JOURNAL OF CLINICAL MICROBIOLOGY, July 2011, p. 2697–2699 Vol. 49, No. 7
0095-1137/11/$12.00 doi:10.1128/JCM.00336-11


# Genotypic Prediction of Human Immunodeficiency Virus Type 1 Tropism by Use of Plasma and Peripheral Blood Mononuclear Cells in the Routine Clinical Laboratory▽

Christian Paar,[1] Maria Geit,[2] Herbert Stekel,[1] and Jörg Berg[1,3]*

*Institute of Laboratory Medicine[1] and Department of Dermatology,[2] General Hospital Linz, Krankenhausstrasse 9, A-4020 Linz, Austria, and Clinical Institute of Medical and Chemical Laboratory Diagnostics, Medical University Graz, Auenbruggerplatz 2-4, A-8036 Graz, Austria[3]*

Received 16 February 2011/Returned for modification 15 April 2011/Accepted 4 May 2011

**We developed a sequencing assay for genotypic HIV-1 tropism determination. The assay allows examination of HIV RNA from plasma and HIV DNA from peripheral blood mononuclear cells (PBMC), including PBMC samples from patients with undetectable viral loads. Assessment of 100 pairs of plasma and PBMC samples showed a high concordance of 90%. With the limitations of population-based sequencing, the assay was found to be robust and suitable for the routine clinical laboratory.**

HIV-1 enters a cell by use of CD4 molecules as primary receptors and either one of two chemokine receptors, CCR5 or CXCR4, as coreceptors (for a review, see reference 3). Depending on coreceptor usage, HIV-1 strains are classified as of R5, X4, or R5X4 (dual/mixed) tropism (1).

The CCR5 antagonist Maraviroc blocks HIV entry and has been introduced into clinical practice (5, 9). As Maraviroc exclusively blocks entry of R5-tropic HIV strains, testing of HIV tropism is required in each patient prior to therapy.

Phenotypic tropism assays, such as the Trofile test, are quite complex, labor-intensive, and require long turn-around times (2, 15, 20, 28). Genotypic tropism assays are less complex, offer rapid turn-around times, and with the increasing availability of nucleic acid sequencing facilities can be performed in routine clinical laboratories (for recent comments, see references 4, 6, 10, 13, and 19). For sequence interpretation, bioinformatics tools are freely available on the World Wide Web (7, 13, 14, 22). Recent genotypic tropism investigations of samples from Maraviroc treatment outcome studies suggested that genotyping may be applicable for HIV tropism determinations in the clinical setting (16, 21).

We describe the development of a genotypic HIV-1 tropism assay for the routine clinical diagnostic laboratory. The setup was chosen to allow testing of HIV RNA from plasma and HIV DNA from peripheral blood mononuclear cells (PBMC), to also allow assessments of samples with low or undetectable viral loads.

The study was performed within the frame of the Austrian HIV-1 cohort study, which was approved by the local ethics committee. Backup EDTA-blood samples ($n = 405$) from routine viral load examinations (COBAS TaqMan 48 HIV-1 test kit [Roche, Vienna, Austria]) were used. The AREVIR HIV-1 subtype panel (K. Korn, Institute of Virology, University of Erlangen, Erlangen, Germany), which contains 12 members of the group M (A to H), was used as a reference (17).

HIV RNA was prepared from 1 ml of plasma with the MagNA pure total nucleic acid isolation kit, large volume, on the MagNA Pure LC instrument (Roche Applied Science, Mannheim, Germany). RNA was eluted with 50 μl (12). PBMCs were prepared by using standard Lymphoprep density centrifugation in 12-ml Leucosep tubes according to the manufacturer's instructions (Greiner Bio-One, Kremsmünster, Austria). DNA from PBMC was isolated by using the Qiacube instrument with the QIAamp DNA blood mini-isolation kit (Qiagen, Hilden, Germany). DNA was eluted with 50 μl.

