Reliability and Clinical Relevance of the HIV-1 Drug-Resistance Test in Patients with Low Viremia Levels

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ABSTRACT

Background: We evaluated reliability and clinical usefulness of genotypic-resistance-testing (GRT), in patients failing combination-antiretroviral-therapy (cART) with viremia levels 50-1000 copies/mL, for whom GRT is generally not recommended by current guidelines.

Methods: Genotyping-success-rate was evaluated in 12828 HIV-1 plasma-samples with viremia >50 copies/mL, tested using the commercial ViroSeq HIV-1 Genotyping-System or a homemade-system. Samples were stratified in 6 groups according to different viremia-levels (50-200; 201-500; 501-1000; 1001-10000; 10001-100000; >100000 copies/mL). Phylogenetic analysis was performed to test the reliability and reproducibility of the GRT also at low-viremia-levels.

Drug-resistance was evaluated in 3895 samples from 2200 treatment-failing-patients (viremia >50 copies/mL) by considering the resistance-mutations panelled in the IAS list (2013).

Results: Overall, success rate of amplification/sequencing was 96.4%. Viremia-levels of 50-200 and 201-500 copies/mL afforded success rates of 67.2% and 88.1%, respectively, reaching 93.2% at 501-1000 copies/mL and ≥97.3% above 1000 copies/mL. Phylogenetic analysis revealed a high homology among sequences belonging to the same subject for 96.4% of patients analyzed.

The overall resistance prevalence was 74%. Drug-resistance was commonly found also at low-viremia-levels. Detection of at least one resistance-mutation was: 50-200 copies/mL=52.8%; 201-500=70%; 501-1000=74%; 1001-10000=86.1%; 10001-100000=76.7%; >100000=63% (P<0.001). Similar bell-shaped results were found when the GRT-analysis was restricted to 2008-2012, though at slightly lower prevalence.

Conclusions: In patients failing cART with viremia-levels 50-1000 copies/mL, HIV-1 genotyping provides reliable and reproducible results, that are informative about emerging drug-resistance also at low-viremia-levels. Results may be helpful for the therapy optimization in patients under virological-failure, to decrease the risk of virological failures with drug-resistance accumulation.
INTRODUCTION

Over the past 15 years, antiretroviral therapy for the treatment of human immunodeficiency virus type-1 (HIV-1) infection has improved; to date, about 90% of HIV-1 infected patients who start a first-line regimen achieve virological suppression [1-10]. However, therapy failures are still observed in clinical practice; particularly at early time points, many are characterized by low viremia levels (LLV).

Standard of care management recommends use of resistance testing to guide further therapy. One area of uncertainty is the evaluation of treatment failure in patients with LLV. Treatment guidelines usually do not recommend genotypic resistance testing (GRT) for plasma HIV RNA <500-1000 copies/mL. This potential limitation of GRT mostly derives from the detection limits of commercial assays, as well as by the technical difficulty of many laboratories in obtaining consistent results with such LLV, yet some studies support the use of GRT, and laboratories increasingly report success in performing genotypes at this level [11-26].

In this study we provide data supporting reliability and usefulness of GRT at viremia levels ≤500-1000 copies/mL by analyzing a large population of HIV-1 patients followed in Central-Italy, who underwent GRT in routine clinical practice. Moreover, we evaluated whether different viremia levels affect the detection of drug-resistance in HIV-1 patients who failed therapy.

MATERIALS AND METHODS

Patients

This retrospective study included 13926 HIV-1 plasma samples that were genotyped over the years 1999-2012 in two clinical centers in Rome (Italy) for routine clinical purposes. Sample information (date of sampling, final results of sequencing, nucleotide sequences obtained, mutations found in each...
sequence), together with the data of patients for whom genotyping was performed (i.e., viro-immunological, clinical, and therapeutical data) were recorded in an anonymous database.

For each sample, viremia value at genotyping was known. We focused our analyses on samples with viremia >50 copies/mL (N=12828) that were stratified in six groups according to different viremia ranks (copies/mL): 50-200, 201-500, 501-1000, 1001-10000, 10001-100000, >100000.

