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

Authors

Howard Newman [1*]

Lukas Breunig[1,2]

Gert van Zyl [1]

August Stich [2]

Wolfgang Preiser [1]

[1]Division of Medical Virology, Department of Pathology, National Health Laboratory Service (NHLS) Tygerberg and Stellenbosch University, Cape Town, Western Cape, South Africa.

[2]Medical Mission Institute and University of Würzburg, Würzburg, Germany

[*]Corresponding author. Email: howard.newman@nhls.ac.za. Tel: +27219389347

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 per millilitre. 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.

Key words

Pooled testing

ART monitoring

HIV drug resistance

Cost effectiveness

Resource limited setting

Abbreviations

ART – Antiretroviral Therapy

WHO – World Health Organization

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

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 Organization (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 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 [11,12,13] 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 RealT*ime* 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.

3.3 Pooling of samples and nucleic acid extraction

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

3.4 PCR amplification

Initially the primers from Tilghman [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 14µl of nuclease free water, 2µl each of Mj3 and Mj4 (10µM), 2µl of Superscript III Taq mix and 25µl of 2X reaction mix (a buffer containing 0.4mM of each dNTP, 3.2mM $MgSO_4$), to give a final reaction volume of 50µl. The second round reaction mixture consisted of 11.25µl of nuclease free water, 5µl of 5X Go Flexi buffer, 0.5µl of dNTPs, 2µl of MgCl (25µM), 2µl each of 5RT long C and 4RT (10µM), 0.25µl of Go Taq polymerase and 2µl 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 100µl of sample and 100µl 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 in previous studies [17] and is currently the diagnostic method at Tygerberg Hospital, using the amplified second-round PCR product and 5RT long C and 4RT (the same forward and reverse primers used for second round amplification, respectively) as sequencing primers. The sequencing platform used was the 3130xl Genetic Analyzer, Applied Biosystems (Foster City, CA, USA). The Stanford HIV db Program, available fromhttp://hivdb.stanford.edu/, was used for sequence analysis and resistance interpretation. This allowed identification of the most common NNRTI and nucleoside reverse transcriptase inhibitors (NRTI) associated mutations [14,17]. Table 1 shows the primer sequences as well as the kits and equipment used.

3.6 Evaluation of pooled testing results

After pooled testing, deconvolution of positive pools and genotypic resistance testing of individual positive samples, results were compared to routine HIV viral load test results. In South Africa, virologic failure is defined as a viral load of 1000 copies per millilitre or greater [3].

4. Results

Table 2 shows the ART data for the study population. The majority of patients were on the current first-line ART regimen in South Africa, consisting of tenofovir, lamivudine and efavirenz.

Twenty-two of the 60 pools were positive. Individual testing revealed 29 (9.7%) samples with a detectable viral load. Of these, 26 samples (8.7%) had viral loads of above 1000 viral RNA copies per millilitre and were therefore defined as failing ART. Pooled testing detected 24 of those 26 patients.The two samples that were missed were retested using the pools and individually, confirming the lack of amplification. Both patients were females on tenofovir-based ART regimens, with viral loads of 8596 copies/ml and 38542 copies/ml, respectively. For the latter, residual extract was available for standard genotyping, which confirmed this particular strain to be genotype C.

The negative predictive value of pooled testing was 99.3% and the sensitivity for detecting patients failing therapy 92%. Table 3 summarises the results of pooled testing.

Most positive samples did have antiretroviral drug resistance-associated mutations. Figure 1 and Figure 2 illustrate the prevalence of various resistance mutations detected for NRTI and NNRTI, respectively. Of the patients failing first-line ART, 83.3% had NRTI resistance and 91.7% had NNRTI resistance. The commonest NRTI mutations were M184V/I and K65R, with 75% and 25% among patients failing ART, respectively, while the commonest NNRTI mutation was K103N in 46% of failing patients.

5. Discussion

The pooled testing algorithm presented here was able to detect 24 of 26 adult patients failing first-line ART according to WHO and South African national criteria and provided drug resistance information on the failing patients.

