Comparison of the LiPA HIV-1 RT test, selective PCR and direct solid phase sequencing for the detection of HIV-1 drug resistance mutations

Jean-Claude Schmit a,*, Lidia Ruiz b, Lieven Stuyver c, Kristel Van Laethem a, Ilse Vanderlinden a, Theresa Puig b, Rudi Rossau c, Jan Desmyter a, Erik De Clercq a, Bonaventura Clotet b, Anne-Mieke Vandamme a

a Rega Institute for Medical Research, Katholieke Universiteit Leuven, Leuven, Belgium
b Foundation IRSI-Caixa and HIV Unit, Hospital Universitari 'Germans Trias i Pujol', Badalona, Spain
c Innogenetics, Ghent, Belgium

Received 5 November 1997; received in revised form 20 February 1998; accepted 20 February 1998

Abstract

The performance to detect drug resistance mutations in the reverse transcriptase gene of HIV-1 was compared for direct solid phase sequencing, selective polymerase chain reaction (PCR) using the amplification refractory mutation system (ARMS) and the new line probe assay (LIPA) HIV-1 RT™. The three tests were undertaken on 50 plasma samples from 25 treatment-experienced patients under combination therapy with dideoxynucleoside analogues. LiPA HIV-1 RT gave interpretable results in 80 to 96% of the samples depending on the codon of interest. In 2% of the samples a failure to amplify resulted in uninterpretable results for sequencing. ARMS gave no result in seven samples (14%). Overall, there was a 73 to 100% concordance between the three methods. In this study, LiPA HIV-1 RT proved to be an accurate and reliable alternative to DNA sequencing for the detection of drug resistance mutations in patient samples. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: HIV drug resistance; DNA sequencing; LiPA HIV-1 RT; Selective PCR

1. Introduction

HIV drug resistance is likely to remain an important issue for the management of patients, despite encouraging preliminary results with three-drug combination therapies (Caesar Coordinating...
Committee, 1997; Hirsch et al., 1997), showing suppression of virus replication for prolonged time periods and delay of the emergence of drug-resistant virus strains. Drug resistance mutations for all food and drug administration (FDA)-approved reverse transcriptase (RT) and protease inhibitors have been described and their relationship with phenotypic drug resistance is now at least partially understood (Schinazi et al., 1996).

In order to optimise individual treatment regimens, information on drug resistance from patient-derived virus is required. To date, several assays for drug resistance have been developed. Assays based on phenotypic drug testing (i.e. growth of the patient virus strain in the presence of various concentrations of the drug to be tested) are expensive, time-consuming and have a limited intra- and inter-assay reproducibility (Japour et al., 1993; Kellam and Larder, 1994; Larder et al., 1996). Enzymatic assays, based on in vitro inhibition of the RT enzyme, have been introduced recently (Tan et al., 1991), but they lack sensitivity, and they fail for some drugs (Larder and Kemp, 1989). Genotypic assays (i.e. detection of drug resistance mutations) are of interest because they give fast results and avoid hazardous work with infectious material. DNA sequencing, the reference procedure for genotyping, is expensive and demanding and thus unsuitable for routine use. Simpler procedures for patient monitoring are needed urgently. Promising results have been obtained for some mutations with the so-called selective polymerase chain reaction (PCR) or amplification refractory mutation system (ARMS) (Larder and Boucher, 1993). Unfortunately, this technique is not standardised and it rapidly becomes labour-intensive when multiple codons are to be examined. Another test based on PCR amplification is the microtitre format point mutation assay (Kaye et al., 1992). It allows accurate quantification of mutant and wild-type virus in cells, plasma and other body fluids and can be applied to a wide range of point mutations.

The new line probe assay (LiPA) HIV-1 RT™ (Murex/Innogenetics, Dartford, UK) (Stuyver et al., 1997), based on the principle of reverse DNA hybridisation, enables the assessment of a large range of resistance-related mutations at reasonable cost in a short period of time. In order to evaluate its performance, LiPA HIV-1 RT was compared with direct solid phase sequencing of PCR products and to selective PCR for the zidovudine resistance mutation T215Y/F in the RT gene.

