Performance of HIV-1 Drug Resistance Testing at Low-Level Viremia and Its Ability to Predict Future Virologic Outcomes and Viral Evolution in Treatment-Naive Individuals

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(See the Major Article by Santoro et al on pages 1156–64, and the Editorial Commentary by Richman on pages 1174–5.)

Background. Low-level viremia (LLV; human immunodeficiency virus [HIV-1] RNA 50–999 copies/mL) occurs frequently in patients receiving antiretroviral therapy (ART), but there are few or no data available demonstrating that HIV-1 drug resistance testing at a plasma viral load (pVL) <1000 copies/mL provides potentially clinically useful information. Here, we assess the ability to perform resistance testing by genotyping at LLV and whether it is predictive of future virologic outcomes in patients beginning ART.

Methods. Resistance testing by genotyping at LLV was attempted on 4915 plasma samples from 2492 patients. A subset of previously ART-naive patients was analyzed who achieved undetectable pVL and subsequently rebounded with LLV (n = 212). A genotypic sensitivity score (GSS) was calculated based on therapy and resistance testing results by genotyping, and stratified according to number of active drugs.

Results. Eighty-eight percent of LLV resistance assays produced useable sequences, with higher success at higher pVL. Overall, 16 of 212 (8%) patients had pretherapy resistance. Thirty-eight of 196 (19%) patients without pretherapy resistance evolved resistance to 1 or more drug classes, primarily the nucleoside reverse transcriptase (14%) and/or non-nucleoside reverse transcriptase (9%) inhibitors. Patients with resistance at LLV (GSS <3) had a 2.1-fold higher risk of virologic failure (95% confidence interval, 1.2- to 3.7-fold) than those without resistance (P = .007). Progressively lower GSS scores at LLV were associated with a higher increase in pVL over time (P < .001). Acquisition of additional resistance mutations to a new class of antiretroviral drugs during LLV was not found in a subset of patients.

Conclusions. Routine HIV-1 genotyping of LLV samples can be performed with a reasonably high success rate, and the results appear predictive of future virologic outcomes.

Keywords. HIV-1; low-level viremia; resistance; outcome; ART.

An increasing proportion of persons living with human immunodeficiency virus (HIV-1) are receiving suppressive antiretroviral therapy (ART) [1, 2]. However, despite stable ART, many patients experience episodes of low-level viremia (LLV), defined as plasma viral load (pVL) measurements between 50 and 1000 HIV-1 RNA copies/mL, which includes both blips and sustained low-level viremia. An increased risk of virologic failure has been associated with episodes of LLV in several studies [3–6], but not in others [7, 8]. In addition, LLV episodes have been associated with higher immune activation [9, 10] and even possible increased mortality [11]. A main factor in increased risk of virologic failure appears to be the accumulation of drug resistance mutations, either released from stable HIV-1 reservoirs [12] and/or from ongoing cycles of replication [7].

However, data on resistance during low-level viremia are limited, in part because US Food and Drug Administration (FDA)–approved genotypic HIV-1
resistance assays require at least 1000 copies/mL (TRUGENE HIV-1 Genotyping Assay) or 2000 copies/mL (ViroSeq HIV-1 Genotyping System) [13]. Another concern in the possibility that amplification of a very small number of HIV-1 copies may render inaccurate genotypic results. Thus, studies of antiretroviral resistance during low-level viremia on patients receiving first-line ART are scarce [14]. The aims of our study were to evaluate emergence of HIV-1 drug resistance mutations during periods of low-level viremia, assess the ability to successfully sequence them using an in-house assay, and evaluate the association of LLV resistance with subsequent virologic outcomes in a cohort of patients beginning their first ART regimen.

MATERIALS AND METHODS

Study Population
We evaluated genotype success rates of all HIV-1-infected adults who enrolled in the British Columbia Drug Treatment Program between 1996 and 2012 with any detectable pVL <1000 copies/mL by Roche COBAS Amplicor HIV-1 Monitor Test version 1.5 (detection limit of 400 copies/mL from 1996 to 1999, and 50 copies/mL from 1999 to 2009) or Roche COBAS TaqMan HIV-1 version 1.0 or 2.0 (detection limit set to 50 copies/mL, and in use from 2009 to 2012). A total of 4915 results were obtained from samples with detectable pVL <1000 copies/mL. These samples came from a total of 2492 patients. Many of these results (27%) were obtained retrospectively for research purposes on stored specimens, as the cutoff for ordering an LLV resistance genotype was initially 500 copies/mL, lowered subsequently to 250 copies/mL, although samples with pVL <250 could be tested by physician request.