**HIV-1 RNA viral load**

Depending on methodologies available over years 1999-2012, plasma viremia was determined using three different assays: the bDNA v3.0 (until January 2009; Bayer Corporation, Diagnostics Division, Tarrytown, New York), the Abbott RealTime HIV-1 (February 2009-February 2012; Chicago, Illinois) and the Roche Cobas CA/CTM v2.0 (starting from March 2012; Mannheim, Germany). These assays quantify HIV-1 RNA over the range of 50-500000 copies/mL, 40-10000000 copies/mL and 20-10000000 copies/mL, respectively. Previous studies demonstrated the results obtained by these assays to be well correlated, with a difference >0.5 log_{10} copies/mL, only for few samples [27-29].

**HIV-1 pol sequencing**

HIV-1 genotype analysis was performed on plasma samples by using either the ViroSeq HIV-1 genotyping system (Abbot Molecular) and/or a homemade system, designed to improve the performance of the ViroSeq system itself [30]. Indeed, genotyping success by this commercial kit is generally guaranteed for samples with viremia ≥2000 copies/mL [31, 32]. Therefore, some steps of the ViroSeq system were modified, in order to test HIV-1 pol sequences also in subjects with viremia <2000 copies/mL. All the details on the amplification and sequencing procedure can be found in Supplementary Methods and Supplementary Figure 1.
Subtyping analysis

All HIV-1 pol sequences were aligned in Bio-Edit and compared to reference sequences for major HIV-1 subtypes and circular recombinant forms (CRFs), available at Los-Alamos database (http://www.hiv.lanl.gov); a phylogenetic tree was performed. To analyze trends in subtype genetic diversity over time, genetic distances were calculated by using maximum-likelihood method of MEGA (http://www.megasoftware.net/), by using Kimura two-parameter model as the best-fitting evolution model for tree reconstruction [33]. The tree was shown by using the graphical user-interface FigTree. Subtype classification was confirmed also by the REGA subtype tool (http://www.bioafrica.net/regagenotype/html/subtypinghiv.html), the COMET subtype tool (http://comet.retrovirology.lu/) and the DataMonkey subtype tool (http://www.datamonkey.org/dataupload_scueal.php). To improve the accuracy of recombinant and unique forms, RDP3 software (http://web.cbio.uct.ac.za/~darren/rdp.html) and Splits Tree software (http://en.bio-soft.net/tree/SplitsTree.html) were used.

Evaluation of genotypic success rate and genotyping reliability

Genotyping success rate was determined on the overall population and according to the different viremia ranks (50-200, 201-500, 501-1000; 1001-10000, 10001-100000, >100000 copies/mL), regardless the genotyping platform upgrades (equipment, kits and reagents) that occurred from 1999 to 2012.

To ensure that there was no cross-contamination of samples analysed and in order to test genotyping reliability for samples with viremia ≤500 copies/mL, a phylogenetic analysis was performed on a subgroup of 1613 pol sequences, obtained from 470 patients with at least 1 GRT performed on samples with viremia ≤500 copies/mL and at least 1 GRT with viremia >1000 copies/mL. The phylogenetic analysis of pol sequences was performed by using the Kimura two-parameter model of MEGA version 5.05, with the same parameters as previously described [33].
Evaluation of resistance in patients who had failed therapy

The prevalence of drug-resistance was evaluated, and stratified according to different viremia levels, in a subset of 3895 samples successfully genotyped from 2200 patients with complete therapeutic history, for whom a GRT was required because of virological failure (defined as viremia >50 copies/mL). Resistance to an antiretroviral drug class was defined by the presence of at least one primary resistance mutation (PRM) included in the mutation list panelled by the International Antiviral Society in 2013 [34], considering the nucleos(t)ide RT inhibitors (NRTIs), non-NRTIs (NNRTIs) and PR inhibitors (PIs). In particular, we have defined: i) the resistance to any drug-class in the overall samples analysed; ii) the resistance to NRTIs among samples from patients who received regimens that contained NRTIs; iii) the resistance to NNRTIs among samples from patients who received regimens that contained NNRTIs; iv) the resistance to PIs among samples from patients who received regimens that contained ritonavir-boosted PIs (PI/r); v) the resistance to PIs among samples from patients who received regimens that contained ritonavir-unboosted PIs.

