Evaluation of genotypic tropism prediction tests compared with *in vitro* co-receptor usage in HIV-1 primary isolates of diverse subtypes

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**Objectives:** To evaluate the sensitivity and specificity of genotypic methods for predicting the co-receptor usage of subtypes B and non-B HIV-1 primary isolates, using as gold standard the infectivity of each primary isolate in GHOST cells stably expressing HIV-1 co-receptors.

**Methods:** Primary isolates were obtained by co-culturing either patient's peripheral blood mononuclear cells (PBMCs) or ultracentrifuged plasma with donor-activated PBMCs. *In vitro* co-receptor usage was determined by infecting GHOST cells. Tropism prediction, based on V3 sequences, was determined with simple rules and bioinformatic tools (Geno2pheno[coreceptor] and WebPSSM).

**Results:** This study includes 102 HIV-1 primary isolates; 23 (22.5%) subtype B and 79 (77.5%) non-B genetic forms. V3 sequences were classified into six subtypes (A–G), although 32 (31.4%) were circulating recombinant forms and 21 (20.6%) were unique recombinant forms. Sixty-nine isolates were R5, 27 R5X4 and 6 X4. The highest levels of sensitivity and specificity for the detection of X4 strains among V3 sequences, between 91% and 100%, were obtained by using PSSM_x4r5, PSSM_x5nsi and the 11/25 rule for sequences of subtypes A, B and G, but not for subtype F. Establishing the recommended cut-off for clinical settings of a 10% false positive rate for Geno2pheno, we obtained 93% specificity and 97% sensitivity.

**Conclusions:** Comparing genotypic assays for HIV-1 co-receptor use with a cell-culture phenotypic assay could provide more reliable results of sensitivity and specificity for the detection of X4 strains than comparing them with recombinant assays, considered as gold standard. In general, except for subtype F isolates, there is a good correlation for tropism prediction.

**Keywords:** V3, maraviroc, CCR5, CXCR4, sensitivity, specificity

**Introduction**

HIV type 1 (HIV-1) enters the cell through attachment of gp120 to CD4, followed by interaction with a co-receptor. Chemokine receptors CCR5 and CXCR4 were identified as the major co-receptors for HIV-1. According to the ability of the virus to use the CCR5 or the CXCR4 co-receptors, HIV-1 tropism is classified as R5, X4 or dual/mixed R5X4.¹

Genetic differences among HIV-1 subtypes might condition tropism predilection. Progression to AIDS is associated with X4 or R5X4 tropism in half of AIDS patients infected with subtype B. With regard to non-B subtypes, previous studies have reported that subtype C isolates obtained at early stages of the disease almost exclusively use the CCR5 co-receptor.²,³ However, some subtype C viruses from either asymptomatic antiretroviral drug-naive individuals or AIDS patients were able to use co-receptors CXCR4, CCR5 or both.⁴,⁵ On the other hand, subtype D viruses from HIV-1 drug-naive patients were dual tropic at a higher frequency (44%) than subtype A or A/D recombinant viruses.⁶,⁷ Moreover, some circulating recombinant forms (CRFs) have been associated with a predominant tropism. CRF14_BG, whose envelope is mainly of subtype B, has characteristic mutations in V3 and primary isolates show R5X4 tropism, even at early stages of infection.⁸,⁹ CRF01_AE and CRF02_AG frequently use CXCR4 at late-stage disease, at frequencies of 73% and 79%, respectively, among AIDS patients.¹⁰ Maraviroc is the first CCR5 antagonist approved for the treatment of HIV-1-infected patients. Patients eligible to receive maraviroc need a previous co-receptor usage test to rule out the presence of X4 strains.¹¹
to express CD4 and one of the co-receptors (CCR5 or CXCR4) have become a useful tool to determine in vitro phenotypic tropism.\textsuperscript{12} Culture-based phenotypic methods are considered to be the most reliable for co-receptor usage determination. However, a selection bias is associated with virus isolation, rendering viral populations that could differ from the original ones present in patients.\textsuperscript{13} Therefore, tropism determination in HIV primary isolates is not validated for clinical purposes. Recombinant and clonal phenotypic tests are other phenotypic methods that have been developed to assign co-receptor usage to clinical samples.\textsuperscript{14–20} The Trofile assay (Monogram Biosciences, South San Francisco, CA, USA) and the Enhanced Sensitivity Trofile Assay (ESTA) were validated for their usage in clinical practice in 2007 and 2008, respectively. Currently, ESTA is considered the gold standard phenotypic method for HIV tropism determination, detecting as few as 0.3% of X4 variants with 100% sensitivity.\textsuperscript{21} However, these tests are expensive, time-consuming and the current version of ESTA is only available in one laboratory, which implies additional shipping costs.

