

Research article

## Analysis of HIV-1 drug resistant mutations by line probe assay and direct sequencing in a cohort of therapy naive HIV-1 infected Italian patients

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### Abstract

**Background:** The routine determination of drug resistance in newly HIV-1 infected individuals documents a potential increase in the transmission of drug-resistant variants. Plasma samples from twenty seven therapy naive HIV-1 infected Italian patients were analyzed by the line probe assay (LIPA) and the TruGene HIV-1 assay for the detection of mutations conferring resistance to HIV-1.

**Results:** Both tests disclosed amino-acid substitutions associated with resistance in a variable number of patients. In particular, two mutations (K70R and V118I), detectable by LIPA and by sequencing analysis respectively, revealed resistance to NRTIs in two plasma samples. At least three mutations conferring resistance to NNRTIs, not detectable by commercial LIPA, able to reveal mutations associated only with nucleoside reverse transcriptase analogues, were disclosed by viral sequence analysis. Moreover, most samples showed mutations correlated with resistance to protease inhibitors. Remarkably, a key mutation, like V82A (found as a mixture), and some "indeterminate" results (9 samples), due the absence of signal on the lines corresponding to a specific probe, was revealed only by LIPA, while a variable number of secondary mutations was detectable only by TruGene HIV-1 assay.

**Conclusion:** Even if further studies are necessary to establish the impact of different tests on the evaluation of drug-resistant strains transmission, LIPA might be useful in a wide population analysis, where bulk results are needed in a short time, while sequencing analysis, able to detect mutations conferring resistance to both NRTIs and NNRTIs, might be considered a more complete assay, albeit more expensive and more technically complex.

### Background

The widespread use of antiretroviral drugs to treat human immunodeficiency virus (HIV-1) infection might re-

sult in an increasing transmission of drug-resistant virus, compromising therapeutic options in newly infected individuals. Since some evidence indicates that viral

resistance and treatment failure are closely linked [1,2], routine determination of drug resistance in drug naive HIV-1 infected individuals might be important to disclose single viral mutations able to influence the initial approach to therapy [3-7]

Several cross-sectional surveys to detect primary infection involving drug resistant virus have shown a variable prevalence (ranging from 0 to 10%) of primary mutations [8,9] conferring resistance to zidovudine, lamivudine or nevirapine resistant virus and protease inhibitors [10-14].

Epidemiological surveys to clarify the prevalence of resistance to HIV-1 isolates from newly infected patients is recommended in the design of initial antiretroviral regimens [11] even if plasma HIV RNA level, CD4 cell count and clinical status, [15-17] also offer important information for therapy initiation.

Several manufacturers have developed genotypic drug resistance assays able to identify the individual combinations of nucleotide substitutions, known to confer resistance to specific antiretroviral agents, and thereby help recognize drugs less likely to be effective. Different methodologies are now available for testing HIV susceptibility to antiretroviral drugs and are able to detect drug-resistance associated with the viral enzyme reverse transcriptase (RT) and protease.

The success of drug therapy depends on the ability to suppress viral replication quickly to preserve the HIV specific CD4+ cell helper response. Complete HIV-1 suppression could be compromised in naive patients who already harbor virus with mutations conferring resistance to antiretroviral drugs, diminishing clinical benefit and fostering broader drug resistance [1,3]. However, determining the prevalence of drug resistant virus in patients with established HIV infection before starting therapy might have important positive implications. Several studies concerning HIV naive subjects have been performed [3-7] even if the choice of assay to disclose drug mutations is not yet well characterized. The aim of our study was to determine the presence of viral mutations in a cohort of naive patients by two different sequence assays [the HIV RT and HIV Protease Line Probe Assay (LIPA, Innogenetics, Alpharetta, GA) and the TruGene HIV-1 assay (Visible Genetics, Toronto, Ontario, Canada)] to study two different methods for detecting the prevalence of drug resistant virus.