HIV-1 RNA Extraction and Drug Resistance Analysis
Drug resistance testing was performed on physician-requested samples with pVL as defined above. In addition to changes in the viral load assay over the years, various methods and equipment have also been used for resistance genotyping in British Columbia. From 1998 to 2006, HIV-1 RNA was manually extracted from frozen plasma samples using guanidinium-based lysis buffer followed by isopropanol/ethanol washes (Qiagen), or automatically using a BioRobot (Qiagen), and from 2006 to 2012 by automated extraction using a NucliSSENS easyMAG (bioMérieux). Amplification of the protease and reverse transcriptase (RT) regions was performed using nested RT–polymerase chain reaction (PCR) followed by sequencing in both the 5’ and 3’ directions on an ABI 3100, 3130, or 3700 sequencer from 1996 to 2006, and an ABI 3730 sequencer from 2006 to 2012. Primers used span all of protease, and to codon 400 of RT (primary PCR product). Amplification was repeated with different primers (spanning to codon 250 of RT; “backup” PCR product) when the first attempt was unsuccessful. Although a second PCR attempt must be made, this backup method simply uses a different primer set that spans a smaller region, so it is no more resource intensive than the primary method. A test was reported as failed when a second attempt with reextraction and backup primers was unsuccessful. Sequence data were analyzed using Sequencher (GeneCodes) from 1996 to 2007 and RECall (British Columbia Centre for Excellence in HIV-1/AIDS) automated sequencing software from 2007 to 2012 [15–17]. Nucleotide mixtures were identified if the secondary peak height exceeded approximately 17.5% of the dominant peak height.

Genotypic Sensitivity Scores
The genotypic sensitivity score (GSS) was obtained using the Stanford HIVdb genotypic resistance interpretation system [18]. The Stanford algorithm generates 5 levels of resistance to a drug, ranging from fully susceptible (ie, wild-type), to low to intermediate resistance, to high-level resistance. Here, we assigned a GSS value of 1 to each drug categorized as susceptible, potential low-level resistance, and low-level resistance; a GSS value of 0.5 to the intermediate resistance category; and a GSS value of 0 to the high-level resistance category. The GSS values for all drugs in a regimen were added together to give a final total GSS. Patients were grouped into 4 categories depending on their GSS scores at LLV, corresponding to the number of active drugs prescribed: 0–0.5; 1–1.5; 2–2.5; and ≥3. In a subsequent analysis, patients were grouped into resistant (GSS <3) and not resistant (GSS ≥3) categories. The virtual phenotypic sensitivity score VircoTYPE HIV-1 was also used to reevaluate the results [19].

Patient Outcome Analysis
To evaluate the effect of LLV resistance on subsequent virologic outcome, further analyses were restricted to previously ART-naive patients who achieved undetectable viral loads but whose virus rebounded with repeated pVL between 50 and 999 copies/mL. Patients were included only if they had not had a previous blip (≥1000 copies/mL), and they were followed as long as they were receiving constant therapy without any changes or interruptions. Many of these results (24%) were obtained retrospectively.

Statistical Analysis
Kaplan-Meier methods were used to monitor time from LLV to virologic failure ≥1000 copies/mL. Subjects without virologic failure were right censored at the date of last observation while on the same therapy. Linear mixed-effect models were used to compare the change in pVL over time between GSS categories. A Cox proportional hazards model was used to estimate the hazard ratios of virologic failure, adjusting for other explanatory variables, such as sex, age, hepatitis C virus (HCV)
infection status, and pVL at LLV. A backward stepwise technique was used in the selection of covariates for an explanatory model. The selection of variables was based on two criteria: Akaike information criterion and type III P values. Tests for statistical significance were performed using Fisher exact test or Pearson $\chi^2$ test for categorical variables and the Wilcoxon rank-sum test for continuous variables. Analyses were performed using SAS software version 9.3 (SAS, Cary, North Carolina).

**Ethics Statement**

This study was approved by the Committee on Human Research and the University of British Columbia/ Providence Health Care Research Ethics Board.