To better understand the clinical relevance of GRT in patients failing with LLV at the time of modern anti-HIV therapies, the prevalence of single PRMs was also evaluated on the 1317 samples from patients for whom a GRT was required because of virological failure in the years 2008-2012. This analysis was performed by dividing the samples into two groups according to viremia levels ≤1000 copies/mL (N=436) or >1000 copies/mL (N=881).

Patient outcome analysis

To evaluate the effect of LLV resistance on subsequent virologic outcome, further analyses were restricted to 51 previously drug-naïve patients at first-line regimen for whom a GRT was requested at viremia levels of 50-1000 copies/mL. Patients were included only if they were followed as long as they were receiving constant therapy without any changes or interruptions.
Statistical analysis

Potential differences among the different viremia groups were evaluated as follows: i) for the categorical variables, by the Chi-squared test for trend (to compare all viremia groups) and Pearson’s Chi-squared test or Fisher’s exact test when expected frequencies were less than 5 (to compare two viremia groups at a time); ii) for the continuous variables, by the Kruskal-Wallis test (to compare all viremia groups). Regarding the virologic outcome, Kaplan-Meier analysis was used to evaluate the probability of reaching viremia >1000 copies/mL after LLV.

In all the analyses performed, *P* values <0.05 were considered as statistically significant. The statistical programs used were R open source software (version 2.15.1) and SPSS (version 19) for Windows (SPSS Inc., Chicago, Illinois).

RESULTS

Study population

Table 1 shows the characteristics of 12828/13926 plasma-samples with viremia >50 copies/mL, processed for genotyping in routine clinical practice from 1999 to 2012. Among them, 4861 (37.9%) were obtained from 4111 drug-naïve patients, and 7967 (62.1%) from 3841 drug-experienced patients. Among drug-experienced patients, viremia levels of 50-1000 copies/mL accounted for 19.2%, (1535/7967) of total genotypic requests (Figure 1). This prevalence significantly increased over time from 1.5% in 1999-2001 to 28.4% in 2012 (*P*<0.001). A consistent proportion of samples with LLV was with viremia 50-500 copies/mL (1158/1535, 75.4%, versus 377, 24.6%, with viremia 501-1000 copies/mL).

Phylogenetic analysis revealed that B subtype was the most prevalent strain (80.1%). All the other subtypes were present with a prevalence <5%; the most prevalent ones were the recombinant form CRF02_AG (4.7%) and the subtypes C (4.3%) and F (3.3%).
Genotyping success rate

Overall success of genotype amplification and sequencing was 96.4%. The rate of success was 93.2% for samples with viremia levels 501-1000 copies/ml, 88.1% for those with viremia 201-500 copies/mL, and decreased to a still relevant 67.2% for viremia 50-200 copies/mL (Table 2). Genotyping success rate was independent of subtype in all viremia groups (Table 2). By focusing the attention on the three most prevalent non-B subtypes analysed (C, F, CRF02_AG), no differences in the success rate were found (data not shown).

Interestingly, the additional use of a nested PCR (or modified amplification protocol; see Supplementary Methods and Supplementary Figure 1) has significantly improved the overall success rate in samples with LLV \( P < 0.001 \). In particular, the nested amplification contributed to 60.4%, 55.3% and the 44.0% of the total genotypic successes with viremia levels 50-200, 201-500 and 501-1000 copies/mL, respectively. In samples with viremia levels >1000 copies/mL, the contribution of nested amplification was less relevant (from 19.2% to 3.6%, data not shown).