The relative advantages and disadvantages of the two assays were evaluated to identify the genetic constitution of virus in designing the best treatment schedules. Optimization of therapies considering all the available informa-

tion, such as viral load, CD4+ T cell count and resistance profile, might have a positive rebound in the choice of therapy.

## Results

### **Viral load analysis in naive HIV-1 patients**

All the patients enrolled in the study showed a high level of viral replication ranging from 1,500 to 540,000 HIV-RNA copies/ml (median initial viral load: 60,000 HIV-RNA copies/ml). However, all the samples could be amplified and the cDNA derived from plasma viral RNA was sequenced to identify RT and protease mutations from all 27 naive patients.

### **Aminoacid substitution associated with resistance to reverse transcriptase inhibitors (RTIs)**

In our experimental conditions, K70R mutation (a mixed pattern showing the presence of WT and MU), the first mutation to appear in zidovudine treated patients, was revealed only by LIPA. Moreover, nine viral samples gave indeterminate results by LIPA, showing codons lacking different amino acid positions (association of M41L + T69K70 + L74V75: 1 case, F214T215: 1 case, M184V: 1 case, T69K70: 2 cases and L74V75: 4 cases).

As expected, the presence of mutations associated with resistance to non nucleoside reverse transcriptase inhibitors (NNRTIs), only revealed by sequencing analysis showed V108I (associated with a reduced drug sensitivity to efavirenz plus nevirapine) [19], V118I (associated with moderate phenotypic lamivudine resistance) [20] and the presence of K103N (key mutation for all the NNRTIs) plus Y181C (with variable effects on susceptibility to efavirenz, besides resistance to nevirapine).

### **Aminoacid substitution associated with resistance protease inhibitors (PIs)**

The overall frequency of secondary mutations [7,11] in the protease region of pol was significantly greater than that observed in RT. Ten patients did not show any mutations conferring resistance to protease inhibitors by either assay. LIPA showed V82A mutation (a mixed pattern showing the presence of WT and MU) correlated with resistance to indinavir and ritonavir, (Table 1) and "indeterminate" results (I50/I54, and L90) were observed in three and two cases respectively. On the other hand, sequence analysis showed some codon substitutions, probably occurring as a spontaneous genetic polymorphism, not correlated with key mutations. In particular, the most frequent substitutions, by genotyping analysis, are present at codon 36 [M36I alone (7 cases) or associated with L10V (2 cases)], at codon 63 [L63P alone (5 cases) or associated with V77I (2 cases)] and at codon 77 [V77I (5 cases)], while L10V was always found in association with A71V or M36I [8,9].

**Table 1: Presence of codon substitutions associated with resistance to antiretroviral drugs (inhibitors of Reverse Transcriptase and Protease) by sequential analysis and by Line Probe assay in untreated HIV-1 patients.**

Mutations associated with RT resistance to			
<b>NNRTIs<sup>1</sup></b>			
Patients' N <sup>o</sup> <sup>3</sup>	Viral load <sup>4</sup>	by sequencing	by Line Probe assay
2	240000	-	K70K/R
4	8900	-	L74/V75 (IR)
5		-	L74/V75 (IR)
8	11000	-	F214T215 (IR)
12	160000	-	M41, T69/K70, T69/K70 (IR)
18	120000	-	T69/K70 (IR)
20	120000	-	L74/V75 (IR)
22	13000	-	L74/V75 (IR)
24	4700	-	T69/K70 (IR)
<b>NNRTIs<sup>2</sup></b>			
9	450000	V108I	-
12	160000	-	-
14	8600	V118I	-
15	24000	K103N, Y181C	-
Mutations associated with protease resistance ( <b>PIs</b> )			
	Viral load	by sequential analysis	by LIPA
1	49000	V77I	-
2	240000	-	V82V/A
3	42000	L101/L, A71V/A	-
8	64000	L63P, V77I	-
9	450000	M36I	-
11	16000	M36I	-
12	160000	L10V, M36I	-
14	8600	L10V, M36I	-
15	24000	L63P	-
16	1500	M36I	V82V/A
17	540000	L63P	-
19	73000	V77I	-
20	120000	M36I	-
23	61000	M36I	-
24	4700	L63P	-
25	5800	L63P, V77I	V82V/A
27	170000	V77I	-