The main tropism determinants are located in the V3 loop of gp120, although other regions, such as V1/V2 and C4 in gp120 and substitutions in gp41, may be involved in viral tropism.\textsuperscript{22–24} Simple rules based on the V3 amino acid sequence, such as a high amino acid net charge or the presence of positively charged amino acids in positions 11/25, have been associated with the use of CXCR4.\textsuperscript{25} Moreover, different combinations of these rules have been proposed as algorithms for better co-receptor usage prediction.\textsuperscript{6,27} Databases generated from phenotypic tropism determination have been used to generate different bioinformatic tools for tropism prediction based on the V3 sequence, including position-specific scoring matrices (PSSM), neural networks, support vector machines (SVM), random forests and logistic models.\textsuperscript{28–30} Genotypic prediction algorithms are being validated in subtype B-infected patients, using Trofile as the gold standard, but there are few data evaluating the performance of these methods for predicting the co-receptor usage of HIV-1 isolates of non-B subtypes.\textsuperscript{31–34} The SVM Geno2pheno and the WebPSSM are considered the two bioinformatic tools most appropriate for use in clinical settings for genotypic prediction of the co-receptor usage.

In the present study, we evaluated the sensitivity and specificity of genotypic methods for predicting the co-receptor usage of subtype B and non-subtype B HIV-1 primary isolates, using as gold standard the infectivity of each primary isolate in cell lines stably expressing the co-receptor.

**Methods**

**Samples**

One hundred and two HIV-1 primary isolates previously obtained in our laboratory were included in the study.\textsuperscript{8,9,35,36} Blood samples were extracted from patients who attended Spanish hospitals, although ≥20% of the patients had acquired HIV-1 infection in other countries.

**Primary isolates**

Primary isolates were obtained by co-culturing either patient’s peripheral blood mononuclear cells (PBMCs) or ultracentrifuged plasma with donor-stimulated PBMCs for 4 weeks, according to the consensus culture protocol developed by the Virology Reference Laboratory (VRL) for the Division of AIDS of the National Institute of Allergy and Infectious Diseases (NIAID).\textsuperscript{37} Supernatants from day 28 of culture were used for both infection of GHOST cells and RNA extraction.

**GHOST cell culture assay**

Co-receptor use was determined by infecting the human osteosarcoma cell line GHOST, which expresses CD4 and either CCR5 or CXCR4, and carries HIV-2 long terminal repeat (LTR)-driven green fluorescent protein (GFP). GHOST-CCR5, GHOST-CXCR4 and parental GHOST cells were seeded in 96-well plates with 5×10\textsuperscript{5} cells per well in 200 μL of Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 7.5% fetal calf serum. The following day, the medium was removed and replaced with 60 μL of fresh medium and a volume equivalent to 5 ng of p24 antigen of culture supernatant of primary isolate. After overnight adsorption, the virus was washed with phosphate-buffered saline and 200 μL of fresh medium was added. On day 4 after infection, GFP expression was observed by fluorescence microscopy. Positive and negative control viruses for each co-receptor were included in the assay.

**RNA isolation, amplification and sequencing**

RNA was extracted from culture supernatant using the Nuclisens Kit (bioMérieux, The Netherlands), according to the manufacturer’s instructions. Single-tube reverse transcription–PCR, followed by nested PCR amplification of either near full-length genome or an env region comprising HXB2 positions 7012–7648, containing the V3 loop, was performed with 45 and 45 primary isolates, respectively. Near full-length genomes were amplified in four overlapping fragments, as previously described.\textsuperscript{38} The env region containing the V3 loop was amplified using EDS/ED12 and ES7b/ES8 as outer and inner primer pairs, respectively, as previously described.\textsuperscript{39} Bulk sequencing was performed with the ABI Prism BigDye Terminator Cycle Sequencing kit and ABI 3700 sequencer (Applied Biosystems, Foster City, CA, USA).

Sequences were assembled with SeqMan 6.1 (DNASTar), edited using BioEdit v7.0 and aligned with MAFFT v6 for subsequent phylogenetic analysis with neighbour-joining trees using MEGA. Intersubtype recombinant patterns were determined by boot scanning using Simplot 3.5.1.