**RESULTS**

**Genotyping Success**

Overall, 4312 of 4915 (88%) LLV assays attempted produced usable sequences (Table 1). When ≥2 different viral strains are amplified within the same sample, >1 mixture will be found in the final sequence. Successful genotypes were obtained more frequently at higher pVL strata approaching 1000 copies/mL. These higher viral load samples tended to have more frequently at higher pVL strata approaching 1000 copies/mL. Successful results were obtained from 74% of samples with viral loads <250 copies/mL and from approximately 90% of samples with viral loads >250 copies/mL. Results were similar regardless of HIV-1 subtype (data not shown). Unsuccessful genotypes and the use of back-up PCR product progressively increased with decreasing viral load (Figure 1A). In addition, we analyzed whether the age of the sample (time from collection to testing) affected the success in testing; we found that with the longer age of the samples, the success in testing was slightly lower (Figure 1B). However, even in samples stored at −20°C for >4 years, success in testing remained around 70%–90%.

**Patient Characteristics and Resistance Testing by Genotyping**

Patient characteristics are shown in Table 2 for 212 previously drug-naive subjects who rebounded with LLV during their first ART regimen. At time of resistance testing, patients had moderately high CD4 counts (median, 415 cells/µL; 25th–75th percentile, 260–580 cells/µL) and low pVL (median, 374 copies/mL; 25th–75th percentile, 267–559 copies/mL). Resistance testing by genotyping before treatment (baseline) and at time of genotype testing are shown in Table 3. Overall, 16 of the 212 patients (8%) had baseline resistance prior to therapy. Of those without baseline resistance (n = 196), 38 patients (19%) evolved resistance to any class of medication at follow-up with LLV, a median of 6.9 months (25th–75th percentile, 3.3–18) after their pVL became undetectable. In these patients, resistance was most common to the nucleoside reverse transcriptase inhibitor (n = 28; 14%) and/or nonnucleoside reverse transcriptase inhibitor (n = 18; 9%) drug classes. Of note, only 2 cases (1%) of emerging protease inhibitor (PI) mutations arose (D30N), both in patients taking nelfinavir, despite 67% of patients receiving a PI. No patients evolved triple-class drug resistance during the study period. The most common mutations found at LLV were M184V/I (10% V, 4% I), K103N (6%), T215Y/F/C/D/E/S/I (4%), M41L (4%), Y181C (3%), K70R/E (3%), and T69D/N/S (3%).

Before treatment, baseline (ie, transmitted) resistance was marginally more common in patients who were male ($P = .02$) and slightly older (median, 46 vs 43 years; $P = .04$). Analyses were then further restricted to the 196 patients without baseline resistance. Those patients who evolved resistance at LLV had a non–statistically significant trend toward higher viral load levels at LLV vs those who did not evolve resistance (median, 472 vs 369 copies/mL; $P = .067$). Moreover, we observed that the prevalence of resistance increased at higher viral load strata at LLV. Only 5% of patients (n = 2) with 50–249 copies/mL at LLV had resistance, whereas 24% (n = 22), 17% (n = 7), and 30% (n = 7) had LLV resistance at 250–499, 500–749 and 750–999 copies/mL, respectively ($P = .041$). Other patient characteristics (risk group, HCV coinfection, CD4, ethnicity, time from undetectable viremia to LLV) were not associated with resistance at LLV ($P \geq .1$).

**Virologic Outcomes**

Kaplan-Meier curves were used to evaluate time to virologic failure (≥1000 copies/mL) while remaining on the same therapy. Figure 2A indicates that patients with resistance (GSS <3) at LLV had significantly increased risk of subsequent virologic failure compared to those without resistance (GSS ≥3) ($P = .007$). Furthermore, linear mixed-effect models showed that progressively

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**Table 1. Success at Low-Level Viremia Genotyping**

<table>
<thead>
<tr>
<th>Input Viral Load, Copies/mL</th>
<th>50–249</th>
<th>250–499</th>
<th>500–749</th>
<th>750–999</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actual input copiesa</td>
<td>1.7–8.3</td>
<td>8.3–16.6</td>
<td>16.7–24.9</td>
<td>25–33.3</td>
</tr>
<tr>
<td>No. attempted</td>
<td>751</td>
<td>2068</td>
<td>1212</td>
<td>884</td>
</tr>
<tr>
<td>No. successful</td>
<td>555</td>
<td>1841</td>
<td>1118</td>
<td>798</td>
</tr>
<tr>
<td>% Successful</td>
<td>74%</td>
<td>89%</td>
<td>92%</td>
<td>90%</td>
</tr>
<tr>
<td>% Sequences with any mixtures</td>
<td>36%</td>
<td>51%</td>
<td>64%</td>
<td>71%</td>
</tr>
<tr>
<td>Median No. of mixtures per sequence</td>
<td>0</td>
<td>1</td>
<td>5</td>
<td>9</td>
</tr>
</tbody>
</table>

a Actual input copies is a 30-fold dilution from the plasma viral load based on the use of 500 µL from 1000 µL of plasma, utilized to obtain 60 µL of RNA extract, from which only 4 µL was finally used for polymerase chain reaction.
lower GSS scores at LLV are significantly associated with an increased change in median pVL over time (overall \( P < .001 \); Figure 2B).