Genotyping reliability

In order to test genotyping reliability for samples with VL \( \leq 1000 \) copies/mL, we performed phylogenetic analysis on 1613 sequences from 470 patients having at least one genotypic sample with viremia 50-1000 copies/mL and at least another with viremia >1000 copies/mL. By evaluating each cluster, we found that sequences belonging to the same subject showed a high homology (bootstrap value >90%) in 96.4% of cases (453/470 patients) (Supplementary Figure 2). Only 25/1613 sequences (1.5%) of the remaining 17 patients did not properly cluster within the same subject.

Evaluation of resistance according to different viremia ranges in patients failing therapy

Prevalence of PRMs was analyzed on 3895 samples from a subgroup of 2200 patients at therapy failure. Patients’ characteristics of this subgroup are reported in Supplementary Table 1. Overall, the
median (interquartile range, IQR) year of genotyping was 2006 (2003-2009) and the proportion of samples from subtype B infected patients was about 86%.

The overall prevalence of samples with at least one PRM was 74% (Table 3). PI-resistance in patients treated with ritonavir-boosted PI (PI/r) was in general less frequent than NRTI- or NNRTI-resistance (40.5%, vs. 66% and 77.7%, P<0.001) (Table 3).

If we consider PI-resistance only in patients who had failed at first-line regimen containing a PI/r, the rate of resistance dropped dramatically to 3.7%. By contrast, PI-resistance in patients treated with unboosted-PI was more similar to that of NRTI/NNRTI (61.7%) and remained high also among patients tested at first-line failure (46.6%).

The prevalence of resistance varied significantly by viremia strata (P<0.001), and was characterized by a bell-shaped curve in which the highest prevalence was in the 1001-10000 copies/mL stratum, with lower prevalence values at lower and higher viremia strata. Detection of resistance was consistent also at LLV. In particular, for viremia levels of 50-200 copies/mL, NRTI-resistance was 41.3%, NNRTI-resistance was 40.2%, PI/unboosted-resistance was 51.6% and PI/r-resistance was 20.8%. For viremia 201-500 copies/mL, rates of resistance were 62.3%, 69.3%, 30.8%, and 28.0% respectively, that increased, for viremia 501-1000 copies/mL, to 67.1%, 79.5%, 79.2%, 39.0% for each respective drug class (Table 3). Therefore, substantial levels of resistance can be detected also at LLV for all drug classes, with higher rates for NRTI and NNRTIs.

The distribution of drug-resistance stratified for viremia was similar also considering samples only from patients failing their first-line regimen. In particular, a consistent proportion of NRTI and NNRTI resistance was found also at viremia levels 50-1000 copies/mL, while PI-resistance was very low in samples from patients failing their first-line PI/r containing regimen (for viremia 50-200 copies/mL: NRTI-resistance, 19.2%; NNRTI-resistance, 13.6%; PI/r-resistance, 4.9%; for viremia 201-500
copies/mL: NRTI-resistance, 38.3%; NNRTI-resistance, 54.5%; PI/r-resistance, 0%; for viremia 501-1000 copies/mL: NRTI-resistance, 59.5%; NNRTI-resistance, 73.3%; PI/r-resistance, 7.1%).

The resistance to NRTI and NNRTI varied according to viremia strata also by restricting the analysis over the years 2008-2012, with a still considerable prevalence of resistance in samples with viremia levels ≤1000 copies/mL (Figure 2). By contrast, the prevalence of PI-resistance was not influenced by viremia strata because it was very limited among all failures and was almost zero in patients failing their first-line PI/r containing regimen.

Finally, by characterizing the prevalence of each single PRM in samples genotyped over the years 2008-2012, no major differences were found by analyzing samples with viremia ≤1000 vs. >1000 copies/mL (Supplementary Table 2). In particular, only the NNRTI PRM K103N was found with a significantly higher prevalence in patients failing with viremia >1000 copies/mL (43.3%) vs. ≤1000 copies/mL (20.2%, \( P<0.001 \), after multiple comparison correction).