Pooled viral load testing has been shown to decrease the cost of virological monitoring in adults on first-line ART who have a low prevalence of failure [14,23]. Qualitative rather than quantitative detection of failure has previously been shown to reduce the cost of virological monitoring [24], and sequencing of the reverse transcriptase coding region of the pol gene alone likewise reduces costs compared to longer sequences [25].

An approach that combines pooled testing, qualitative detection of virological failure and reverse trancriptase-only sequencing for detection of first-line NNRTI-based ART regimen failure was developed by Tilghman et al in 2012 [20]. Pools consisting of five individual specimens are tested by qualitative PCR, which could be followed by deconvolution of positive pools and sequencing of individual positive specimens. In their study, deconvolution of positive pools was not done due to insufficient sample volumes, but this proof-of-principle experiment has shown promise in the USA [20]. However, this approach may in fact be needed more urgently in resource-constrained settings where virological monitoring is not available widely at present. We therefore validated this approach in a Southern African context, where HIV-1 subtype C is most prevalent and patient characteristics differ markedly from industrialised countries. We found poor amplification when using the primers from Tilghman et al, 2012, in our setting where subtype C dominates, which can be explained by too many mismatches when aligned with HIV-1 subtype C *in silico*. We therefore designed primers, 5RT long C and 4RT, primarily for subtype C but also considering other group M subtypes, as our inner primers, while using previously described primers, Mj3 and Mj4 [21], as outer primers.

We were able to achieve a sensitivity of 92% and a positive predictive value of 89.7% for detecting patients who were failing ART according to WHO criteria (i.e.

with a viral load of > 1000 copies / ml), missing two patients out of a total of 26 with ART failure, with a specificity of 98.9%. The most likely reason for the lower sensitivity is probably primer-template mismatches, which are a common problem with amplification and sequencing of a highly divergent virus [26,27], where intra-clade differences in the RT region for subtype C are nearly 6% [26], possibly explaining the reason for the slightly lower sensitivity for detecting ART failure compared to the Tilghman study in North America, where subtype B is predominant. However, with a negative predictive value of greater than 99%, and being able to provide drug resistance information for patients failing first-line ART, our study provides proof of principle that the concept developed by Tilghman et al. is viable in a Southern African context and possibly beyond.

Table 4 highlights the potential cost savings and benefits of the pooling algorithm over individual viral load monitoring. Although there may be potential cost savings based on reagent costs and number of tests, the most substantial benefit is likely to be earlier and appropriate switching to second-line ART, limiting switching of patients to those who indeed have antiretroviral drug resistance and avoiding switching to more expensive and less well tolerated second-line ART unnecessarily in the absence of resistance mutations. A recent WHO report stated that in approximately 70% of first-line ART failure cases, at least one drug resistance mutation was detected. Conversely, in the remaining 30%, no resistance mutations were detected [18,28]. In countries where DRT is not available, these patients may have been switched unnecessarily to more expensive second-line treatment. Therefore, preventing the latter could potentially result in a cost saving.

In addition, some studies have shown that delaying switching to second-line ART after first-line failure is associated with increased risk of failing second-line ART, with

poorer patient outcomes [29,30], whereas other studies suggest that NRTI resistance may not matter in this regard [31,32]. This notwithstanding, there is uncertainty regarding the long term durability of second-line ART in the presence of significant NRTI resistance mutations.

One of the limitations of this study stems from a lack of a full cost effectiveness analysis. This would be needed in future to prove the cost benefit and convince policymakers in poorer countries that the qualitative pooling algorithm including resistance testing could allow for cheaper, more efficient, and more clinically useful ART monitoring.

Another limitation is that in the interest of sensitivity and sequencing using only two primers, two major reverse transcriptase resistance associated mutations were excluded: M41L, which confers low level resistance to AZT or D4T, and M230L, a major NNRTI mutation. In our setting, M41L occurs in only 2.8% of patients failing a first-line regimen containing a NNRTI and a thymidine analogue and was the only thymidine analogue mutation in only ¼ of these patients [17]. M230L occurs in 6.5% of patients who fail an EFV-based regimen [17], however it very rarely (0.1%) occurs as the only NNRTI mutation (G. van Zyl, 2014, unpublished results from 858 sequences from patients failing an EFV regimen). The exclusion of these mutations therefore should have minimal impact on the clinical predictive value of our genotyping results.

