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Pooled Nucleic Acid Testing to detect Antiretroviral Treatment Failure in HIV-infected Patients in Mozambique

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Abstract

Background—In resource-limited settings, viral load monitoring of HIV-infected patients receiving antiretroviral therapy (ART) is not readily available due to high costs. Here, we compared the accuracy and costs of quantitative and qualitative pooled methods to standard viral load testing.

Methods—Blood was collected prospectively from 461 patients receiving first-line ART in Mozambique who had not been evaluated previously with viral load testing. Screening for virologic failure of ART was performed quantitatively (i.e. standard viral loads) and qualitatively (one and two rounds of polymerase chain reaction; PCR). Individual samples and minipools of 5 samples were then analyzed using both methods. The relative efficiency, accuracy and costs of each method were calculated based on viral load thresholds for ART failure.

Results—Standard viral load testing of individual samples revealed a high rate of ART failure (19–23%) across all virologic failure thresholds, and the vast majority of the patients (93%) with viral loads >1,500 copies/ml had genotypic resistance to drugs in their ART regimen. Pooled quantitative screening and deconvolution testing had positive and negative predictive values exceeding 95% with cost savings of \$11,250 compared to quantitative testing of each sample individually. Pooled qualitative screening and deconvolution testing had a higher cost savings of \$30,147 for one PCR round and \$25,535 for two PCR rounds compared to quantitative testing each sample individually. Both pooled qualitative PCR methods had positive and negative predictive values 90%, but the pooled one-round PCR method had a sensitivity of 64%.

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Conclusions—Given the high rate of undiagnosed ART failure and drug resistance in this cohort, it is clear that virologic monitoring is urgently needed in this population. Here, we compared alternative methods of virologic monitoring to standard viral load testing of individual samples and found these methods to be cost saving and accurate. The test characteristics of each method will likely need to be considered for each local population before it is adopted.

Keywords

HIV; virologic monitoring; viral loads; resource limited settings

Introduction

Routine virologic monitoring of patients on antiretroviral therapy (ART) is recommended [1] in order to limit the **clinical consequences of ART failure and the** development and transmission of drug-resistant HIV [2]. However, viral load testing is expensive and requires commercial laboratory equipment, trained technicians and specialized maintenance that are not widely available in most resource-limited settings, where disease burden is high [3-5]. Alternative immunological, hematological, and clinical parameters have been suggested and utilized in some resource-limited settings to monitor patients for ART failure. For example, at our study site CD4 counts used for immunological monitoring cost \$5 per patient, while viral loads cost \$75 per patient sample, but CD4 counts are not as sensitive for detection of ART failure as viral load testing[5, 6]. To reduce costs of virologic monitoring, we prospectively evaluated the use of pooled nucleic acid testing using both quantitative (i.e. viral loads) and qualitative polymerase chain reaction (PCR) testing to detect virologic treatment failure in patients receiving first-line ART in Maputo, Mozambique.

Methods

Study population

Study subjects were consecutively recruited from the Centro de Saúde Primeiro de Maio in Maputo, Mozambique. **Any patient who was** receiving first-line ART, including nucleoside and non-nucleoside reverse transcriptase (RT) inhibitors (NRTIs and NNRTIs), **was eligible for the study**. None of the study subjects had received viral load testing previously, and study personnel enrolling subjects did not target enrollment based on previous laboratory or clinical measures, since these measures were not known by study personnel. Blood was collected via standard phlebotomy in the clinic by trained nurses.

Ethics approval

Ethics committee approval was obtained from the University of California San Diego Human Research Protections Program and the Mozambican National Committee of Bioethics for Health, and each participant signed an approved informed consent prior to enrollment in the study.

Individual viral load testing

Blood plasma was separated from collected whole blood (10 mL), aliquoted into 1-mL vials, and cryopreserved at -80°C . No aliquots were subjected to more than one freeze-thaw cycle.

The viral load of each individual patient sample was measured using the Abbott m2000 Real Time HIV-1 RNA Assay (Abbott Laboratories, North Chicago, Illinois), which has a lower limit of detection of 75 copies/mL, according to manufacturer's instructions.

Pooled nucleic acid testing

Based on previously described methods[3, 4, 7-11], blood plasma samples were combined to form minipools that were evaluated using both quantitative (i.e. viral load) and qualitative PCR assays[12]. Specifically, minipools were constructed by combining 100 μ L of blood plasma from 5 individual patient samples, for a total volume of 500 μ L per minipool. Two duplicate minipools were made for each group of 5 patient samples in order to evaluate both quantitative and qualitative testing.

Qualitative PCR—For each minipool, HIV RNA extraction, reverse transcription and two rounds of qualitative PCR amplification of the HIV-1 RT coding region were performed, as previously described[12].

