Pooling Strategies to Reduce the Cost of HIV-1 RNA Load Monitoring in a Resource-Limited Setting

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Background. Quantitative human immunodeficiency virus (HIV) RNA load testing surpasses CD4 cell count and clinical monitoring in detecting antiretroviral therapy (ART) failure; however, its cost can be prohibitive. Recently, the use of pooling strategies with a clinically appropriate viral load threshold was shown to be accurate and efficient for monitoring when the prevalence of virologic failure is low.

Methods. We used laboratory request form information to identify specimens with a low pretest probability of virologic failure. Patients aged >15 years who were receiving first-line ART had individual viral load results available were eligible. Blood plasma, dried blood spots, and dried plasma spots were evaluated. Two pooling strategies were compared: minipools of 5 samples and a 10 χ 10 matrix platform (liquid plasma specimens only). A deconvolution algorithm was used to identify specimens(s) with detectable viral loads.

Results. The virologic failure rate in the study sample was <10%. Specimens included were liquid plasma specimens tested in minipools (n = 400), of which 300 were available for testing by matrix, and specimens tested with minipools only: dried blood spots (n = 100) and dried plasma spots (n = 185). Pooling methods resulted in 30.5%–60% fewer HIV RNA tests required to screen the study sample. For plasma pooling, the matrix strategy had the better efficiency, but minipools of 5 dried blood spots had the best efficiency overall and were accurate at a >95% negative predictive value with minimal technical requirements.

Conclusions. In resource-constrained settings, a combination of preselection of patients with low pretest probability of virologic failure and pooled testing can reduce the cost of virologic monitoring without compromising accuracy.
therapy, and other patients retained on a failing first-line ART regimen, which is composed of a nonnucleoside reverse-transcriptase inhibitor (NNRTI) and 2 nucleoside reverse-transcriptase inhibitors (NRTIs), in many resource-limited settings, resulting in clinical deterioration and the accumulation of resistance mutations. Indeed, when CD4 cell count and/or clinical monitoring is used to identify ART failure, a greater percentage of patients harbor both thymidine analogue mutations and mutations such as K65R[10] than when VL monitoring is used[11–14]. Thus, unrecognized failure could compromise second-line ART, which in South Africa currently is composed of ritonavir-boosted lopinavir plus 2 NRTIs, lamivudine, and either tenofovir or zidovudine[15]. Although VL testing is extremely valuable for monitoring patients receiving ART, it is unaffordable in many countries that rely on inadequate patient monitoring with CD4 cell count and clinical criteria alone.

Strategies that reduce the cost of VL monitoring could therefore increase access to VL monitoring. One such strategy is the pooled testing of samples. Pooled HIV RNA testing is the performance of HIV RNA testing of pools made from multiple patient specimens, which is efficient in screening blood donors[16] and for diagnosing acute HIV infection[17–19]. Recently, pooled testing using a clinically appropriate VL threshold has been shown to be an accurate and efficient method for detecting virological failure in patients receiving ART[20]. The efficiency of a pooling strategy is, however, largely dependent on the pretest probability of ART failure[21]. If failure in a population is >25%, the saving achieved through pooling decreases to <30%[21]. Therefore, in this study we first evaluated how basic demographic information recorded on standard laboratory request forms can be used to select patients receiving ART who have a low probability of virologic failure (<10%) and then compared minipool and matrix strategies to reduce costs of virologic monitoring. To maximize the usefulness of procedures in resource-limited settings, we also evaluated minipools using dried blood spots (DBS) and dried plasma spots (DPS).