In dealing with the dramatic consequences of the HIV pandemic, early detection of the presence or absence of drug resistance mutations in patients failing ART is vitally important to minimise the cost of delayed or unnecessary switching to second-line therapy, especially in resource limited settings. The pooling algorithm first presented by Tilghman et al in 2012 [20], and here adapted for a Southern African

context, is a potential solution to this problem, and provides an affordable approach to detecting first-line therapy failure and simultaneously differentiating poor adherence to medication from major drug resistance mutations as the cause of failure.

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Competing Interests

We hereby declare that there is no conflict of interest.

Ethical Approval

The study protocol was approved by the health research ethics committee of Stellenbosch University (reference S12/03/064).

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Table 1.Primers, reagents and equipment.

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

RT-PCR – reverse transcription polymerase chain reaction

Table2. ART data for n= 300 patients included in the study

CD4 count (nX10^6 per litre)	**Males n= 87 (%)**	**Females n= 213 (%)**	**Total n= 300 (%)**
> 500	20 (23)	109 (51)	129 (43)
351 - 500	34 (39)	45 (21)	79 (26)
200 - 350	21 (24)	44 (20)	65 (22)
< 200	12 (14)	15 (7)	27 (9)
ART Regimen			
ART 1	52 (60)	111 (52)	163 (54)
ART 2	2 (2)	17 (8)	19 (6)
ART 3	1 (1)	27 (13)	28 (9)
ART 4	20 (23)	22 (10)	42 (14)
ART 5	10 (11)	8 (4)	18 (6)
ART 6	2 (2)	28 (13)	30 (10)

ART regimen 1 = tenofovir, lamivudine, efavirenz
ART regimen 2 = zidovudine, lamivudine, efavirenz
ART regimen 3 = zidovudine, lamivudine, nevirapine
ART regimen 4 = stavudine, lamivudine, efavirenz
ART regimen 5 = stavudine, lamivudine, nevirapine
ART regimen 6 = tenofovir, lamivudine, nevirapine

Table 3.Results of pooled testing.

No. of sample tested in total	300
No. of individual samples tested negative	271
No. of individual samples tested positive	29
No. of individual samples with VL > 1000 cps/ml	26
No. of pools tested in total	60
No. of pools tested negative	38
No. of pools tested positive	22
No. of samples with VL > 1000 cps /ml detected by pooled testing	24
Sensitivity for detecting samples with > 1000 cps/ml	92.0 %
No. of true negative individual samples	269
Negative predictive value	99.3%
VL –HIV viral load ; cps/ml – copies per millilitre	

Table 4. Potential cost saving and benefits of pooling assay

Current ART monitoring in South Africa	Pooling Algorithm
Approximate reagent cost 300 patients → 300 viral load tests Reagent cost per reaction ~ R100 Therefore, total reagent cost ~ R30000	**Approximate reagent cost** 300 patients → 60 pools ; 22 positive pools 22 X 5 samples per positive pool = 110 Total no. of reactions = 170 Reagent cost per PCR reaction ~ R100 Therefore total reagent cost for PCR ~ R17000 Reagent cost per test for DRT ~ R172 Therefore total reagent cost for 29 tests ~ R4988 Therefore total reagent cost for pooling algorithm = R21988
Advantages Gold standard for virological monitoring	**Advantages** Potentially cheaper than viral load monitoring Drug resistance information provided Excellent negative predictive value May allow for earlier switching to second-line therapy May prevent unnecessary switching to second-line therapy
Disadvantages Expensive No drug resistance information	**Disadvantages** Not as sensitive for detecting patients with viral loads > 1000 copies/ml

Figure 1: Prevalence of NRTI resistance mutations detected using pooling method algorithm

