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Pooled nucleic acid testing strategy for monitoring HIV-1 treatment in resource limited settings

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ABSTRACT

Background: : Virological monitoring (VM) and drug resistance (DR) analysis are crucial for effective HIV management. Due to the high cost of commercial assays, VM and DR analysis is not performed in resource-limited-settings.

Objective: : The objective of this study is to develop a pooling based algorithm for the combined identification of virologic treatment failure (VTF) by nucleic acid testing (NAT) and DR by sequencing - NAT + DR assay.

Study Design: : We enrolled 559 participants on first-line therapy and analyzed for VTF. The virologically suppressed participants were followed-up to see the VTF prevalence (> 1000 copies/mL) and DR by the NAT + DR pooling. Each pool comprising 5 plasma samples were amplified by targeting reverse transcriptase gene, if found positive, the pool was deconvoluted and samples were individually tested for HIV RNA and DR. Assay characteristics of NAT + DR assay were calculated in comparison with commercial assay.

Results: : Of 559 participants, 67 had VTF at baseline and were excluded. Of the remaining 478 participants, 325 returned for follow-up and NAT + DR assay was performed for them. Of 65 pools tested, 13 pools were positive. On deconvolution 14 individuals were found to have VTF. Sensitivity, specificity, positive predictive value and negative predictive value was 100%, relative efficiency was 59% and 87% & 85% cost was saved for identifying VTF and combined identification of VTF and DR, respectively.

Conclusions: : Pooled NAT + DR assay is likely a good strategy to drastically reduce the cost and sustainability of the VM and can thereby facilitate the scale-up of successful HIV treatment programs, and reduce unnecessary switching to second-line drugs in resource-limited-settings.

1. Background

Globally 36.9 million people are living with HIV (PLWH), and about 21.7 million of these people receive HIV treatment [1]. With this, the scale up of antiretroviral treatment (ART) over the past couple of decades, HIV incidence has declined [2,3]. Thus, UNAIDS launched an ambitious 90-90-90 target by 2020, to end the AIDS epidemic by 2030 [4]. The third '90' is to have 90% of PLWH on HIV treatment and achieving virological suppression, which is a crucial piece of the plan. One of the major challenges in achieving this '90' target of virological suppression is the emergence of HIV drug resistance (DR) and lack of viral load testing in resource limited settings.

Some PLWH receiving treatment can experience loss of viral suppression. Such treatment failure (TF) is often associated with the emergence of HIV DR, which can hamper the efficacy of ongoing ART and lead to accumulation of DR mutations (DRMs) and cause cross-resistance to other ART class and affect future ART options [5]. The timely detection of TF is performed through the regular monitoring of viral loads [6,7]. Though WHO has recommended viral load based monitoring as the primary methodology for ART monitoring and emphasize DR testing before ART initiation and TF, viral load and DR testing cannot be adopted in many resource limited settings due to the cost of the assay. In view of this, we developed and validated a cost-effective pooling based approach for the combined identification of

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virologic TF (VTF) and HIV DR testing (nucleic acid testing (NAT) and DR by sequencing - NAT + DR assay).

2. Objective

The objective of this research work is to develop and validate NAT + DR assay for the combined identification of virological failure and drug resistance and to evaluate the cost-effectivenss of the assay compared to the conventional method of testing

3. Study design

3.1. Study population

We consecutively enrolled participants living with HIV who were on first-line ART regimens that included two nucles(t)ide reverse transcriptase inhibitors (NRTI) and one non-nucles(t)ide reverse transcriptase inhibitors (NNRTI),at YRG CARE, Chennai. These participants were followed until their next visit to YRG CARE to study the rate of VTF (> 1000 copies/ml), and to develop pooling based combined nucleic acid testing (NAT) and drug resistance (DR) assay (NAT + DR assay). The study participants who had viral load > 1000 copies/mL at enrollment (VF, WHO 2013) were not included for further follow-up. For each participant, HIV-1 plasma viral load was performed at the time of enrollment and at the time follow-up. If participant was identified as TF, DR testing was performed (Fig. 1).

3.2. Ethical approval

This study was approved by the institutional review board of YRG CARE and institutional ethical committee of University of California San Diego Human Research Protections Program

3.3. HIV-1 plasma viral load (Individual testing)

Quantitative HIV-1 plasma viral load was performed with Abbot m2000sp HIV-1 viral load assay (Abbott molecular inc, Illinois, USA) following 0.6 mL protocol following manufacturer's instruction.