Deconvolution of positive minipools

For quantitative testing minipools were deconvoluted based on the Measurement Enhanced Pooling Assay Calculator or MEPAC[10]. In brief, each individual sample in the pool was tested individually and each quantitative viral load result was entered into the calculator consecutively until the algorithm had accounted for all positive samples in the pools. Since numerical results were not available for the qualitative PCR assay, all patient samples included in positive pools were retested individually.

Drug Resistance

Genotyping was performed from HIV RNA extracted from individual samples with positive viral loads using the High Pure Viral RNA Kit protocol (Roche Diagnostics, Indianapolis, Indiana), as previously described[12]. The HIV-1 RT coding region was reverse transcribed using the Ambion® RETROscript kit (Applied Biosystems, Foster City, California), according to the manufacturer's instructions, and then the resultant target DNA was amplified and sequenced using the following hemi-nested primers:

First round:

CI-POL1 GGAAGAAATCTGTTGACTCAGATTGG (Forward)

3RT ACCCATCCAAAGGAATGGAGGTTCTTTC (Reverse)

Second round:

5RT AAATCCATACAATACTCCAGTATTTGC (Forward)

3RT ACCCATCCAAAGGAATGGAGGTTCTTTC (Reverse)

Population based sequencing of amplified product was performed as described previously [12]. GenBank Accession numbers (KP711655 - KP711747). Sequences were assessed for resistance associated mutations by entering them into the Stanford University HIV Drug Resistance Database[13].

Efficiency, Accuracy, and Cost Savings

Test characteristics of efficiency and accuracy were determined for pooled quantitative and qualitative testing, and both were compared to individual viral load testing. Relative efficiency was defined as the percentage of assays saved by using pooling methods rather than performing individual viral loads. For the qualitative PCR assay, relative efficiencies were determined for one and two PCR rounds. Accuracy of quantitative and qualitative pooled testing was calculated as sensitivity, specificity and positive and negative predictive values based on different thresholds of virologic failure (**500, 1000 and 1500** HIV RNA copies/mL). The costs of the viral load assays and of one or two rounds of the qualitative PCR assay were used to estimate the costs associated with each virologic monitoring method relative to individual viral load testing (Abbott m2000 Real Time HIV-1 RNA Assay), as previously described[8, 11, 14].

Results

Study Population

Study subjects (n=461) were recruited from **one clinic** in Maputo, Mozambique, **and** were receiving first-line ART, **At enrollment**, the study cohort was 61% female with a mean age of 39 years (**IQR 16**) and mean CD4 count of 358 cells/mL (**IQR 263**). Roughly half of participants (**54%**) started ART in 2010 or after, and almost two-thirds were receiving trimethoprim-sulfamethoxazole prophylaxis. Another two-thirds had history of opportunistic infections, the most common of which was CMV retinitis (29%, see Table 1). Based on individual viral load testing, 105 of the 461 patients (23%) had detectable viremia based on individual viral load testing (range 75-1,023,293 copies/mL), and most had viral loads >1,500 copies/ml (82%) (**Figure 1A**). Of these 105 individuals, the HIV-1 RT coding region was amplified from HIV RNA extracted from blood plasma in 93 individuals, and 89% had genotypic evidence of resistance to nevirapine and 83% to lamivudine (**Figure 1B**). The average viral load of those individuals for whom sequencing failed was 2,943 copies/ml (range: 98-10,233).

Pooled Quantitative (Viral Load) Testing

Viral load testing was performed on a total of 92 minipools of 5 individual samples (i.e. 460 patients), and the remaining sample was tested individually. One-third of the 92 pools (n=30) and the single individual sample had undetectable viral load (<75 copies/mL). The average relative efficiency was around one third regardless of the threshold used to define ART failure (i.e. threshold of 500, 1,000 and 1,500 copies/mL) (Table 2). The average relative efficiency was 37% at a threshold of 3,000 copies/ml, which is the current definition of virologic failure in Mozambique. In other words, pooled quantitative testing would have required around 150 fewer viral load assays than individual testing of all 461 samples. Based on **our** reagent and supply cost of \$75 per viral load assay, the cost of screening all 461 patients with pooled viral load testing would have resulted in saving around \$11,250 compared to the \$34,575 for individual viral load testing. The positive predictive values of pooled viral load testing were 100%, and the negative predictive values were above 95% regardless of the threshold value used to define ART failure (500, 1,000 and 1,500 copies/mL, see **Table 2**). Overall, the lack of variation based on viral load thresholds was

likely because almost all individuals with virologic failure had viral loads of 1,500 copies/ml or more (**Figure 1**).