Legend: **NRTIs<sup>1</sup>** nucleoside reverse transcriptase inhibitors **NNRTIs<sup>2</sup>**: nucleoside reverse transcriptase inhibitors <sup>3</sup>Only patients who showed codon changes are reported; <sup>4</sup>Viral load: N<sup>o</sup> of HIV-RNA copies/ml. <sup>5</sup>IR: indeterminate results.

In our experimental conditions we did not find mixed populations by sequencing analysis.

### Discussion

Results obtained showed that both tests disclosed the presence of amino-acid substitutions associated with resistance in a variable number of patients. Even if our study, limited to a relatively small cohort, showed discordant results, our aim was to verify the presence of circulating virus in a group of seropositive treatment naive patients using two different tests.

First, LIPA represents "a key mutation concept" able to monitor a maximum of 13 codon changes and must be seen as an early warning of clinically relevant emerging drug resistance [20,21]. Second, LIPA is more sensitive for the detection of a mixed virus population but, the limited number of codons and the high number of indeterminate results represent major limitation [3,19,22–26]. On the other hand, the identification of K70R, a zidovudine resistance mutation, and V82A, indinavir and ritonavir resistance mutation, probably confirm the detection of mutant(s) present in a low proportion [16,27,28] and might explain the failure of sequence testing.

It is well known that LIPA might represent a valid method able to determine the presence of drug resistance mutations rapidly and simultaneously in some but not all codons. Bearing these data in mind, LIPA revealed at least one mutation where sequencing analysis failed. On the other hand, we do not underestimate the possibility of sequencing analysis by TRUGENE to detect key mutations (at least two – in our experimental conditions) conferring resistance to NNRTIs, even if sequencing analysis presents a greater complexity (i.e. the sequence alignment, editing and interpretation of results require constant operator care and attention).

We must also take into account that secondary mutations might generate future problems in assessing drug regimens since the plasma viral population early may be relatively homogeneous and representative of the transmitted species early in the infection [6] Since a high viral load has frequently been detected in most naive patients, a correct identification of secondary mutations is important before embarking on an anti-retroviral therapy.

From this point of view, sequence analysis must be considered a more complete assay, than LIPA and might be useful in a wide population analysis where bulk results are needed in a short time.

### Conclusion

The discrepancies observed in results between assays need further in depth evaluation to identify potentially

clinically relevant mutations able to compromise future treatment, in an era where the widespread use of protease and RT-inhibitors requires a more comprehensive analysis of viral genotyping resistance

However, since the analysis of drug resistant viruses might have serious implications in terms of recommendations in clinical practice and epidemiological surveys, the study of emerging viruses by ever newer assay technology, with protocol feasibility, data interpretation and sources of data variation, together with the knowledge of the limitations of each assay, the cost and the time consumed to have a result, all play a role in selecting the optimal genotyping assay for the microbiology laboratory. Even if the ultimate decisions regarding antiretroviral therapy also incorporate information regarding patients' clinical and virologic response to therapy and a thorough ascertainment of treatment history, our data confirmed the importance of monitoring the prevalence of mutations in HIV-1 naive patients.

### Materials and Methods

#### Patients

Twenty seven heterosexual HIV-1 seropositive patients never treated with antiretroviral compounds, were analyzed for the presence of mutation associate with drug resistance. All the patients were white, 20 men and 7 women, aged 21 to 38 years, enrolled in this study 1–12 months after seroconversion (mean time:  $5.4 \pm 3$  months) documented by immunoenzymatic assay (Vidas HIV Duo, BioMerieux, Marcy-L'Etoile, France) and Immunoblot (Chiron RIBA, HIV-1/HIV-2, Chiron Co., Emeryville, CA, USA). Moreover, peripheral blood CD-4 lymphocytes, counted by flow cytometry (FACScan, Becton & Dickinson, Mountain View, CA) using commercially available monoclonal antibody (Becton-Dickinson) showed a mean baseline value of  $532 \pm 127/\text{mm}^3$ .