MATERIALS AND METHODS

Study Population and Screening Criteria

The study was approved by the Stellenbosch University Committee for Human Research. To determine selection criteria for identifying a study population with low (<10%) probability of having virologic failure and thus eligible for pooled VL testing, we performed a retrospective, descriptive analysis with use of information entered on routine laboratory request forms by health care providers. Four representative weeks (ie, excluding holiday seasons) from August 2008 through August 2009 were randomly selected. All VL tests performed during these weeks by the routine virology laboratory at the National Health Laboratory Service in Tygerberg, South Africa, were included, and information entered on the routine laboratory request forms was collected. The VL threshold for defining ART failure throughout this investigation was defined a priori as VL >1000 HIV RNA copies/mL, which is the current threshold for virologic failure in South Africa[15]. The simplest combination of variables was selected that would predict a low pretest probability of having virologic failure. The criteria, thus identified, were applied to prospectively select patient specimens for all subsequent pooled VL testing, excluding only specimens with insufficient specimen volumes remaining after individual VL testing had been performed.

Pooled Testing of Liquid Blood Plasma Specimens

The effectiveness and accuracy of routinely performed individual VL testing were compared with those of pooled VL testing using blood plasma specimens. Specimens stored at −80°C were selected according to the previously established criteria. Two different pooling strategies were used: minipools of 5 samples and 10×10 matrix pools. At a VL threshold of 1000 HIV RNA copies/mL for defining ART failure for an individual specimen, the VL threshold applicable to pools is 200 HIV RNA copies/mL for the minipool platform and 100 HIV RNA copies/mL for the matrices. Times required to constitute the pools were recorded. Resolution of pools to identify which samples were above the virologic threshold was done using a previously published search-and-test algorithm, implemented in the Measurement Enhanced Pooling Assay Calculator of the University of California, San Diego, available at http://mepac.ucsd.edu/ [20]. Briefly, for matrix pools this algorithm uses results of horizontal and vertical pools to resolve some individual specimens and directs one to test the remaining individual specimens in the most efficient order, thereby reducing the number of tests needed. For minipools, this algorithm subtracts the contribution of sequentially tested individual samples from the pooled VL until the pool is resolved below the defined level of virologic failure, at which stage no further testing of individual specimens is needed. For each of these strategies, we calculated the negative predictive value and number of assays performed relative to testing each sample individually (ie, efficiency and the potential cost differential between individual and pooled VL testing strategies).

Pooled Testing with DBS and DPS

Pooling of specimens was also evaluated in the minipool platform using DBS (n = 100) and DPS (n = 185). DBS and DPS were prepared from specimens meeting the aforementioned criteria and that had individual VL test results from liquid plasma specimens. Spots were dried overnight, and cards were kept in plastic bags with desiccants at room temperature until tested within 3 days after preparation. Only minipools of 5 were constituted, as previously described for
liquid plasma[20–21]. The following experiments were conducted: one DBS per patient was eluted in 3 mL lysis buffer, and the pool VL results were examined at thresholds of 133, 200, 400, and 1000 HIV RNA copies/mL to define a positive pool; 2 DBS per patient eluted in 10 mL lysis buffer were examined at thresholds of 200 and 300 copies/mL; and one DPS per patient eluted in 3 mL lysis buffer was examined at thresholds of 133 and 200 copies/mL. The threshold of 200 was chosen on the basis of this corresponding to an individual VL of 1000 HIV RNA copies/mL when 5 patient specimens are pooled, and 133 was chosen on the basis of correcting for the amount of eluant absorbed by the DBS (≈1mL of the original 3mL was absorbed). The other thresholds were multiples of 200.

Figure 1. Retrospective evaluation of laboratory form information to identify patient specimens suitable for pooled viral load testing. The strategy used to select demographic information on routine laboratory request forms that can be used as criteria to identify specimens with a low pretest probability of having HIV RNA loads >1000 copies/mL (ie, virologic failure as defined in this study). Specimens were excluded from pooled testing on the basis of the form containing no antiretroviral therapy information, the inclusion of a protease inhibitor (PI) in the regimen, or the patient being <15 years of age. Specimens that were eligible for pooled viral load testing were from patients at least 15 years of age who were receiving a nonnucleoside reverse-transcriptase inhibitor regimen.
**Results**

Establishing Screening Criteria for Patients with Low Probability of Virologic Failure

The process of identifying suitable selection criteria for patients with a low probability of failure is shown in Figure 1. The 2 criteria needed to identify a target population with virologic failure prevalence <10% on individual testing were age ≥15 years and treatment with a first-line (NNRTI-based) regimen. The evaluated retrospective sample had a failure rate of 14.2% before and 8.7% after applying these criteria (see Figure 1). The characteristics of the patient specimens included in the prospective study of pooled testing are shown in Table 1.

