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A qualitative PCR minipool strategy to screen for virologic failure and antiretroviral drug resistance in South African patients on first-line antiretroviral therapy

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ABSTRACT

Background: The high cost of commercial HIV-1 viral load tests for monitoring of patients on antiretroviral treatment limits their use in resource-constrained settings. Commercial genotypic antiretroviral resistance testing is even more costly, yet it provides important benefits.
Objectives: We sought to determine the sensitivity and negative predictive value of a qualitative PCR targeting partial reverse transcriptase for detection of virologic failure when 5 patient specimens are pooled.
Study design: A total of 300 South African routine patient samples were included and tested in 60 pools of 5 samples each. A qualitative nested PCR was optimised for testing pools and individual samples from positive pools. All positive samples were sequenced to detect drug resistance-associated mutations. Results were compared to those of conventional viral load monitoring.
Results: Twenty-two of 60 pools tested positive. Individual testing yielded 29 positive individual samples. Twenty-six patients had viral loads of above 1000 copies/ml. The pooling algorithm detected 24 of those 26 patients, resulting in a negative predictive value of 99.3%, and a positive predictive value of 89.7%. The sensitivity for detecting patients failing therapy was 92%, with a specificity of 98.9%. Of the patients failing first-line ART, 83.3% had NRTI and 91.7% NNRTI resistance mutations.
Conclusions: The pooled testing algorithm presented here required 43% fewer assays than conventional viral load testing. In addition to offering a potential cost saving over individual viral load testing, it also provided drug resistance information which is not available routinely in resourced-limited settings.

1. Background

The enormous increase in the number of patients receiving antiretroviral therapy (ART) worldwide is a remarkable success story but also poses a considerable burden on resource-limited countries due to the cost of drugs, medical care and laboratory monitoring [1].

As part of its public health approach, the World Health Organisation (WHO) recommends non-nucleoside reverse transcriptase inhibitor (NNRTI)-based first-line ART regimens for adult patients and for those failing first-line ART a boosted protease inhibitor (PI)-based second-line regimen [2,3]. This approach has been adopted by many countries, including South Africa [4]. ART failure may be determined in different ways. Monitoring of HIV viral load detects failure very early and is more specific than clinical and immunological (CD4 count) monitoring; therefore routine viral load monitoring is the gold standard and recommended where available [2,5]. However, the technical complexity of available viral load assays and their cost hamper access to viral load monitoring in most resource-limited settings [6,7].

Dried blood spots (DBS) prepared from blood obtained through skin pricks are widely used to diagnose HIV infection in infants [8], and can also be used for viral load monitoring [7,9,10], which may

Abbreviations: ART, antiretroviral therapy; WHO, World Health Organisation; PI, protease inhibitor; DBS, dried blood spot; ADR, antiretroviral drug resistance; NHLS, National Health Laboratory Service; RT, Reverse Transcriptase Coding Region of Pol Gene; NNRTI, non-nucleoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; DRT, drug resistance testing.

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reduce overall cost and increase access. However the high cost of viral load testing still remains an obstacle.

Pooled viral load testing can save costs by reducing the number of tests needed [11–13] and can be combined with the use of DBS [14,15]. However, a viral load value above a clinical threshold (in South Africa currently 1000 viral RNA copies/ml in line with current WHO guidelines) cannot ascertain the cause of treatment failure [16], which may be due to suboptimal drug levels secondary to insufficient adherence or drug and food interactions etc., or due to drug resistant virus [14,17]. According to a recent WHO report, approximately 30% of patients failing first-line ART have no detectable resistance mutations [18] but are at risk of being unnecessarily switched to more costly and less well tolerated second-line ART regimens.

In resource-rich countries, antiretroviral drug resistance (ADR) testing is used to determine whether virological failure is due to drug resistance. However, although the need for ADR testing has been recognised, and despite recently published guidelines, a lack of testing capacity and budget constraints seriously hamper their application [19].

Recently, a novel approach combining testing of pooled samples by qualitative PCR with sequencing of positive samples was proposed [20]. If a pool tests positive, each sample in that pool is tested individually with the same assay and any positive sample is sequenced using the amplified product. This approach is very elegant in that it is more affordable than individual viral load monitoring through the use of pooling and of a qualitative instead of a quantitative PCR, while providing valuable additional information on the presence of resistance mutations for those who are failing treatment.