An intensive medical evaluation excluded a history of drug abuse and transmission was established to be by sexual contact in all subjects.

#### HIV-RNA viremia

All the plasma samples were analyzed for HIV-1 RNA virus using the "Quantiplex HIV-RNA-3.0" assay (Chiron Corporation, Emeryville, CA, USA), according to the manufacturer's instructions. HIV RNA levels were expressed as copy number for ml of plasma and the lower detection limit of the assay was 50 copies/ml. Plasma samples were separated from the cell fraction by centrifugation at  $700 \times g$  for 10 minutes and then frozen at  $-70^\circ\text{C}$  until tested for HIV-1 RNA.

### Nucleic Acid extraction

Viral RNA was isolated from EDTA treated plasma samples by using the QIAmp viral extraction kit (Qiagen Inc., Chatsworth, California USA) according to the manufacturer's instructions

### Genotyping Assays

Briefly, the TRUGENE HIV-1 Genotyping Assay was used in combination with the open gene automated DNA sequencing System (Visible Genetics Inc. Toronto Canada) to sequence protease and RT region of HIV-1 cDNA. Testing involved simultaneous clip sequencing of the protease and codons 35–244 of RT from amplified cDNA in both the 3' and 5' directions. Sequences were aligned and compared with a lymphadenopathy-associated virus type 1 (HIV-B-LAV1) consensus sequence using Visible Genetics Librarian software. We focused on mutations at positions in the *pol* gene known to be associated with the most commonly used drugs against HIV-1 [8,9].

The commercially available LIPA (Innogenetics Inc. Alpharetta, Georgia, USA) was used to detect the presence of wild type, mutant RT (position 41, 69, 70, 74, 184 and 215\*) and protease (position 30, 46/48, 50 54, 82/84 and 90\*\*) codons, according to the test protocol recommended by the manufacturer.

After HIV-RNA isolation, an RT-nested PCR was performed with the biotinylated primers included in the test. After denaturation, amplified RT and protease fragments of viral RNA were incubated with LIPA membrane test strips at 39°C for 30 min, onto which selected oligonucleotide probes had been fixed. Following hybridization a colorimetric reaction provided visual indication of the presence or absence of wild type and mutated codons.

A clear visible line is considered a positive reaction. A wild type (WT) or mutant (MU) codon was established when a clearly visible line was detectable where WT or MU probes were fixed. The presence of wild type (WT) or mutant (MU) strains at the same codon was considered a viral strain containing WT and MU simultaneously. Results were considered "indeterminate" (missing codon) for a codon when no reactivity was observed with the WT and the MU probes, while the results of the other codons were read independently.

The DNA sequences obtained from the HIV-infected patient samples were compared to the HIV consensus sequences derived from GenBank sequences of accession number K02013 [18].

Footnote\*: Positions: **41** [from Met to Leu (ATG to TTG or CTG)], **69** [from Thr to Asp (from ACT to GAT)] **70** [(from Lys to Arg (from AAA to AGA)], **74** [from Leu to Val (from TTA to GTA)], **184** [from Met to Val (from ATG to GTG)], **214** [from Leu to Phe (from CTT to TTT)] and **215** [from Thr to Tyr/Phe (from ACC to TAC/TTC)]

Footnote\*\*: Positions: **30** [from Asp to Asn (from GAT to AAT)], **46/48** [from Met to Ile (from ATG to ATA) and from Gly to Val (from GGG to GTG)], **50** [from Ile to Val (from ATC to GTC)], **54** [from Ile to Val (from ATC to GCT)], 82/84 [from Val/Ile to Phe/Ala/Thr (from GTC/ATC to TTC/GCC/ACC and from He to Val (from ATA to GTA)] and **90** [from Leu to Met (from TTG to ATG)].

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