**Prediction algorithms for co-receptor usage**

**V3 net charge**

The V3 net charge was calculated by subtracting negatively charged residues (D+E) from positively charged ones (R+K) in the V3 loop. A virus with a V3 net charge ≥+5 was considered to be of X4 phenotype.

**11/25 rule**

This rule predicts X4 co-receptor usage in isolates with positively charged amino acids (R or K) at any of these positions (11 and/or 25) of the V3 loop.

**Geno2pheno[coreceptor]**

Available at http://coreceptor.bioinf.mpi-inf.mpg.de/index.php.\textsuperscript{28} For each co-receptor prediction, the different false positive rates (FPRs) indicating the percentage of R5 samples misclassified by the program as X4 (1%, 2.5%, 5%, 10%, 15% and 20%) were used.

**WebPSSM**

Available at http://indra.mullins.microbiol.washington.edu/webpssm/.\textsuperscript{30} Two matrices for determining scores were used: (i) x4r5, calculated using sequences of known co-receptor phenotype; and (ii) syncytium-inducing/
non-syncytium-inducing (s/i/nsi), calculated using sequences of known syncytium-inducing phenotypes in the MT2 cell line.

The sensitivity was calculated as the percentage of samples predicted as X4 of the total CXCR4 using samples in the GHOST cell assay. The specificity was calculated as the percentage of samples predicted as R5 of the total R5 samples in the GHOST cell assay.

Results

This study includes 102 HIV-1 primary isolates, 23 (22.5%) of subtype B and 79 (77.5%) of non-B genetic forms. The genetic classification is shown in Table 1. The V3 sequences were classified into six subtypes (A–G), although, when analysing along the genomes, 32 (31.4%) samples were CRFs (CRF02_AG, CRF05_DF, CRF12_BF, CRF14_BG, CRF20_BG, CRF24_BG and CRF47_BF) and 21 (20.6%) were diverse unique recombinant forms (URFs). The classification of co-receptor usage in GHOST cells is also indicated in Table 1. Sixty-nine (68%) isolates were R5 strains, 27 (26%) were R5X4 and 6 (6%), all of them CRF14_BG, were classified as X4 strains.

Overall, using as gold standard the phenotypic tropism observed in the GHOST cell infection assays, we obtained high levels of sensitivity and specificity for the detection of X4 isolates with Geno2pheno set at an FPR >1%. Both matrices of PSSM, V3 net charge and the 11/25 rule. The best results in terms of both sensitivity and specificity were obtained using PSSM s/i/nsi (94% sensitivity, 100% specificity), PSSM x4r5 (91% sensitivity, 100% specificity), Geno2pheno 2.5% FPR (91% sensitivity, 100% specificity) and the 11/25 rule (94% sensitivity, 98% specificity). Establishing the recommended cut-off for clinical settings of 10% FPR for Geno2pheno, we obtained 97% sensitivity for the detection of X4 or R5X4 strains and also a high specificity of 93% (Figure 1a). The sensitivity and specificity were also high (94% and 96%, respectively) when the 5.75% FPR recommended in the MERIT clinical trial was used. These values were 88% and 99%, respectively, for the 2% FPR used in the retrospective analyses of the MOTIVATE trial.

The comparative analysis of sensitivity for the detection of X4 strains among V3 sequences of different subtypes (Figure 1b–f) showed that the highest levels, between 91% and 100%, were detected using PSSM s/i/nsi, PSSM x4r5 and the 11/25 rule for sequences of subtypes A, B (from recombinant forms) and G, but not for subtype F, for which values between 50% and 75% were obtained.

A decrease in the specificity was detected when false positive results were obtained from the analysis of any of the 69 R5 isolates assayed. Two isolates of subtypes A and G, respectively, were predicted as being of X4 phenotype by Geno2pheno set at 5% FPR (97% specificity). At a setting of 10% FPR, we obtained 5 false positive results (93% specificity) with samples of subtypes A, B, F and G; at 15% FPR, 9 false positives (87% specificity) were obtained.