**Virological outcome**

By Kaplan-Meier analysis, we found that the probability of reaching viremia >1000 copies/mL after LLV was significantly higher in patients with resistance than in those without resistance, as follows: at 24 weeks, 49.7% versus 4.2%; at 48 weeks, 58.1% versus 8.7%; at 72 weeks, 72.1% versus 15.2% (\( P<0.001 \), data not shown).

**DISCUSSION**

This study aimed at evaluating the reliability and usefulness of GRT in HIV-1 infected patients with detectable LLV, in a large dataset of samples tested in two clinical centers in Italy. Our results showed that the genotyping success rate was 96% for the overall population. In particular, this success rate was very high also for viremia above 200 copies/mL (about 88%), reaching about 93% at 501-1000 copies/mL and greater than 97% above 1000 copies/mL. Reasonable results in terms of success rate
were obtained also for samples with viremia between 50 and 200 copies/mL. The ability to easily detect samples with LLV is mainly due to the improvement of the amplification step performed in our laboratories. The success of sequencing was very similar in B and non-B strains, thus suggesting that the subtype diversity does not represent a limit. Our findings are in agreement with those recently obtained in other studies, showing a high success of amplification and sequencing also at LLV [16, 19, 21, 26]. Our results with LLV may not reflect the true population, but rather reflect founder effects, especially when nested amplification is needed. Nevertheless, phylogenetic analysis confirmed the reliability and reproducibility in our laboratories of genotypic tests at different viremia levels. Indeed, by evaluating 1613 pol sequences obtained from 470 patients with at least two GRTs performed at different times and with different viremia levels (ranging from <50 to >100000 copies/mL), very high similarity among sequences from the same patient was observed.

It should be emphasized that the additional step of the nested PCR does not affect the total cost of genotyping test because the reagents used (see Supplementary Methods) are inexpensive. Indeed, by adding the Nested PCR step, the total amount of HIV-1 genotyping costs is increased only of about 10-15 Euros per sample performed. Therefore, we can conclude that the use GRT for treatment optimization in HIV-infected patients with treatment failure at LLV is in any case cost-effective. The clinical relevance of our findings is related to the fact that in the last few years there has been an increased demand for GRTs for drug-experienced patients failing with LLV (mainly ≤500 copies/mL, as shown in our analysis; see Figure 1), explained by a greater tendency to closely monitor patients in terms of response to treatment and drug-resistance. In our dataset the proportion of requests from patients failing with LLV has been about 30% since 2009.

Moreover, our results corroborate the already discussed recruitment about drug-resistance presence also at viremia levels ≤1000 copies/mL [26, 35, 36, 39], underlining the importance of GRTs also at LLV for the optimization of therapy in patients under virological failure. In this regard, it should be emphasized
that the optimization of the sequencing protocol in the last years has led to a higher accuracy in
detecting the PRMs for each viremia level. In our study, a considerable prevalence of resistance was
found also at LLV among the samples analyzed from patients failing therapy. This finding proves that
the detection of drug-resistance is not a rare event in these low viremia ranges.
A decline in the prevalence of PRMs was observed also at the very high viremia strata among drug-
experienced individuals. This decline is likely to reflect suboptimal medication adherence, with lower
drug resistance selection [35].
A considerable prevalence of resistance to NRTIs and NNRTIs at LLV was found also when the
analysis was restricted to 1317 samples from patients failing therapy in the last few years. This
prevalence can be due to the large usage of low genetic barrier drugs such as lamivudine/emtricitabine
or efavirenz/nevirapine. By the evaluation of the effect of LLV resistance on subsequent virologic
outcome, we found that the probability of reaching viremia >1000 copies/mL by 72 weeks after LLV
was significantly higher in patients with resistance than in those without resistance (). This strongly
suggests that the early detection of resistance (when viremia is still below 1000 copies/mL) may
prevent the evolution toward a) a virological failure with higher viremia and b) the accumulation of
additional mutations, thus affecting the choice of future therapeutic regimens. A potential limitation of
this analysis could be it was performed only on a very small dataset of patients. In line with our data, a
recent study, performed in a larger cohort of patients, confirmed that the LLV resistance is predictive of
subsequent virological failure [39]. Taken together these results reinforce the concept that GRT may be
useful in the management of failure even at LLV.
Data presented in our study, in agreement with previous articles [35, 36] and with data recently
presented [26, 38, 39], suggest that newer guidelines may reconsider the importance of GRT in clinical
practice even at LLV. Indeed, in spite of the technical improvements achieved in the last few years,
treatment guidelines still do not usually recommend GRT in patients with a plasma viral load ranging between >50 and 1000 copies/mL [2, 4].