2. Materials and methods

Blood sample collection and nucleic acid extraction. Blood samples on EDTA were obtained at the HIV Unit, University Hospital ‘Germans Trias I Pujol’, Badalona, Spain, from 25 patients who had received a combination therapy with zidovudine (ZDV) and didanosine (ddI) or ZDV and zalcitabine (ddC) for at least 6 months followed by a combination treatment with lamivudine (3TC) (Ruiz et al., 1996; Schmit et al., 1998). Plasma samples before (week 0) and after addition of 3TC (week 24) were stored at $-80^\circ$C. Viral RNA was extracted using the QIAamp Viral RNA kit (Qiagen, Hilden, Germany) following the Manufacturer’s instructions.

Selective PCR for RT codon 215 (ARMS 215). Selective PCR for codon 215 was performed as described previously (Larder and Boucher, 1993) with the following modifications. Briefly, cDNA was synthesised using specific priming (primer NE-1(35) with the RNA core kit (Perkin Elmer, Brussels, Belgium)) (Vandamme et al., 1995). A total of 10 μl of cDNA were used in an outer PCR with primers A35 and NE-1(35) to generate a DNA fragment of 810 bp under the following conditions: MgCl$_2$ 2.5 mM, Perkin Elmer 9600 thermocycler, 94°C for 30 s, 55°C for 30 s, 72°C for 1 min, 25 cycles. Two nested PCR were performed with either primer B and primer 215 W (specific for the wild-type sequence ACC at codon 215) or primer B and primer 215 M (for the mutant sequence TTC or TAC at codon 215) under the following conditions: hotstart using Mg$^{2+}$-containing waxbeads for a final reaction concentration of 1.5 mM (Invitrogen, The Netherlands), Biometra Trithermobloc (Westburg, Leusden, The Netherlands), initial denaturation 94°C for 1 min, then 94°C for 30 s, 45°C for 30 s, 72°C for 1 min, 25 cycles. A total of 8 μl of the
amplification product were visualised on PAGE after EtBr staining. Results were scored as wild-type or mutant depending on the presence of amplification product in the wild-type or mutant reaction. Detection limit and selectivity of the assay were evaluated (Vandamme et al., 1998) on a dilution series of wild-type HIV-IIIb (ACC at codon 215) and mutant HIV RTMF (TAC at codon 215) (Larder et al., 1991).

**Direct solid phase sequencing of nucleic acids.** cDNA synthesis, amplification and sequencing of the RT gene on a semi-automated sequencer (ALF, Pharmacia, Uppsala, Sweden) was done as described previously (Schmit et al., 1996) using a T7 polymerase (Sequenase, Pharmacia) reaction with fluorescent-labelled primers.

**LiPA HIV-1 RT.** LiPA HIV-1 RT is based on the reverse hybridisation principle. Biotinylated HIV-1 RT PCR product is hybridised with specific oligonucleotide probes for wild-type or mutant sequences at RT codons 41, 69, 70, 74, 184, 214 and 215. Streptavidin labelled with alkaline phosphatase is added and binds to any biotinylated hybrid previously formed. Incubation with bromochloroindolyphosphate (BCIP)–nitroblue tetrazolium (NBT) chromogen results in a purple–brown precipitate. The presence of a clearly visible line is considered a positive reaction. For the purpose of this study a pre-commercial evaluation kit, provided by Innogenetics, Ghent, Belgium was used. The procedure was as recommended (Stuyver et al., 1997) with small modifications in the DNA amplification step: 2 μl product of the outer PCR reaction for ARMS (see above) was used to do a nested PCR with the primers enclosed in the kit (MgCl₂ 1.5 mM, cycler conditions: Biometra Triothermobloc (Westburg), 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min). A total of 8 μl of amplification product were run on a 6% PAGE, and the presence of a 600 bp band was checked after EtBr staining.

**Analysis of data:** For each codon of interest, results were scored with the three methods as wild-type, mutant, a mixture of both, or absence of interpretable results. Concordance was defined as obtaining the same interpretable result for a codon found with the different methods.

