amplifications to test 152 patients included in the GenoTropism study to assess the concordance between the V3 genotype and two phenotypic assays. Single PCR detected minor X4 variants accounting for 3%–15% of the virus population as assessed by pyrosequencing. Single PCR detected minor X4 variants in only two samples, whereas duplicate PCR detected them in the same samples and in three more samples (data not shown). However, the lower sensitivity of the single PCR approach may also be due to small differences between the different laboratories. Nevertheless, all the laboratories undergo annual quality controls, including genotypic determinations of HIV tropism.

The genotyping of samples with a low HIV RNA load can be increased by triplicate amplification. In the GenoTropism study, duplicate PCR seemed to improve the genotyping of samples with both high and low plasma HIV-1 RNA loads. Besides the efficiency of the genotypic algorithms for interpreting the V3 sequence, optimized analytical conditions could also improve the performance of the genotype-based determination of HIV-1 tropism.

The sensitivity of a triplicate PCR strategy, based on three separate PCR amplifications and three bulk sequencings, is 67.4% for predicting CXCR4-using viruses. However, this strategy is costly and time consuming. We obtained a very similar sensitivity using duplicate PCR amplification and bulk sequencing of the pooled products. The additional cost of our strategy is the price of only one RT- and nested-PCRs compared with a single PCR strategy. A recent study showed that ultra-deep sequencing detected more than twice as many maraviroc recipients as having non-R5 viruses than did OTA. In samples where ultra-deep sequencing detected the presence of non-R5 viruses accounting for 2%–20% of the virus population, 37% of them were also identified as having non-R5 virus by the triplicate strategy and 58% by Trofile. We therefore compared our duplicate approach and triplicate PCR plus three bulk sequencings in selected samples in which X4 minor variants accounted for <20% of the virus population, as assessed by pyrosequencing. The proportions of X4 viruses detected by the two strategies were the same (2/6) (data not shown).

In conclusion, our data suggest that the sensitivity of genotype-based detection of CXCR4-using viruses can be improved by duplicate amplifications of the HIV envelope gene and sequencing the pooled PCR products. This is a good compromise between improved sensitivity and reasonable cost for genotype-based determinations of tropism.

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HIV-1 V3 genotyping using duplicate PCR

Transparency declarations
None to declare.

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