Bivariate analysis of patient characteristics associated with virologic failure after LLV indicated that higher pVL at LLV \( (P = .02) \), history of injection drug use \( (P = .03) \), HCV coinfection \( (P = .04) \), and female sex \( (P = .05) \) were associated with an increased likelihood of a subsequent pVL \( \geq 1000 \) copies/mL. To determine predictors associated with the hazard of virologic failure, unadjusted and adjusted Cox proportional hazards
models were applied to all the variables related to virologic failure. Among covariates considered, only sex and pVL at LLV were included in the final model. Adjusted hazard ratios were 2.12 (95% confidence interval [CI], 1.23–3.66) for patients with resistance at LLV (GSS <3), 2.34 (95% CI, 1.11–4.93) for patients with pVLs 500–749 copies/mL vs 50–249 copies/mL, and 1.64 (95% CI, .97–2.78) for patients reporting female sex. The other pVL strata had P values >.1 compared with pVLs 50–249 copies/mL.

Additionally, in a subset of 29 patients maintaining LLV with follow-up resistance test results, there was no evidence of acquisition of additional resistance mutations to a new class of anti-retrovirals (data not shown), suggesting that the selection of resistance to an additional family drug during a LLV episode on the same regimen may not be a common event, although the follow-up times were relatively short (8 months). Moreover, when the evolutionary distance between the first and last genotype was analyzed by the TN93 model [20], a slight trend toward increased genetic diversity over time was observed. However, this did not reach statistical difference, probably because of the low number of patients (data not shown). This trend may suggest that even with no apparent resistance evolution, HIV-1 may be able to evolve at LLV.

Data were also stratified by whether or not any nucleotide mixtures were observed in the sequence chromatograms. An absence of mixtures suggested that only a single molecule may have been successfully reverse transcribed and amplified during sample processing. As is shown in Figure 3, the time to virologic failure curves are different between patients with resistance and no resistance, whether or not mixtures were observed (P = .007). Cox proportional hazards model adjusting for an indicator for mixture still showed a significant P value for resistance (P = .007), implying that even clonal products with only 1 sequence (no mixtures) gave useful results for predicting virologic failure. All the above analyses were repeated using VircoTYPE virtual phenotype interpretation instead of the Stanford algorithm, and very similar results were obtained (Supplementary Data).

**DISCUSSION**

We have shown that routine HIV-1 genotyping of LLV samples can be performed with a reasonably high success rate (74% in samples <250 copies/mL and 90% >250 copies/mL). In addition, we have shown that genotyping of LLV samples is predictive of future virologic outcomes in treatment-naive patients on their first ART regimen.

Diagnosis and management of emerging drug resistance during LLV is a clinical challenge, as FDA-approved genotyping assays require at least 1000 copies/mL [13] and some standard genotypic tests (including ours) have higher failure rates at
amplifying HIV-1 RNA at LLV [21]. However, the in-house PCR method used in this study shows a high success rate for genotyping LLV samples (about 74% at 50–249 copies/mL and 90% at 250–999 copies/mL), suggesting that genotyping can be performed routinely on clinical LLV samples. Actually, based on the pVL and the volume used for testing, genotypes

![Figure 2. A. Time to virologic failure was more rapid in patients with resistance during low-level viremia (LLV). Kaplan-Meier curve of time to virologic failure >1000 copies/mL while remaining on constant therapy, as a function of whether patients had resistance at LLV (genotypic sensitivity score [GSS] <3) or not (GSS ≥3). Patients with resistance at LLV had a more rapid rate of virologic failure (P = .007). B. More extensive resistance at LLV leads to higher viral load rebounds. Progressively lower GSS scores at LLV were significantly associated with a higher increase in median plasma viral load over time in patients while remaining on constant therapy (overall P < .001).]
sequences were obtained ranging from 2 to 33 actual input copies. In addition to lower assay success in samples with especially low pVL levels, we found that the longer time elapsed between sample collection and testing also negatively affected success of genotyping. However, success was still relatively high, at 70%–90%, even for samples >4 years old. The relatively high success rate of resistance genotyping at LLV has also been demonstrated by an independent laboratory, which reported strikingly similar success rates to ours [22].