**Pooled Testing Using Blood Plasma**

A total of 400 stored specimens were available to be tested with the minipool—and 300 of these with the 10 × 10 matrix strategy. Specimens were tested in 80 minipools, and the mean time required to constitute 20 minipools (100 individual specimens) was 35.5 min. VL test results of 3 minipools were invalid; thus, all those 15 individual specimens required individual testing. In total, only 69.5% of the 400 tests that would have been needed for individual testing were used in the minipool strategy. This 30.5% reduction of tests used would have resulted in a reagent cost savings of US$1220 per 100 specimens, based on the cost for one VL test of US$40 (ZAR 300).

For the 10 × 10 matrix strategy, 300 patients were tested in 3 matrices. The mean time to constitute one matrix consisting of 100 individual samples was 45 min, ~10 min more than preparing minipools for the same number of specimens. Using the Measurement Enhanced Pooling Assay Calculator for deconvolution, only 59% of the tests needed for individual testing would have to be performed, resulting in a reagent savings of US$1640 per 100 specimens. The negative predictive value for both methods was >95% (Table 2).

**Pooled Viral Load Testing Using DBS and DPS**

When one DBS was eluted into 3 mL of lysis buffer, pooling efficiencies as compared with individual testing ranged from −10% to 20% depending on the VL threshold used to define a positive pool (133, 200, 400, and 1000 HIV RNA copies/mL). When 2 DBS were eluted in 10 mL of lysis buffer, pooling efficiencies were 5% and 25% for pool VL thresholds of 200 and 400 HIV RNA copies/mL, respectively (Table 3). The slight increase in efficiency was, however, offset by a decrease in negative predictive value when using the larger eluant volume (Table 3). The efficiency of DPS pooling, however, remained similar (57% vs 60%) for pool thresholds of 133 and 200 HIV RNA copies/mL. The associated increase in positive predictive value from 92% to 100% was attributable to a single pool with a VL of 190 copies/mL testing false-positive at the 133 HIV RNA copies threshold but not at 200. Only one DPS pool tested false-negative, and this pool had 1 specimen with a low VL of 1200 copies/mL (Table 3).

**Discussion**

The long-term clinical success of ART depends on regular VL monitoring inpatients, and the long-term sustainability of ART programs in resource-constrained countries will require the availability of VL testing. Although novel approaches, from point-of-care VL assays [22] to alternatives to the commercially available laboratory-based VL assays, are urgently needed [23], pooled testing could be implemented rapidly to substantially reduce the reagent costs of VL testing and thereby make VL testing more affordable. Currently available commercial assays require specialized equipment, well-equipped laboratories, and skilled staff; therefore, these assays are only viable in a few centralized laboratories, which could, nevertheless, improve their use of expensive resources through innovative but accurate testing strategies.

In our study, the 10 × 10 matrix pool strategy had a higher efficiency than the 5-specimen minipool strategy (41% reduction in VL tests needed, compared with 30.5%), most likely because of 3 minipools with invalid VL tests. However, the minipool strategy was easier in practice, in both time and expertise required to constitute and deconvolute the pools.
Moreover, the $10 \times 10$ matrix requires 100 samples to be available before any can be tested, whereas minipools require multiples of 5 specimens only, which could be preferable in terms of turnaround time enabling rapid clinical decision making. Furthermore, due to the difficulty of correctly constituting the $10 \times 10$ matrix, it may require experienced laboratory personnel, which could greatly limit its use in resource-constrained settings.