Sample RNA (10 μl) was treated for 5 min at 65°C, cooled to 50°C, and amplified by nested reverse transcription-PCR (RT-PCR) with the One-Step RT-PCR kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions in 50 μl with 0.2 μM each of our manually defined primers V3-forward (5′-GTCAGCACAGTACAATGYACACATGG-3′; GenBank accession number K03455; nucleotides [nt] 6948 to 6973) and V3-reverse (5′-AGTGCTTCCTGCTGCTCCYAA GAACCC-3′; GenBank accession number K03455; nt 7785 to 7811), 400 μM deoxynucleoside triphosphates (dNTPs) each, 10 U RNase Out (Invitrogen, Karlsruhe, Germany). Buffer, Q solution, and the RT-PCR enzyme mixture were added according to the manufacturer's instructions. RT-PCR was run on a Biometra thermocycler T*personal* (Biometra, Göttingen, Germany) under the following cycling conditions: 30 min at 50°C, 15 min at 95°C, and then five touch-down cycles (−1°C annealing temperature/cycle), starting with 30 s at 95°C, 30 s at 62°C, and 90 s at 72°C. Then, 35 cycles with 30 s at 95°C, 30 s at 57°C, and 90 s at 72°C were added. Final extension was 10 min at 72°C.

HIV DNA was amplified by nested primer PCR (50-μl volume). The first PCR was performed with GoTaq Flexi polymerase (Promega, Mannheim, Germany) and with the same primers and concentrations as for the RT-PCR. PCR buffer, dNTPs, and $MgCl_2$ were added according to the manufacturer's recommendations. The temperature program was as follows: 2 min at 95°C, five touch-down cycles (−1°C/cycle) with

* Corresponding author. Mailing address: Institute of Laboratory Medicine, General Hospital Linz, Krankenhausstrasse 9, A-4020 Linz, Austria. Phone: 43-732-7806-73845. Fax: 43-732-7806-741174. E-mail: joerg.berg@akh.linz.at.

▽ Published ahead of print on 18 May 2011.

TABLE 1. Tropism determinations of the AREVIR HIV-1 subtype panel members by V3 loop sequencing and g2p sequence interpretation

| Panel member[a] | Reference tropism[b] | FRP score (%) | Predicted tropism[c] |
|---|---|---|---|
| A | SI | 0.5 | R5X4/X4 |
| B | NSI | 86.1 | R5 |
| C | NSI | 38.4 | R5 |
| D1 | SI | 0.0 | R5X4/X4 |
| D2 | NSI | 8.5 | Ambiguous |
| D3 | SI | 0.1 | R5X4/X4 |
| E1 | NSI | 13.0 | Ambiguous |
| E2 | NSI | 33.0 | R5 |
| F1 | NSI | 44.5 | R5 |
| F2 | SI | 0.7 | R5X4/X4 |
| G | NSI | 39.7 | R5 |
| H | NA | 8.5 | Ambiguous |

[a] The letter of the panel member name is equivalent to the respective HIV-1 subtype (K. Korn, University of Erlangen, personal communiation).
[b] The AREVIR reference tropism as determined by phenotypic assay. SI, syncytium inducing; NSI, non-syncytium inducing; NA, data not available.
[c] Clinical interpretation according to the g2p prediction following the "German recommendations." R5X4/X4, ≤5%; ambiguous, 5 to 20%; R5, >20%.

30 s at 95°C, 45 s at 67°C, and 90 s at 72°C, followed by 35 cycles with 30 s at 95°C, 45 s at 62°C, and 90 s at 72°C. Final extension was 5 min at 72°C. The second nested PCR (50-μl volume) was performed with GoTaq Flexi DNA polymerase (Promega, Mannheim, Germany) using our manually defined primers V3-nested-for (5′-TGTTAAATGGCAGYCT AGCAG-3′; GenBank accession number K03455; nt 7003 to 7023) and V3-nested-rev (5′-TGGGAGGGGCATACATT G-3′; GenBank accession number K03455; nt 7522 to 7539) (0.2 μM each). The temperature program consisted of three touch-down cycles (−1°C/cycle) starting with 30 s at 95°C, 30 s at 62°C, and 60 s at 72°C, succeeded by 27 cycles of 30 s at 95°C, 30 s at 57°C, 60 s at 72°C, and 5 min at 72°C.