### 3. Results

**Amplification failures.** Only one sample (2%) gave no PCR product for DNA sequencing. Selective PCR for codon 215 resulted in seven samples with no signal (14%). Finally, for LiPA HIV-1 RT one sample did not amplify (2%).

**DNA sequencing.** Sequencing reactions were always interpretable for the region of interest (RT codons 1–259) once amplification product had been obtained. The frequency of the different drug resistance mutations is summarised in Table 1. Full sequence data is available under Genbank–EMBL accession numbers (Z99298 to Z99338). All sequenced virus strains belonged to clade B (Felsenstein, 1989). Interestingly, two
virus strains presented the Q151M mutation associated with resistance to multiple dideoxynucleoside analogues (Schmit et al., 1996).

Selective PCR. Selective PCR was done only for codon 215. Twelve samples scored as wild-type, 26 as mutant and five as a mixture of wild-type and mutant. For seven samples no result was obtained (i.e. amplification failures).

LiPA HIV-1 RT. LiPA tests were not interpretable for 18% of the RT codons. Out of the 49 samples that could be amplified, 39 samples (80%) were interpretable for codon 41, 41 samples (84%) for codons 70, 39 samples (80%) for codon 74, 47 samples (96%) for codon 184 and 39 samples (80%) for codon 215. In the tests undertaken, all codons on a same strip were interpretable for 28 tests out of 49 (57%). The results obtained with LiPA HIV-1 RT are summarised in Table 1.

**Comparison of the genotyping methods.** Overall, there was a degree of concordance ranging from 73.7 to 100% between the DNA sequencing and LiPA, depending on the codon of interest (Table 2). LiPA HIV-1 RT found more often mixtures of wild-type and mutant, especially for codons 41 and 70. No contradictory results were found (i.e. wild-type in one method and mutant in the second or vice versa). Selective PCR for codon 215 gave a 83.3% concordance with sequencing. When comparing selective PCR to LiPA for codon 215, again a high concordance level (94.2%) was found.

### Table 2
Comparison of genotyping results obtained with the LiPA HIV-1 RT test, selective PCR for codon 215 (ARMS 215) and DNA sequencing

<table>
<thead>
<tr>
<th>Method/Codon</th>
<th>Samples with sequencing and LiPA HIV-1 RT or ARMS results*</th>
<th>Concordant results compared to sequencingb</th>
<th>Discordant results compared to sequencingc</th>
</tr>
</thead>
<tbody>
<tr>
<td>LiPA 41</td>
<td>38</td>
<td>28 (73.7%)</td>
<td>10 (26.3%)</td>
</tr>
<tr>
<td>LiPA 70</td>
<td>40</td>
<td>33 (82.5%)</td>
<td>7 (17.5%)</td>
</tr>
<tr>
<td>LiPA 74</td>
<td>38</td>
<td>38 (100%)</td>
<td>0</td>
</tr>
<tr>
<td>LiPA 184</td>
<td>46</td>
<td>45 (97.8%)</td>
<td>1 (2.5%)</td>
</tr>
<tr>
<td>LiPA 215</td>
<td>38</td>
<td>32 (84.2%)</td>
<td>6 (15.8%)</td>
</tr>
<tr>
<td>ARMS 215</td>
<td>42</td>
<td>35 (83.3%)</td>
<td>7 (16.7%)</td>
</tr>
</tbody>
</table>

NOTE. a Samples for which both methods give interpretable results; b Same results for both methods (LiPA or ARMS compared to sequencing); c Discordant results were in all cases due the detection of mixtures of wild-type and mutant sequences in LiPA, whereas only one of both could be detected by sequencing.