Pooled Qualitative PCR Testing

As with the quantitative pooled method, the qualitative PCR assay was performed on 92 minipools of 5 individual samples and one individual sample. Out of these 93 screens, 41 (44%) had detectable viremia after one round of PCR, and 63 (68%) had detectable viremia after two rounds of PCR. Deconvolution of pools revealed that 67 and 100 patients had virologic failure (>75 copies/ml) using one and two rounds of PCR, respectively. The specificity and positive predictive value of the pooled qualitative assay were both 100% using only one round of PCR and >99% with second round PCR. The sensitivity and negative predictive values were lower at 64% and 90% for only one round of PCR, but both remained high at >90% for two rounds of PCR (Table 2). The relative efficiency of the qualitative pooled assay was 47% using one PCR round and 6% using two PCR rounds. In other words, 215 qualitative assays would have been saved by pooling samples for one round of PCR rather than testing samples individually, and only 20 assays would have been saved by pooling samples with two rounds of PCR. When two round PCR was used to screen each test individually, the specificity and positive predictive value were both 100%, and the sensitivity was 90% and negative predictive value 97%.

Cost Analysis of Qualitative Methods

Based on reagent and supply costs of (\$6) HIV RNA extraction, (\$12) RT PCR for first round PCR, and (\$2.5) for second round PCR, we calculated the costs of each rounds of PCR. We did not include labor costs in any of our estimates, since this would vary widely across the world. Qualitative PCR to screen all 461 samples individually with one round PCR was \$8,298 (461 samples $\times$ \$18/sample) and \$9,451 (461 samples $\times$ \$20.5/sample) for two rounds of PCR, and the pools of 461 samples (93 pools) with only the first round of PCR cost \$1,674 (93 pools $\times$ \$18/pool) and \$1,907 (93 pools $\times$ \$20.5/pool) for second round PCR screening. Costs of deconvolution of positive samples for first round PCR screening was \$2,754 (153 samples $\times$ \$18/sample) and \$7,134 (348 samples $\times$ \$20.50/sample) for second round PCR. Therefore, to screen the pools of 461 samples with deconvolution when a pool was found positive, then the one round PCR screening would cost \$4,428 (\$1,674 for screening + \$2,754 for deconvolution testing) and the two round PCR screening assay would cost \$9,041 (\$1,907 for screening + \$7,134 for deconvolution testing). When compared to testing each sample with a viral load (\$75/assay; \$34,575 for 461 samples), we would have saved \$26,277 if we screened each sample with one round PCR (\$34,575 – [461 samples $\times$ \$18/sample]) and \$25,125 with two round PCR (\$34,575 – [461 samples $\times$ \$20.5/sample]). With pooled screening and deconvolution testing, we would have saved \$30,147 with one round PCR (\$34,575 – [93 pools + 153 deconvolution samples] $\times$ \$18/sample) and \$25,535 with two round PCR (\$34,575 + [93 pools + 348 deconvolution samples] $\times$ \$20.5/sample). See **Table 2**. Parity in costs would be achieved with a viral load cost of \$20/assay.

Conclusion

Although viral load testing has been established as the standard of care for monitoring of patients on ART for treatment failure[1], its implementation in most resource-limited settings has been impeded by a variety of barriers, like sample processing, sample transport, and technical capacity. At our site the main barrier has been high costs[15, 16], **although these costs have been decreasing**. In this study, two pooling strategies were evaluated as a means to decrease the cost of virologic monitoring of a Mozambican cohort for ART failure. This is the first study to prospectively evaluate and compare such pooling methods with individual testing in a resource-limited setting that could benefit from such methods.

Mozambique was selected as an ideal setting in which to evaluate these methods, because the adult HIV prevalence is high (9.9%-12.9% compared to 0.8% global prevalence [17]), and viral load testing is currently recommended there but rare in actual practice due to costs. Further, virological monitoring maybe an urgent need given that in the capital Maputo, a transmitted drug resistance rate of <5% was calculated from multiple surveys[18]. We were especially interested in further validating a pooled qualitative PCR assay in Mozambique that would allow for subsequent genotyping to assess for drug resistance in cases of virologic failure[12].

This study found a high rate of virologic failure (>20%) among a large cohort of HIV-infected individuals who were enrolled from one clinic in Maputo sequentially based on receiving first line ART. There were no further selection criteria that would bias participants to have an increased likelihood of virologic failure. Further, most individuals with virologic failure had very high viral loads, with 82% having >1500 HIV RNA copies/ml in their blood plasma. Such high rates of virologic failure would be expected to impact the relative efficiency of the pooling strategies[9], and the relative efficiencies of these methods were lower than in those of other studies, ranging from 6-47%. The two extremes were among the qualitative pooling platforms, while the quantitative platforms with various thresholds to define virologic failure were all around 30%. Despite a range of relative efficiencies, all methods demonstrated at least some cost savings with excellent positive and negative predictive values and specificity. The qualitative pooling platform using only one round of PCR had the best relative efficiency (47% representing \$26,277 cost savings) but it also had the poorest sensitivity (64%), basically missing 38 individuals with viral loads >75 HIV RNA copies/ml. The qualitative pooling platform using two rounds of PCR had a cost savings of \$25,535 with a sensitivity of >90%, but this cost savings, as compared to individual viral loads, was similar (\$25,125) to performing two rounds of qualitative PCR on each individual sample, i.e. pooling only saved an extra \$410 in reagent and supply costs.