PCR products were purified with ExoSAP reagent and sequenced with the BigDye Terminator v3.1 kit (Applied Biosystems, Darmstadt, Germany). Sequence analysis was performed on the ABI 3130 instrument with the SeqScape v2.6 analysis software (Applied Biosystems). Coreceptor tropism was determined by use of the online bioinformatics tool geno2pheno$_{[coreceptor]}$ (g2p; http://coreceptor.bioinf.mpi-inf.mpg.de/index.php) (14, 25). Submitted sequences returned a score termed the false-positive rate (FPR, a percentage), which was defined as the probability of incorrectly classifying an R5 strain as X4, i.e., the greater the value of the FPR, the greater the probability of R5 tropism. Samples were analyzed according to the clinical interpretation scheme "German recommendations," which, according to the latest g2p consortium resolution, classifies X4 tropism at an FPR of ≤5%, ambiguous tropism with FPRs between 5% and 20%, and R5 tropism at an FPR of >20% (R. Kaiser, Institute of Virology, University of Cologne, Germany, personal communication, 1 December 2010).

Examination of the AREVIR HIV-1 subtype panel led to clear sequences of each panel member. With the clinical interpretation scheme, exactly matched tropisms were obtained in 9 of the 11 members with available phenotype data (Table 1). Two members (D2 and E1) of the non-syncytium-inducing phenotype were classified as ambiguous. Panel member H, for which phenotype data were not available, was also classified as ambiguous. With the FPR cutoff at 5% according to the findings of McGovern et al. (16), matching tropisms were observed in all 11 members with available phenotypic tropism data, and panel member H was classified as R5 (Table 1).

TABLE 2. Tropism determination of paired plasma HIV RNA and PBMC HIV DNA samples[a]

| PBMC HIV DNA tropism | No. of plasma samples with HIV RNA tropism of: | | |
|---|---|---|---|
| | R5 | Ambiguous | R5X4/X4 |
| R5 | 53 | 3 | 1 |
| Ambiguous | 2 | 24 | 0 |
| R5X4/X4 | 2 | 2 | 13 |

[a] R5X4/X4, ≤5%; ambiguous, 5 to 20%; R5, >20%.

Accuracy of the sequencing process was assessed with 8 identical subtype B samples at about 1,000 copies/ml, which were tested in 8 separate runs. Identical results were obtained in 6 runs. In one run one wobble nucleotide and in a second run three wobble nucleotides, such as M and R, were recorded. The wobble positions did not affect the tropism determination. The minimum viral load to allow consistent sequencing of HIV RNA was 500 copies/ml.

The assay was evaluated by examining 145 plasma and 260 PBMC samples. Those samples covered HIV-1 group M subtypes A to G, including CRF01 and -02. With 150 of the 260 PBMC samples, viral loads were undetectable. Of the plasma samples, 85 (59%), 35 (24%), and 25 (17%) were R5, ambiguous, or R5X4/X4, respectively, and from the PBMC samples 144 (56%), 61 (21%), and 55 (23%) were obtained as R5, ambiguous, and R5X4/X4, respectively. Among the PBMC samples with undetectable viral loads, 88 (55%), 30 (19%), and 42 (26%) were found to be R5, ambiguous, and R5X4/X4, respectively. In order to show specificity, 5 PBMC samples from healthy individuals were examined, and these did not return any sequencing result. Participation in the first European coreceptor proficiency panel test showed 100% agreement with the respected syncytium-forming or non-syncytium-forming phenotype of the proficiency test samples (data not shown) (11).

The established sequencing assay yielded consistent tropism determinations from HIV RNA and HIV DNA for a wide spectrum of HIV group M subtypes, exhibited reliable sequence reproducibility, and, for plasma testing, resulted in a viral load limit comparable to those for sequencing assays for the detection of HIV drug resistance mutations (6, 8, 17). In addition, PBMC samples from patients with undetectable viral loads were also able to be examined and yielded a slightly higher percentage of R5X4/X4 tropisms than plasma samples or PBMC samples with detectable viral loads.

To specifically compare HIV RNA with HIV DNA testing, we examined 100 pairs of plasma and PBMC samples obtained from identical collection tubes. Matching R5, ambiguous, and R5X4/X4 tropisms were obtained for 90 pairs, resulting in 90% concordance (Table 2). Two discrepant sample pairs showed FPR scores just above and beneath the cutoff at 5%. One sample pair returned differing HIV subtypes, possibly due to coinfection. By analyzing the sample pairs with an FPR cutoff

of 5%, according to the findings of McGovern et al., 95% concordance was obtained (16). Our observations are in agreement with three studies on 38, 34, and 100 patients, which also showed high concordances (82% to 90%) between plasma and PBMC samples (18, 23, 24). The observed high concordances between HIV RNA and HIV DNA assessments may indicate that HIV DNA-derived tropisms also by and large reflect the major HIV tropism within a patient. HIV DNA tropism testing in patients with undetectable viral loads may offer a further treatment option, may assist in the simplification of treatment regimens, and may contribute to fewer side effects (24, 26).