We also explored the use of DBS and DPS for pooled VL testing. Other studies [24–29], one of them using the same platform for VL testing [27], found similar results with DBS having a mean VL lower than in liquid plasma; however, this will most likely depend on the proportion of patients who have virological failure, where cell-associated HIV RNA may contribute more or less to the measured VL in a DBS, compared with that in liquid plasma. This could be especially relevant when DBS are pooled, as a larger volume of blood, containing more cells, is tested compared with individual DBS VL testing. Furthermore, the use of DBS is complicated by the variable plasma fraction due to differences in hematocrit between patients. The combination of these factors and the high variability could result in a falsely high VL when compared with individual plasma testing and could explain the observed poorer specificity and efficiency of pooled DBS testing. In contrast, pooled testing of DPS yielded less variability and excellent efficiency and accuracy at both pool thresholds of 133 copies and 200 HIV RNA copies/mL. The only measured difference between these 2 thresholds was due to one negative pool that had a VL result of 190 HIV RNA copies/mL and was thus false-positive at 133 copies and true-negative at the 200 HIV RNA copies/mL threshold. The one pool that tested false-negative had a specimen with a relatively low VL of 1,200 HIV RNA copies/milliliter.

The study sample evaluated with DPS did have a lower prevalence of virologic failure than the DBS sample (7.6% vs 9%). However, it is unlikely that this alone would account for the difference in observed efficiency between the methods, which most likely should be ascribed to the absence of cellular HIV RNA and less variability in the plasma, compared with whole blood input. In addition, the observed efficiency difference could be due to effects of inhibitory substances such as hemoglobin in DBS but not in DPS. The excellent efficiency of DPS pooling of about 60% could have resulted in cost savings of about US$2400 per 100 specimens screened for virologic failure.

### Table 2. Comparison of Minipools and Matrix Pools

<table>
<thead>
<tr>
<th>Method</th>
<th>Prevalence of Failure (&gt;1,000 RNA copies/mL)</th>
<th>Negative Predictive Value</th>
<th>Relative Efficiency (reduction in tests needed)</th>
<th>Cost Savings per 100 Specimens (US$40 per viral load test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 matrices ($n = 300$ specimens)</td>
<td>11%</td>
<td>98%</td>
<td>41%</td>
<td>US$1,640</td>
</tr>
<tr>
<td>80 minipools ($n = 400$ specimens)</td>
<td>9.50%</td>
<td>100%</td>
<td>30.5%</td>
<td>US$1,220</td>
</tr>
</tbody>
</table>

### Table 3. Efficiency and Accuracy of Minipool Viral Load Testing of Dried Blood Spots (DBS) and Dried Plasma Spots (DPS)