In conclusion, our study, carried out in standard clinical practice, confirms that drug resistance mutations can be detected even at low viral load, regardless of the antiretroviral target genes, and can remarkably reduce the current therapeutic options for further regimens. Our findings emphasize the importance of using the genotypic test at the first failures even at low viremia, to guide the choice of an effective alternative regimen.
FIGURE LEGENDS

Figure 1. Genotypic requests for 7967 plasma samples from drug experienced patients over the years 1999-2012. The proportions of genotypic requests stratified by different viremia ranges are represented in different shades from black to white. The differences of genotypic requests over the years in patients with viremia levels 50-1000 copies/mL vs. patients with viremia levels >1000 copies/mL were evaluated by Chi-squared test for trend. P values <0.05 were considered significant.

Figure 2. Resistance to NRTI, NNRTI or PI/r classes in samples collected from January 2008 to December 2012 stratified for plasma viremia ranges. Analysis performed on 1317 samples from patients under NRTI, NNRTI or ritonavir-boosted PI failure. Resistance was defined as the presence of at least one primary NRTI, NNRTI or PI resistance mutation among those paneled by the International AIDS Society-USA [2013, Johnson et al 2013]. Potential differences in the percentage of resistance among the different viremia ranges were evaluated by Chi-squared test for trend. P values <0.05 were considered significant. Abbreviations: N, total samples analyzed per each group; NRTI, nucleos(t)ide reverse transcriptase inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; PI/r, ritonavir-boosted protease inhibitor.
Conflict of Interest Statement

Maria Mercedes Santoro has received funds for attending symposia, speaking and organizing educational activities from Abbott, Bristol Myers Squibb, Merck Sharp & Dohme, and Janssen.

Adriana Ammassari has received funds for advisory board membership from Merck Sharp & Dohme.

Emanuele Nicastri received funds for attending lectures (including services on speakers bureaus) and grant research support from Janssen, Pfizer, Merck Sharp & Dohme, ViiV Healthcare.

Nicola Petrosillo has received funds for attending lectures from Pfizer, Novartis, MSD, Astellas, Carefusion, Johnson & Johnson.

Massimo Andreoni has received funds for attending symposia, sponsoring, organizing educational activities and grant research support from Abbvie, Bristol Myers Squibb, Gilead Sciences, ViiV Healthcare.

Francesca Ceccherini-Silberstein has received funds for attending symposia, speaking and organizing educational activities from Abbott, Merck Sharp & Dohme, Gilead, Janssen, ViiV Healthcare, Roche, and Virco.

Andrea Antinori has received funds for attending symposia, speaking, grant research support and consultancy from Abbvie, Bristol Myers Squibb, Gilead Sciences, Merck Sharp & Dohme, Janssen, ViiV Healthcare.

Carlo-Federico Perno has received funds for attending symposia, speaking, organizing educational activities, grant research support, consultancy and advisory board membership, from Abbvie, Bristol Myers Squibb, Gilead, Merck Sharp & Dohme, Janssen, Pfizer, Roche, ViiV Healthcare.

The other authors declare no competing interests.

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REFERENCES


The figure shows the prevalence of resistance by viremia range for different classes of antiretrovirals. The table provides the numbers of patients in each viremia range for NRTI, NNRTI, and PI classifications.