As our sequencing is population based, minority R5X4/X4 variants are usually not detected, even at low viral loads, unless they rise to levels above 10% to 20%. The first studies with massive parallel sequencing technology for tropism testing showed promising detection levels for minorities (27). Apart from this, population-based genotypic tropism testing was found to be equally successful as phenotypic tropism testing in predicting virological response to Maraviroc (16, 21).

The g2p coreceptor tool has been used in a majority of studies on genotypic HIV tropism predictions, including reanalysis of samples from Maraviroc treatment outcome studies (10, 16). g2p was shown to yield comparable tropism determination results as phenotypic assays (7, 16, 19, 21–23). Of note, sensitivities and specificities of the Trofile test were found to be quite similar as for the genotypic test at an FPR cutoff of 5% (16). However, for clinical applications, the g2p consortium implemented the interpretation scheme "German recommendations" to address patient safety issues (R. Kaiser, personal communication). Interpretation schemes and cutoff values might need adjustment, e.g., when data from prospective studies emerge or recommendations change. As the g2p algorithm returns FPR values, such adjustments can conveniently be implemented.

In conclusion, we developed a population-based sequencing assay for genotypic HIV-1 tropism determinations in conjunction with the g2p interpretation tool. The assay allows examination of plasma and PBMC samples, including PBMC samples from patients with undetectable viral loads, and covers the spectrum of HIV-1 group M subtypes. Testing of 100 pairs of plasma and PBMC samples from identical collection tubes showed high concordance of HIV RNA and HIV DNA tropisms. With the limitations of population-based sequencing, the assay proved robust and was found suitable for the routine clinical laboratory.

We are grateful to Klaus Korn, Institute of Virology, University of Erlangen, Erlangen, Germany, for his generous gift of the AREVIR HIV-1 subtype panel. We warmly thank Rolf Kaiser, Institute of Virology, University of Cologne, Cologne, Germany, for his helpful discussions. The expert technical assistance of Roland Mayr, Christina Palmetshofer, and Brigitte Schartner is gratefully acknowledged.