<table>
<thead>
<tr>
<th>Subtype V3</th>
<th>Genetic form</th>
<th>HIV-1 isolates</th>
<th>Co-receptor usage (GHOST cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>R5</td>
</tr>
<tr>
<td>A</td>
<td>A</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>CRF02_AG</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>URF_AG</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Total A</td>
<td>18</td>
<td>16 (89%)</td>
<td>2 (11%)</td>
</tr>
<tr>
<td>B</td>
<td>B</td>
<td>23</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>CRF14_BG</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>URF_BG</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>CRF47_BF</td>
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<td>2</td>
</tr>
<tr>
<td>Total B</td>
<td>46</td>
<td>23 (50%)</td>
<td>17 (37%)</td>
</tr>
<tr>
<td>C</td>
<td>C</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Total C</td>
<td>8</td>
<td>8 (100%)</td>
<td>0</td>
</tr>
<tr>
<td>D</td>
<td>URF_DFG</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>URF_ADG</td>
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<td>1</td>
</tr>
<tr>
<td>Total D</td>
<td>4</td>
<td>1 (25%)</td>
<td>3 (75%)</td>
</tr>
<tr>
<td>F</td>
<td>F1</td>
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<td>3</td>
</tr>
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<td></td>
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<td>2</td>
</tr>
<tr>
<td></td>
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<td>1</td>
</tr>
<tr>
<td></td>
<td>URF_BF</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>Total F</td>
<td>14</td>
<td>10 (71%)</td>
<td>4 (29%)</td>
</tr>
<tr>
<td>G</td>
<td>G</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>CRF20_BG</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>CRF24_BG</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Total G</td>
<td>12</td>
<td>11 (92%)</td>
<td>1 (8%)</td>
</tr>
<tr>
<td>Total</td>
<td>102</td>
<td>69 (68%)</td>
<td>27 (26%)</td>
</tr>
</tbody>
</table>
(a) Total primary isolates (N=102)
(b) Subtype B (N=23)
(c) BG recombinants (subtype B^{V3}) (N=21)
(d) Subtype A^{V3} (N=18)
(e) Subtype F^{V3} (N=14)
(f) Subtype G^{V3} (N=12)

% Sensitivity % Specificity
observed and at 20% FPR, 13 false positives (81% specificity) were detected. PSSM was 100% specific, predicting correctly the tropism of all 69 R5 isolates with any matrix. No R5 isolate had positively charged amino acids at positions 11 and/or 25, and the highest net charge of R5 isolates was +5, found in only three isolates of subtype A (one isolate) and B (two isolates).

A decrease in the sensitivity was detected when false negative results were obtained from the analysis of any of the 33 R5/X4 or X4 isolates assayed. Tropism prediction of all the six X4 strains was correct with any method used. False negative predictions were obtained in one isolate of subtype F with Geno2pheno 10% FPR. When FPR was decreased to 5%, two additional isolates (one subtype F and one subtype B) had false negative predictions, although PSSMsi/nsi correctly predicted the X4 phenotype of the subtype B sample. Seventeen out of 27 (63%) dual/mixed R5X4 samples resulted in false negative predictions of CXCR4 usage with Geno2pheno 1% FPR.

Analysing our data according to the V3 loop subtype, the main discrepancies were found in the lack of sensitivity of the genotypic methods for the detection of CXCR4-using variants of subtype F. Only two of the four R5X4 strains of subtype F were correctly detected.

Discussion

The purpose of this study was to compare the performance of different genotypic tropism prediction methods in different genetic forms of HIV-1. The reference phenotypic method for our comparisons consisted of the infection of GHOST cell lines expressing each co-receptor with HIV-1 primary isolates. HIV-1 variants obtained in cell culture could be driven by a selection bias and be different from the viruses circulating in plasma. Consequently, this technique is not suitable for routine clinical determination of co-receptor usage. Nevertheless, for our purpose, possible selection bias was not an obstacle, because we just compared the tropism predictions derived from the V3 sequence obtained from the primary isolate with the co-receptor usage shown by each isolate infecting GHOST co-receptor cell lines.