The results obtained in this study suggest that resistance detected during LLV may be clinically relevant to future outcomes, and this information should be provided to healthcare practitioners to better monitor their patients. In fact, use of resistance testing by genotyping testing has been shown to improve virologic outcomes in several prospective studies of patients failing ART [23–25]. To infer resistance in this study, we used the Stanford HIVdb genotype resistance interpretation system [18]. Other rules-based systems such as ANRS, Rega, and AntiRetroScan are available, and similar results are often obtained regardless of the system used [26, 27]. In addition, all our results were confirmed by using the VircoTYPE HIV-1 as an alternative classification algorithm. Although some studies have reported that GSS had no impact on viral responses [28–30], several other investigations support genotypic or phenotypic sensitivity scores as predictors of viral response [4, 26, 27, 31, 32].

Here we show a strong association between risk of virologic failure and GSS scores (P < .007). This is in agreement with a previous study analyzing LLV of 50–500 copies/mL [33]. We also found a weak association between virologic failure and injection drug use history, HCV coinfection, and female sex. This may be explained because all 3 of these patient characteristics coassociate in British Columbia and have been previously linked to suboptimal adherence to ART [34]. However, after adjusting the model for these variables, the only variables independently associated with virologic failure were GSS <3 (P = .007) and pVL 500–749 copies/mL vs 50–249 copies/mL (P = .026), whereas female sex was no longer significant (P = .069).

Only 1% of patients evolved resistance to PIs (specifically D30N mutations, while taking nelﬁnavir), despite 67% of patients receiving a PI. This result conﬁrms that emerging PI resistance rarely occurs during LLV, probably due to their high genetic barrier to resistance [35]. Also, our results showed that patients with higher pVL at LLV were more likely to have resistance mutations (mainly M184V/I and K103N), which is in agreement with a previous study analyzing 59 patients with LLV on first-line therapy [12].

A previously unaddressed concern of genotyping LLV samples has been whether the higher likelihood of amplifying only 1 viral input copy would still be informative. To clarify that concern, we analyzed our results by whether or not mixtures were found in the sequence chromatograms (A) or not (B) (P = .007).

Figure 3. A, Time to virologic failure (patients with >1 viral input copies amplified, and sequence mixtures). B, Time to virologic failure (patients with 1 viral species amplified, and no sequence mixtures). Time to virologic failure curves are different between patients with resistance and no resistance whether virologic mixtures were observed in the sequence chromatograms (A) or not (B) (P = .007).
Strengths of our study include its large sample size (about 5000), the duration of the study (from 1996 to 2012), and the duration of clinical follow-up (up to 8 years). The analysis of only previously ART-naive patients achieving undetectable viral load before LLV and remaining on the same regimen during the entire follow-up were also major strengths of the study. Moreover, when calculating the GSS, we considered only intermediate and high-level resistance mutations, leaving aside low-level and potential low-level resistance mutations, which have a weaker influence on regimen efficacy. Furthermore, different from other groups [4], we considered only the resistant mutations found at sampling and did not assume that mutations detected in any previous analysis (eg, before therapy) were still present even if not detected [37]. All these factors provide a more consistent and cleaner picture of LLV resistance.

Nevertheless, our study has several limitations. It was an observational study, and some of our results were obtained retrospectively. A randomized controlled trial would be more definitive in proving that resistance testing at LLV is a useful strategy. We did not exclude patients based on the therapies they were receiving, so 2 patients with suboptimal regimens (<3 full-dose drugs) were included in our study. Resistance was assessed only through sequencing of the protease and RT regions of HIV-1, although other regions may also play a role in drug susceptibility [38]. We also did not address adherence in this study. Nevertheless, although adherence levels may play a hypothetical role in LLV, a previous study showed that adherence levels did not modify the associations between LLV resistance and virologic outcome [31]. Furthermore, the low viral loads of these patients likely indicate some level of adherence.

In conclusion, we show that routine HIV-1 genotyping of LLV samples can be performed with a reasonably high success rate; the results obtained appear predictive of future virologic outcomes.

### Supplementary Data

Supplementary materials are available at Clinical Infectious Diseases online (http://cid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

### Notes

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### Potential conflicts of interest

All authors: No reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

### References