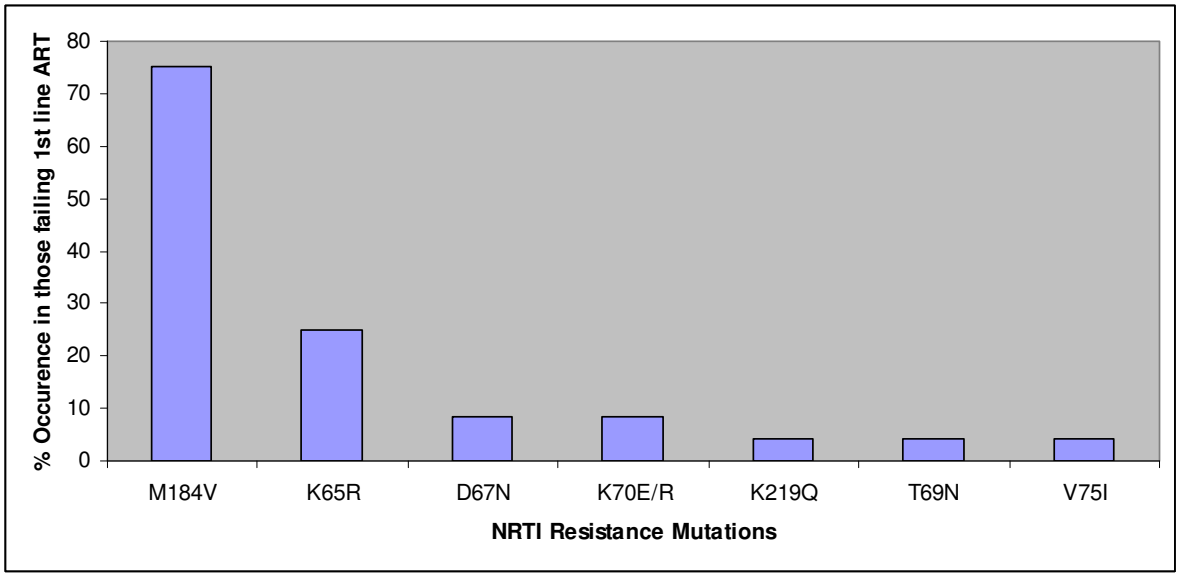
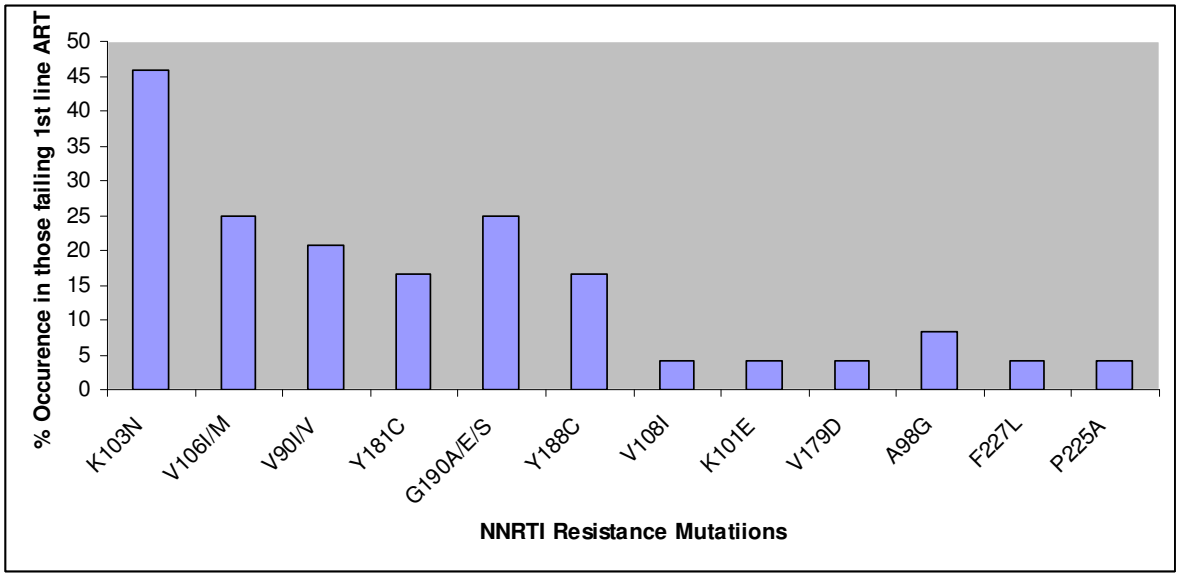


Figure 2: Prevalence of NNRTI resistance mutations detected using pooling method algorithm



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