## REFERENCES

1. **Berger, E. A., et al.** 1998. A new classification for HIV-1. Nature **391:**240.
2. **Coakley, E., et al.** 2009. Comparison of human immunodeficiency virus type 1 tropism profiles in clinical samples by the Trofile and MT-2 assays. Antimicrob. Agents Chemother. **53:**4686–4693.
3. **Corbeau, P., and J. Reynes.** 2009. CCR5 antagonism in HIV infection: ways, effects, and side effects. AIDS **23:**1931–1943.
4. **Delobel, P., et al.** 2007. Population-based sequencing of the V3 region or *env* for predicting the coreceptor usage of human immunodeficiency virus type 1 quasispecies. J. Clin. Microbiol. **45:**1572–1580.
5. **Dorr, P., et al.** 2005. Maraviroc (UK-427,857), a potent, orally bioavailable, and selective small-molecule inhibitor of chemokine receptor CCR5 with broad-spectrum anti-human immunodeficiency virus type 1 activity. Antimicrob. Agents Chemother. **49:**4721–4732.
6. **Erali, M., S. Page, L. G. Reimer, and D. R. Hillyard.** 2001. Human immunodeficiency virus type 1 drug resistance testing: a comparison of three sequence-based methods. J. Clin. Microbiol. **39:**2157–2165.
7. **Garrido, C., et al.** 2008. Evaluation of eight different bioinformatics tools to predict vital tropism in different human immunodeficiency virus type 1 subtypes. J. Clin. Microbiol. **46:**887–891.
8. **Grant, R. M., et al.** 2003. Accuracy of the TRUGENE HIV-1 genotyping kit. J. Clin. Microbiol. **41:**1586–1593.
9. **Gulick, R. M., et al.** 2008. Maraviroc for previously treated patients with R5 HIV-1 infection. N. Engl. J. Med. **359:**1429–1441.
10. **Harrigan, P. R., and A. M. Gerretti.** 2011. Genotypic tropism testing: evidence-based or leap of faith? AIDS **25:**257–264.
11. **Heger, E., et al.** 2011. European coreceptor proficiency panel test. Rev. Antivir. Ther. Infect. Dis. **2:**25.
12. **Hölzl, G., M. Stöcher, V. Leb, H. Stekel, and J. Berg.** 2003. Entirely automated quantification of human immunodeficiency virus type 1 (HIV-1) RNA in plasma by using the ultrasensitive COBAS AMPLICOR HIV-1 monitor test and RNA purification on the MagNA pure LC instrument. J. Clin. Microbiol. **41:**1248–1251.
13. **Jensen, M. A., et al.** 2003. Improved coreceptor usage prediction and genotypic monitoring of R5-to-X4 transition by motif analysis of human immunodeficiency virus type 1 *env* V3 loop sequences. J. Virol. **77:**13376–13388.
14. **Lengauer, T., O. Sander, S. Sierra, A. Thielen, and R. Kaiser.** 2007. Bioinformatics prediction of HIV coreceptor usage. Nature Biotechnol. **25:**1407–1410.
15. **Low, A. J., L. C. Swenson, and P. R. Harrigan.** 2008. HIV coreceptor phenotyping in the clinical setting. AIDS Rev. **10:**143–151.
16. **McGovern, R. A., et al.** 2010. Population based V3 genotypic tropism assay: a retrospective analysis using screening samples from the A4001029 and MOTIVATE studies. AIDS **24:**2517–2525.
17. **Paar, C., et al.** 2008. Genotypic antiretroviral resistance testing for human immunodeficiency virus type 1 integrase inhibitors by use of the TruGene sequencing system. J. Clin. Microbiol. **46:**4087–4090.
18. **Parisi, S. G., et al.** 2011. A study of HIV coreceptor tropism in paired plasma PBMC and cerebrospinal fluid isolates from antiretroviral naive subjects. J. Clin. Microbiol. **49:**1441–1445.
19. **Poveda, E., et al.** 2009. Design and validation of new genotypic tools for easy and reliable estimation of HIV tropism before using CCR5 antagonists. J. Antimicrob. Chemother. **63:**1006–1010.
20. **Raymond, S., et al.** 2010. Development and performance of a new recombinant virus phenotypic entry assay to determine HIV-1 coreceptor usage. J. Clin. Virol. **47:**126–130.
21. **Recordon-Pinson, P., et al.** 2010. Evaluation of the genotypic prediction of HIV-1 coreceptor use versus phenotypic assay and correlation with virological response to Maraviroc: the ANRS GenoTropism study. Antimicrob. Agents Chemother. **54:**3335–3340.
22. **Sánchez, V., et al.** 2010. Performance of genotypic algorithms for predicting HIV-1 tropism measured against the enhanced-sensitivity Trofile coreceptor tropism assay. J. Clin. Microbiol. **48:**4235–4239.
23. **Saracino, A., et al.** 2007. HIV-1 biological phenotype and predicted corecpetor usage based on V3 loop sequence in paired PBMC and plasma samples. Virus Res. **130:**34–42.
24. **Seclén, E., M. del Mar González, C. De Mendoza, V. Soriano, and E. Poveda.** 2010. Dynamics of HIV tropism under suppressive antiretroviral therapy: implications for tropism testing in subjects with undetectable viraemia. J. Antimicrob. Chemother. **65:**1493–1496.
25. **Sing, T., et al.** 2007. Predicting HIV coreceptor usage on the basis of genetic and clinical covariates. Antivir. Ther. **12:**1097–1106.
26. **Soulié, C., et al.** 2010. Factors associated with proviral DNA HIV-1 tropism in antiretroviral therapy-treated patients with fully suppressed plasma HIV viral load: implications for the clinical use of CCR5 antagonists. J. Antimicrob. Chemother. **65:**749–751.
27. **Swenson, L. C., et al.** 2011. Deep sequencing to infer HIV-1 co-receptor usage: application to three clinical trials of Maraviroc in treatment-experienced patients. J. Infect. Dis. **203:**237–245.
28. **Whitcomb, J. M., et al.** 2007. Development and characterization of a novel single-cycle recombinant virus assay to determine human immunodeficiency virus type 1 coreceptor tropism. Antimicrob. Agents Chemother. **51:**566–575.