Genotypic assays are more practical alternatives to phenotypic assays for the study of CCR5 antagonists as a treatment option in clinical routine. Comparing genotypic assays for HIV-1 co-receptor use with a cell-culture phenotypic method, as in this study, could provide more reliable results of sensitivity and specificity for the detection of X4 strains than comparing them with recombinant assays. In this study, very high levels of sensitivity and specificity for the detection of X4 strains were obtained, them with levels ranging between 91% and 100% for sensitivity, except for Geno2pheno 1% FPR (which was 51%), and between 82% and 100% for specificity. These values are higher than those obtained in other previous comparative studies, in which the first-generation Trofile was considered as the gold standard. Overall, the best results in terms of both sensitivity and specificity to detect X4 variants were obtained using PSSMsi/nsi, PSSMx4r5, Geno2pheno 2.5% FPR and the 11/25 rule. It is to be noted that V3 net charge and 11/25 rules have performances similar to Geno2pheno and PSSM genotypic prediction tools. In this regard, comparative analysis of cell culture-based tropism studies and V3 sequence-based prediction considering the 11/25 and V3 net charge rules has been described for CRF02_AG and CRF02_AG-containing recombinants, and showed 93% comparable results. With regard to the Geno2pheno algorithm, it is important to determine the most advisable FPR for the detection of X4 isolates. A higher FPR enables the detection of more X4 viruses, but with less specificity. A recent evaluation of the genotypic prediction of HIV-1 co-receptor use versus a phenotypic assay performed in patients included in the Maraviroc Expanded Access Program suggests that a 10% FPR results in a good balance between sensitivity and specificity, and shows highest correlation with virological response to maraviroc treatment. Both PSSM matrices, x4r5 and si/nsi, are suitable for use in the prediction of viral tropism, having been shown to be similar in terms of sensitivity and specificity compared with Trofile.

V3-based genotypic algorithms could be less suitable for predicting the tropism of non-B viruses, because they were built using datasets of genotypy-phenotype correlations from subtype B viruses. Previous studies have shown lower values of sensitivity for identifying X4 variants for non-B subtypes using Geno2pheno 20% FPR, Geno2pheno 10% FPR and PSSMx4r5 compared with the phenotypic assay HIV-1 Phenoscript Env (ViralBinance, Paris, France), and also for CRF02_AG strains using both PSSM matrices (x4r5 and si/nsi). Recently, it has been described that the genotypic determinants of co-receptor usage for HIV-1 subtype C were mainly in V3 and were globally similar to those previously reported for subtype B viruses, and that the main genotypic algorithms built from subtype B viruses perform well when applied to subtype C viruses.

The present study includes 102 HIV-1 primary isolates, of which 23 are of subtype B and 79 are non-B genetic forms, including pure subtypes, CRFs and URFs. The comparative analysis of sensitivity and specificity for the detection of X4 strains among V3 sequences of different subtypes showed that the highest levels, between 91% and 100%, were obtained by using PSSMsi/nsi, PSSMx4r5 and the 11/25 rule for all subtypes A, B and G, but not for subtype F. However, there is a limitation in the number of primary isolates in our study for each genetic form and for each tropism, for which reason it is advisable to analyse additional primary isolates to confirm the current results. With regard to subtype F, it is important to note that infections with HIV-1 of this subtype have epidemiological relevance in South America, Romania and Spain. Eleven (22%) of 49 CRFs described to date are BF intersubtype recombinants and subtype F is present in the V3 regions of 9 CRFs (8 CRF_BFs and 1 CRF05_DF). Moreover, infections with subtype F of HIV-1 are currently actively transmitted in Spain. In the present study, only 4 out of 14 subtype F samples showed RSX4+ phenotype and the genotypic prediction tests failed in two of the four samples, as well. In this regard, a lack of correlation in genotypic
prediction for subtype F has been reported previously. Further analyses of viral determinants of co-receptor use outside of the V3 loop could contribute to the explanation of these discrepancies.

Co-receptor antagonists are currently in use for antiretroviral treatment of HIV-1-infected patients and it is necessary to identify strains on the basis of their co-receptor use, in order to provide the optimal therapeutic regimens including these drugs. Since the activity of CCR5 antagonists is limited to patients in whom only CCR5-tropic viruses are detected, viral tropism determination is required before prescription of maraviroc or other CCR5 antagonists. A genotypic clinical model for predicting HIV-1 tropism in treatment-experienced patients combining the interpretation of V3 loop sequences and clinical parameters has been defined recently.49

In conclusion, in the present study, co-receptor usage prediction by genotypic tools was in good agreement with GHOST cell culture-based phenotype determination, showing very high levels of sensitivity and specificity for the detection of X4 strains in subtype B and non-subtype B primary isolates, except for subtype F viruses. To our knowledge, this is the first study comparing the genotypic prediction of co-receptor usage with cell culture-based phenotype determination of HIV-1 primary isolates of different genetic forms. Understanding the co-receptor tropism of HIV-1 strains of B and non-B subtypes is essential to assess the potential usefulness of newer antiretroviral drugs among HIV-1-infected populations, for which it is still necessary to improve the information on the correlation between phenotypic and genotypic methods for viral tropism determination in patients infected with non-B genetic forms of HIV-1.

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Transparency declarations
None to declare.

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