4. Discussion

In the present study, LiPA HIV-1 RT proved to give reliable results when compared to DNA sequencing for the most important resistance codons in the RT gene. Sequencing failed to provide results in 2% of the cases, overall LiPA failed to provide results in 18% of the codons. Failures were exceptionally due to the absence of amplification product in the PCR reaction (2%), but could be explained in 50–71% of the samples by a sequence divergence for specific codon regions between the LiPA probes (Stuyver et al., 1997) and the HIV-1 strain. In fact, all virus strains belonged to clade B, but minor sequence variations (i.e. single point mutations) were often found next to the codons of interest. Due to the test principle of specific nucleotide sequence hybridisation under stringent conditions, LiPA HIV-1 RT is particularly sensitive to sequence polymorphism. Thus, inclusion into the test of additional DNA probes reflecting HIV-1 variability around the codons of interest are expected to improve the performance of the test. Moreover, in order to define the possible routine clinical applications of LiPA HIV-1 RT, studies with larger numbers of samples are still needed.

Standard genotyping methods like DNA sequencing and selective PCR produced failures, too. This was always due to the absence of PCR amplification product. This result could possibly be improved by using primer sets selected in more conserved regions of the HIV genome.
Overall there was a good concordance between the results generated by the three methods. Only a few discordances were found when comparing LiPA to sequencing or selective PCR. They were especially frequent for codon 41 (26.3%) and were uncommon for codons 74 (0%) and 184 (2.5%). Discordant results can be partially explained by the fact that LiPA detected more often mixtures of strains than did sequencing. It is likely that these represent true mixtures of viral strains and not non-specific reactions of the test. We have shown previously that in our hands, sequencing is not able to identify a mixture when less than 25% of mutant or wild-type template is present (Vandamme et al., 1998). LiPA HIV-1 RT has been demonstrated to detect mixtures at concentrations as low as 2–4% (Stuyver et al., 1997). In the same way, due to a lack of specificity of the selective PCR for codon 215, a dilution procedure of the outer PCR product with a repetition of the selective PCR is performed to ensure that a strain scored as a mixture is a true mixture (Vandamme et al., 1998). The consequence of using ten-fold dilution steps is that a mixture of less than 10% wild-type or mutant is diluted out until it is resolved as mutant or wild-type, respectively. However, further improvements in PCR using a hotstart technique may help to score mixtures more accurately (Vandamme et al., 1998).

In conclusion, LiPA HIV-1 RT proved to be a rapid and simple alternative to DNA sequencing and provides more complete information on drug resistance than a simple selective PCR. The higher rate of detection of mixtures by LiPA HIV-1 RT is consistent with previously reported higher sensitivity of LiPA HIV-1 RT compared to sequencing. Clinically it could be important to detect the emergence of genotypic resistance earlier during therapy and to detect pre-existing genotypic resistance which sequencing may miss. The performance of the LiPA test is likely to be improved by the inclusion of additional DNA probes reflecting the high variability of the HIV-1 gene. Based on the current evolution in clinical practice and the tendency to prescribe combination therapy including at least one HIV protease inhibitor or one non-nucleoside RT inhibitor (NNRTI) in association with two nucleoside analogues, an updated version of the test should also include the most important HIV protease inhibitor and NNRTI resistance mutations. This new version should also include additional mutations as for instance the multi-ddN resistance mutation Q151M.

Acknowledgements

We are grateful to Dr H. Holmes, AIDS Reagent Project, Medical Research Council, UK, for providing virus strain RTMF, contributed by Drs B. Larder and S. Kemp, GlaxoWellcome, UK. This work was supported partially by the Biomedical Research Programme of the European Union (EU Biomed 2 grant BMH4-CT-95-1634), the Belgian Geconcerteerde Onderzoeksacties (project GOA 95/5) and the Belgian Nationaal Fonds voor Wetenschappelijk Onderzoek (NFWO grant G.3304.96). J.C. Schmit acknowledges a grant from the European Union Biomed 1 Programme (grant BMH-CT-94-5599).

References


Larder, B.A., Kohli, A., Bloor, S., et al., 1996. Human immunodeficiency virus type 1 drug susceptibility during zidovudine (AZT) monotherapy compared with AZT plus 2',3'-dideoxyinosine or AZT plus dideoxycytidine combination therapy. J. Virol. 70, 5922–5929.