If the viral load assay approached \$20 per test, then it would be on par with the cost of the most efficient pooled platform. However, this calculation does not include the cost of the commercial viral load platform, which is variable throughout the world but always considerably more than the generic thermocyclers and centrifuges needed for the qualitative PCR method, unless provided by donation. Additionally, the current study had excellent technical staff that performed both viral loads and qualitative PCR, so it remains unclear whether variation in technical capacity would impact costs and efficacy of the methods. It is

clear, however, that the pooling methods would require additional technician time and capability that could impact costs and efficacy. This would need to be considered when choosing a method for monitoring virologic control of prescribed ART.

Concerning drug resistance, this cohort of patients were almost all receiving the same ART regimen of nevirapine, lamuvidine and zidovudine (96%) when they were evaluated, and when viral loads were detectable above 75 HIV RNA copies/ml and virus was amplifiable with two rounds of PCR, 97% of evaluable patients had at least some viral resistance to their prescribed therapy. This observation suggests that in locations where resources are limited, drug resistance testing perhaps is not necessary when viral loads are detectable in the setting of prescribed ART that contains nevirapine and lamuvidine. Lack of adherence to their prescribed ART was likely the cause of no detectable resistance in the remaining 3 individuals. Either way, the prescribed ART was not meeting these patients' needs (e.g. resistance, tolerability, adherence), and probably required a change.

With the high rate of undiagnosed ART failure in this cohort, this study clearly identified that virologic monitoring is urgently needed in Mozambique. If the reason for the lack of such monitoring currently is cost, then the methods evaluated in this study could help. This study presents a range of choices that could be adopted for the local health system. On costs alone, the pooled one round of PCR screening method would be the least expensive. Two rounds of qualitative PCR on individual samples or minipools represented considerable cost savings over individual viral load testing, and both had higher sensitivity than pooled one round of PCR screening. Once a regular virologic monitoring is in place, the rate of virologic failure should decrease, as failures are identified, adherence is encouraged and therapy is modified[19].

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Figure 1.
Distribution of Viral Loads (A) and Resistance Associated Mutations (B) during Therapy Failure.

Table 1

Study Cohort Demographics and Clinical Factors

Average Age (years)	39, range: 18-73
Female	61%
Education	
None	5.34%
Literate	4.91%
Primary	52.56%
Secondary	31.41%
College	3.85%
CD4 Counts (range)	
Average current	358 (1-554)
Average nadir (%)	169 (1-60)
Start of ART	
2001	0.2%
2002	0.2%
2003	0.8%
2004	4.2%
2005	4.0%
2006	5.2%
2007	7.3%
2008	8.4%
2009	15.7%
2010	14.9%
2011	19.9%
2012	19.3%
Current ART	
Stavudine	2.9%
Zidovudine	96.8%
Lamuvudine	100.00%
Nevirapine	97.5%
Efavirenz	2.5%
Prophylaxis	
Cotrimoxazole	62.4%
Dapsone	1.0%

Average Age (years)	39, range: 18-73
History Opportunistic Infections	
Cytomegalovirus Retinitis	29.49%
Cryptococcal meningitis	0.95%
Kaposi sarcoma	13.23%
Extrapulmonary Tuberculosis	7.94%
Mycobacterium Avium Intracellular	0.2%
Pneumocystis jiroveci	3.78%
Pulmonary Tuberculosis	13.61%
Toxoplasmosis	2.46%
Herpes Zoster	17.01%
None	38.75%

Table 2

Pooled Testing

	Quantitative (Virologic Failure Threshold)			Qualitative (PCR)	
	500 (copies/mL)	1000 (copies/mL)	1500 (copies/mL)	PCR 1st round	PCR 2nd round
Relative Efficiency (%)	31	32	32	47	6
Sensitivity (%)	85	86	83	64	95
Specificity (%)	100	100	100	100	100
Positive Predictive Value (%)	100	100	100	100	99
Negative Predictive Value (%)	96	97	96	90	99
Cost Savings Compared to Individual Viral Load Testing *	\$11,400	\$11,250	\$11,250	\$26,277	\$25,125

* Viral load testing each sample: \$34,575, \$: US dollars