<table>
<thead>
<tr>
<th>Viremia range (copies/mL)</th>
<th>NRTI (N=1268)</th>
<th>NNRTI (N=309)</th>
<th>PI (N=923)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50-200</td>
<td>82</td>
<td>27</td>
<td>13</td>
</tr>
<tr>
<td>201-500</td>
<td>123</td>
<td>27</td>
<td>63</td>
</tr>
<tr>
<td>501-1000</td>
<td>196</td>
<td>27</td>
<td>106</td>
</tr>
<tr>
<td>1001-10000</td>
<td>233</td>
<td>87</td>
<td>209</td>
</tr>
<tr>
<td>&gt;100000</td>
<td>313</td>
<td>83</td>
<td>240</td>
</tr>
</tbody>
</table>

P-values indicate statistical significance:
- NRTI vs. NNRTI: P=0.016
- NRTI vs. PI: P=0.008
- NNRTI vs. PI: P=0.445
Table 1. Characteristics of plasma samples with HIV-RNA >50 copies/mL at genotypic resistance test

<table>
<thead>
<tr>
<th>Variable</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients, N=7518</td>
<td></td>
</tr>
<tr>
<td>Patients with only one sample</td>
<td>4950 (65.8)</td>
</tr>
<tr>
<td>Patients with more than one sample</td>
<td>2568 (34.2)</td>
</tr>
<tr>
<td>Samples, N=12828</td>
<td></td>
</tr>
<tr>
<td>From drug-naive patients</td>
<td>4861 (37.9)</td>
</tr>
<tr>
<td>From drug-experienced patients</td>
<td>7967 (62.1)</td>
</tr>
<tr>
<td>Samples with subtype information available(a)</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>10212 (80.1)</td>
</tr>
<tr>
<td>CRF02_AG</td>
<td>598 (4.7)</td>
</tr>
<tr>
<td>C</td>
<td>545 (4.3)</td>
</tr>
<tr>
<td>F</td>
<td>422 (3.3)</td>
</tr>
<tr>
<td>BF(b)</td>
<td>312 (2.4)</td>
</tr>
<tr>
<td>G</td>
<td>173 (1.4)</td>
</tr>
<tr>
<td>A</td>
<td>157 (1.2)</td>
</tr>
<tr>
<td>Other</td>
<td>326 (2.6)</td>
</tr>
<tr>
<td>Samples according viremia ranges (copies/mL)</td>
<td></td>
</tr>
<tr>
<td>50-200</td>
<td>769 (6.0)</td>
</tr>
<tr>
<td>201-500</td>
<td>489 (3.8)</td>
</tr>
<tr>
<td>501-1000</td>
<td>444 (3.4)</td>
</tr>
<tr>
<td>1001-10000</td>
<td>2435 (19.0)</td>
</tr>
<tr>
<td>10001-100000</td>
<td>4845 (37.8)</td>
</tr>
<tr>
<td>&gt;100000</td>
<td>3846 (30.0)</td>
</tr>
</tbody>
</table>

\(a\): Subtype information was available for 12745/12828 (99.4%) samples.

\(b\): Including CRF12, CRF17, CRF28, CRF29, CRF40.
Table 2. HIV-1 genotyping resistance success rate according to different viremia levels

<table>
<thead>
<tr>
<th>Viremia ranges (copies/mL)</th>
<th>Overall N (% success)</th>
<th>B Subtype N (% success)</th>
<th>Non-B Subtype N (% success)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall</td>
<td>12828 (96.4)</td>
<td>10212 (97.1)</td>
<td>2533 (96.1)</td>
<td>.683</td>
</tr>
<tr>
<td>50-200</td>
<td>769 (67.2)</td>
<td>583 (72.9)</td>
<td>139 (66.2)</td>
<td>.115</td>
</tr>
<tr>
<td>201-500</td>
<td>489 (88.1)</td>
<td>369 (90.0)</td>
<td>113 (86.7)</td>
<td>.330</td>
</tr>
<tr>
<td>501-1000</td>
<td>444 (93.2)</td>
<td>329 (94.5)</td>
<td>112 (92.0)</td>
<td>.328</td>
</tr>
<tr>
<td>1001-10000</td>
<td>2435 (97.3)</td>
<td>1967 (98.0)</td>
<td>456 (96.9)</td>
<td>.153</td>
</tr>
<tr>
<td>10001-100000</td>
<td>4845 (99.2)</td>
<td>3969 (99.3)</td>
<td>867 (99.2)</td>
<td>.812</td>
</tr>
<tr>
<td>&gt;100000</td>
<td>3846 (99.5)</td>
<td>2995 (99.6)</td>
<td>846 (99.2)</td>
<td>.159</td>
</tr>
</tbody>
</table>