2. Objectives

This study aimed to validate and adapt this approach for a Southern African setting, where HIV-1 subtype C is most prevalent, with the specific aim of determining the sensitivity and negative predictive value the qualitative PCR targeting partial reverse transcriptase for detection of virologic failure when 5 patient specimens are pooled, and to determine the prevalence of mutations detected by sequencing the PCR product of individual specimens from positive pools.

3. Study design

3.1. Study population

Samples received at the Diagnostic Virology Laboratory of the National Health Laboratory Service (NHLS) Tygerberg in Cape Town, South Africa, between May 2013 and June 2013 for routine HIV viral load testing were sequentially selected if they met the following inclusion criteria: adult patient; on first-line ART; no HIV viral load testing in the previous 4 months; sufficient specimen volume left after routine testing.

3.2. Specimens

Routine viral load testing, using the Abbott RealTime HIV-1 assay with a limit of detection of 40 copies/ml, was performed on all samples as requested and residual specimens then used for the study. A total of 300 samples were included, pooled into 60 pools of 5 samples each.

The person performing the study testing was blinded to the routine HIV viral load result which was revealed only once the pooled testing algorithm was completed.

Table 1
Primers, reagents and equipment.

First round one-step RT-PCR	Second round PCR
Forward Primer (Mj3)	Forward primer (5RT long C)
5′-AGTAGGACCTACACCTGTCA-3′	5′CTGAAAATCCATATAACACTCCAATATTTGY-3′
HXB2 position – 2480 → 2499	HXB2 position – 2704 → 2734
Reverse Primer (Mj4)	Reverse Primer (4RT)
5′-CTGTTAGTGCTTTGGTTCCTCT-3′	5′-GATGGAGTTCATACCCCATCCA-3′
HXB2 position – 3399 → 3420	HXB2 position –3234 → 3255
Kit:	Kit:
SuperScript™ III One-Step RT-PCR System, Invitrogen (Carlsbad, CA, USA)	GoTaq® DNA Polymerase, Promega (Madison, WI, USA)

RT-PCR – reverse transcription polymerase chain reaction.

3.3. Pooling of samples and nucleic acid extraction

Pools consisted of 100 μl of each of 5 individual samples, resulting in a total pool volume of 500 μl. Nucleic acid extraction from pooled samples was performed using the NucliSENS® easyMAG® system (Biomérieux, Marcy l'Etoile, France). Of an elution volume of 100 μl, 5 μl were used for reverse transcription and first-round PCR.

3.4. PCR amplification

Initially the primers from Tilghman et al. [20] and a single-round PCR were used, but due to insufficient sensitivity for detecting viral loads below 10,000 copies/ml of HIV-1 subtype C from pooled specimens, this approach was abandoned and a new PCR designed and optimised.

This nested PCR uses previously described primers Mj3 and Mj4 [21] as outer primers and a new set of nested (inner) primers, 4RT (amino acid position in RT 229 → 236) and 5RT long C (amino acid position in RT 52 → 62) [22]. These were designed to amplify with high sensitivity a conserved region in HIV-1 group M viruses including subtype C, and for the short amplification product to allow efficient sequencing using the nested primers while including the most important resistance-related mutations in the reverse transcriptase coding region (RT) of the pol gene. Primer sequences and positions are shown in Table 1.

The first round is a one-step RT-PCR, with the reaction mixture consisting of 14 μl of nuclease free water, 2 μl each of Mj3 and Mj4 (10 μM), 2 μl of Superscript III Taq mix and 25 μl of 2× reaction mix (a buffer containing 0.4 mM of each dNTP, 3.2 mM $MgSO_4$), to give a final reaction volume of 50 μl. The second round reaction mixture consisted of 11.25 μl of nuclease free water, 5 μl of 5× Go Flexi buffer, 0.5 μl of dNTPs, 2 μl of MgCl (25 μM), 2 μl each of 5RT long C and 4RT (10 μM), 0.25 μl of Go Taq polymerase and 2 μl of amplified product from the first round. Thermal cycling was performed on the GeneAmp® PCR System 9700, Applied Biosystems (Foster City, CA, USA). The presence or absence of amplified product was assessed using agarose gel electrophoresis.

3.5. Deconvolution of positive pools and genotypic resistance testing

If a pool tested positive, all five individual samples contained in the pool were tested separately. For this, samples were re-extracted individually with the NucliSENS® easyMAG® system, using 100 μl of sample and 100 μl elution volume. First and second round reactions were carried out as for the pooled testing. Any positive individual sample was subjected to standard Sanger sequencing using a validated in-house protocol (validated against the ABI Prism Dye Terminator Cycle sequencing kit), which has also been used

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