<table>
<thead>
<tr>
<th>Summary Results</th>
<th>At Pool Threshold (HIV RNA copies/mL)</th>
<th>Efficiency</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Positive Predictive Value</th>
<th>Negative Predictive Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>DBS</td>
<td>1 DBS in 3mL (20 minipools of 5)</td>
<td>133</td>
<td>–10.00%</td>
<td>100.00%</td>
<td>20.00%</td>
<td>100.00%</td>
</tr>
<tr>
<td></td>
<td>Efficiency</td>
<td>–5.00%</td>
<td>100.00%</td>
<td>30.00%</td>
<td>40.00%</td>
<td>53.33%</td>
</tr>
<tr>
<td></td>
<td>Sensitivity</td>
<td>5.00%</td>
<td>87.50%</td>
<td>40.00%</td>
<td>60.00%</td>
<td>53.85%</td>
</tr>
<tr>
<td></td>
<td>Specificity</td>
<td>5.00%</td>
<td>87.50%</td>
<td>40.00%</td>
<td>60.00%</td>
<td>60.00%</td>
</tr>
<tr>
<td></td>
<td>Negative Predictive Value</td>
<td>100.00%</td>
<td>100.00%</td>
<td>80.00%</td>
<td>75.00%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 DBS in 10mL(20 minipools of 5)</td>
<td>–5.00%</td>
<td>5.00%</td>
<td>45.45%</td>
<td>60.00%</td>
<td>60.00%</td>
</tr>
<tr>
<td></td>
<td>Efficiency</td>
<td>25.00%</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Sensitivity</td>
<td>88.89%</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Specificity</td>
<td>72.73%</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Positive Predictive Value</td>
<td>72.73%</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Negative Predictive Value</td>
<td>96.00%</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>DPS</td>
<td>1 DPS in 3 mL(37 minipools of 5)</td>
<td>133</td>
<td>57.30%</td>
<td>95.83%</td>
<td>92.31%</td>
<td>95.83%</td>
</tr>
<tr>
<td></td>
<td>Efficiency</td>
<td>60.00%</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Sensitivity</td>
<td>92.31%</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Specificity</td>
<td>100.00%</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Positive Predictive Value</td>
<td>100.00%</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Negative Predictive Value</td>
<td>96.00%</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>
The efficiencies for the liquid plasma minipool and matrix pooling experiments were lower than previously reported [20], where efficiencies for both strategies were above 50%. This could be due to the higher mean VL in our sample (log_{10}VL of 2.88 vs. 2.33 copies/mL) or in the case of the minipools, the 3 pools that had invalid VL test results. For DPS pooling, however, the efficiency was 60% and therefore comparable to this previous study. It is unlikely that the low prevalence of virologic failure (7.6%) of the sample of specimens included in the DPS experiment alone accounts for the better efficiency of DPS pooling than liquid plasma pooling. The improvement observed with DPS pooling probably results from the lower plasma volume input in the DPS and therefore higher detection limit, which could contribute to the observed lower false-positive rate (0%–2.7% for DPS minipools vs. 16.25% for liquid plasma minipools). This increased the overall efficiency, as fewer false-positive pools needed deconvolution compared with liquid plasma pooling using minipools.

One limitation of this study was that it was retrospective, in the sense that we compared the results of pooled VL testing of stored specimens to results of previously conducted routine individual VL testing. Specimens were thus exposed to one cycle of freezing and thawing between individual testing and pooled testing. Another limitation was that we did not compare the DBS- or DPS-pooled VL to DBS or DPS specimens tested individually but, rather, to known (previously tested) liquid plasma VL. These will need to be evaluated in a future prospective study. Furthermore the cost-efficiency of pooling is dependent on the variable costs of labor, reagents, and equipment and the possible different health impacts of VL monitoring in various settings, depending on the ART regimens that are available.

In this study, simple laboratory request form information was found to be valuable in identifying patients who have a low probability of virologic failure. This could be crucial for identifying which specimens are suited for pooled or individualized testing. We have also shown that in a resource-constrained setting, a combination of preselecting patients with low pretest probability of virologic ART failure followed by pooled VL testing can reduce costs without compromising accuracy, and that DPS minipooling was the most efficient method. Taken together, this could be very valuable when considering a centralized laboratory equipped with automated liquid-handling systems for preparing pools and software to assist with the deconvolution of positive pools. This would allow an increase in the number of patients tested without needing more VL tests, testing equipment, or staff.

Acknowledgments
We thank Dr A. Stich and the IRTG 1522 in Würzburg, Germany, for their approval and support, and Josué Pérez Santiago and Winston Tilghman, for their technical expertise.

Financial support. This study was supported by the National Health Laboratory Service, Tygerberg, South Africa, through the K-fund mechanism; National Institutes of Health (AI69432, MH62512, MH083552, AI077304, AI36214, AI047745, AI74621, and AI080353 to D.S.); the Veterans Affairs Healthcare System, San Diego (to D.S.); National Institute of Allergy and Infectious Diseases (K24 AI64086 to R.H.); and University of California, San Diego, Center for AIDS Research (AI36214 to R.H.).

Potential conflicts of interest. D. Smith has received grant support from ViV Pharmaceuticals.

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