The success of the genotypic resistance test in plasma samples from HIV-1 infected patients was evaluated on the overall population with viremia >50 copies/mL (N=12828) and according to subtype (B vs. non-B), by stratifying for viremia ranges. The rate of genotyping success in patients with viremia <50 copies/ml was 17.5%.

* Potential differences in the rate of genotypic success in B and non-B subtypes were evaluated by Chi-squared test (corrected for the population size, as appropriate) or Fisher’s exact test, as appropriate. *P values* < 0.05 were considered as statistically significant.

Abbreviations: P, P value.
Table 3. Resistance to at least one antiretroviral class and to NRTI/NNRTI/PI classes stratified for plasma viremia ranges

<table>
<thead>
<tr>
<th>Viremia ranges (copies/mL)</th>
<th>All samples</th>
<th>Samples from patients taking NRTI</th>
<th>Samples from patients taking NNRTI</th>
<th>Samples from patients taking ritonavir-boosted PI</th>
<th>Samples from patients taking ritonavir-unboosted PI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>%</td>
<td>P*</td>
<td>PRMs number</td>
<td>N</td>
</tr>
<tr>
<td>Overall ranges</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3895</td>
</tr>
<tr>
<td>50-200</td>
<td></td>
<td></td>
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<td></td>
<td>396</td>
</tr>
<tr>
<td>201-500</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>287</td>
</tr>
<tr>
<td>501-1000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>242</td>
</tr>
<tr>
<td>1001-10000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1102</td>
</tr>
<tr>
<td>10001-10000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1212</td>
</tr>
<tr>
<td>&gt;100000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>656</td>
</tr>
</tbody>
</table>

The percentage (%) of drug-resistance and the median (interquartile range, IQR) number of primary resistance mutations (PRMs) were evaluated according to viremia ranges in 3895 patients with known therapeutic history and with at least one genotypic resistance test at failure. Genotypic resistance tests were performed between May 1999 and December 2012; median year (IQR) of genotyping was 2006 (2003-2009) (see Table 3).

Resistance to an antiretroviral drug class was defined by the presence of at least one PRM included in the mutation list panelled by the International AIDS Society in 2013 [34], considering the NRTIs, NNRTIs and PIs. In particular, we have defined: i) the resistance to any drug-class in the overall samples analysed; ii) the resistance to NRTIs among samples from patients who received regimens that contained NRTIs; iii) the resistance to NNRTIs among samples from patients who received regimens that contained NNRTIs; iv) the resistance to PIs among samples from patients who received regimens that contained ritonavir-boosted PIs; v) the resistance to PIs among samples from patients who received regimens that contained ritonavir-unboosted PIs.

A: Potential differences in the percentage of resistance among the different viremia ranges were evaluated by the Chi-squared test for trend.

B: Potential differences in the number of PRMs among the different viremia ranges were evaluated by the Kruskal-Wallis test.

In all the analyses performed, P values < 0.05 were considered as statistically significant.

Abbreviations: IQR, interquartile range; N, total samples analyzed per each group; NNRTI, non-nucleoside reverse transcriptase inhibitor; NRTI, nucleos(t)ide reverse transcriptase inhibitor; P, P value; PI, protease inhibitor; PRM, primary resistance mutation; %, proportion of samples with at least one PRM according to drug class.