3.4. Pooling based NAT + DR assay

The pooling PCR was developed to identify VF from a pool of 5 participants, assuming if, at least one participant has VL of 1000 copies/mL and others have undetectable viral load, the pooling based NAT should be reliable to amplify 200 copies/mL [8]. This was evaluated by serially diluting the plasma samples for which viral load is already known and or diluting or directly including the copy controls obtained from virology quality assurance (VQA) [9]. Pooling was done based on the previously described methods.200 µL plasma sample were pooled from a consecutively enrolled 5 participants to form a minipool (1 mL). Each minipool was subjected to NAT as follows: RNA was extracted from 1 mL blood plasma using Promega RNA extraction kit (04J70-26). The extracted RNA was subjected to cDNA conversion with 10 µL RNA as described by Saravanan et al [10]. Then, 10 µL of cDNA was subjected to 1st round PCR with 1X Buffer, 200µM dNTPs, 0.4µM of forward (NA) NA–5′- AAG CCA GGA ATG GAT GGA CCA-3′and reverse (NNE) NNE– 5′-CCA TTT ATC AGG ATG GAG TTC-3′primers and 1.25U of Taq enzyme (Intron, South Korea). Next, the 5 µL of 1st round product was subjected to nested PCR with 1X Buffer, 200µM dNTPs, 0.5µM forward (OutFC) 5′-CCT ATT GAA ACT GTA CCA GT-3′and reverse (OutRC) 5′-ACTGTCCATTTATCAGGATG-3′ primers and 1.25U of Taq polymerase enzyme (Intron, South Korea). The final PCR product was checked for band in 1% agarose gel electrophoresis; if band was seen at the appropriate size (636 base pairs), then the pool was considered positive.

3.4.1. Deconvolution of positive pool

The positive pool was deconvoluted by individual testing, i.e., 1 mL plasma from each participant's plasma in the pool was subjected to NAT as explained above, with little modifications, 5 µL RNA was used instead of 10 µL RNA in cDNA reaction and 5 µL cDNA product was used instead of 10 µL cDNA product in the first-round reaction. The amplified nested product was purified by GeneJet PCR purification kit (Thermo Scientific, CA, USA). The purified product was subjected to sequencing PCR with nested PCR primers, using BigDye terminator V3.1 (ABI, CA, USA) and sequenced with ABI 3100 Avant Genetic analyzer (ABI, CA, USA). Subtyping of HIV-1 sequence was done with REGA HIV-1 subtyping tool v3.0, DRM was analyzed by Stanford HIV drug resistance database v8.7 and quality analysis of the sequence was done by Calibrated Population Resistance tool.

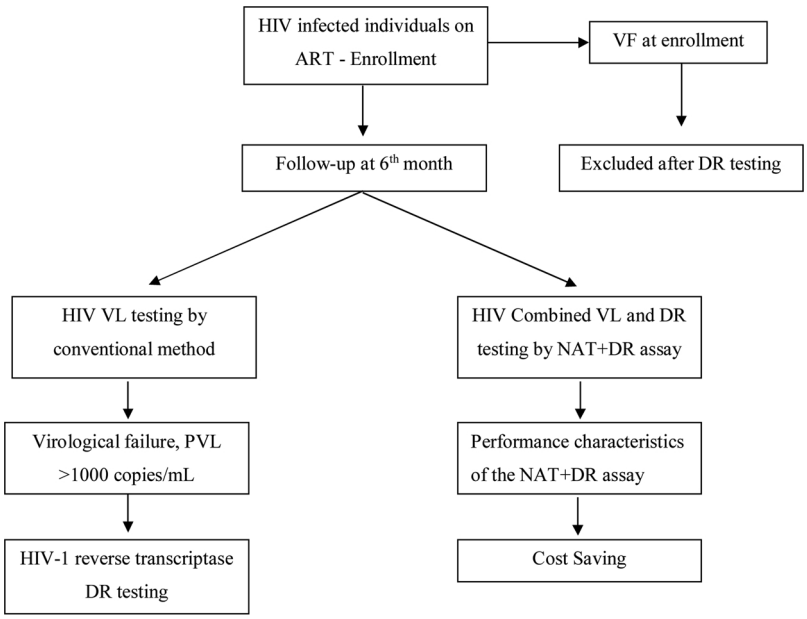

Fig. 1. Flowchart depicting study design.

3.4.2. Performance characteristics of NAT + DR assay

The performance characteristics such as sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and Relative efficiency was calculated for NAT + DR assay in identifying VTF in comparison with standard viral load assay as described by May et al [11].

3.4.3. Cost saving

Cost saving was calculated as: (i) the price in $ required to perform NAT assay in identifying VF, compared to standard viral load assay, and (ii) the price in $ required for combined identification of VF and DR by NAT + DR assay, compared to the individual viral load screening followed by DR testing, which is the standard of care method.

The continuous variables are recorded as median and interquartile range (IQR) and categorical variables are recorded as number and percentage. All statistical analysis was performed with GraphPad Prism 5.0 (GP Prism, CA, USA)

4. Results

In this study, 559 participants were enrolled, their median age was 39 years (IQR 33–44), and most participants were female (56%) (Table 1). Of 559 participants, 67 had VTF detected at enrollment. The DR testing of these 67 participants revealed, 9 (13%) had no DRMs, 43 (64%) had NRTI DRMs and 58 (87%) had NNRTI DRMs. Major NRTI mutations seen among these participants were M184 V 35 (52%), K65R 17 (25%) and K70E 12 (18%) and major NNRTI mutations seen were K103 N 36 (54%) and V106 M 25 (37%). The participants who had VTF at their first visit were excluded from the further analysis. The remaining 478 participants were eligible for the next part of the study and 325 returned for follow-up and evaluated by the proposed NAT + DR platform. The median age of these 325 participants was 39 years (IQR 33–34) and 277 (55%) were female.

Of the 325 samples, 65 pools were subjected to NAT + DR assay. Of the 65 pools tested, 13 pools were found to be NAT positive, and all the samples in the positive pools were tested individually. Of the 13 positive pools, one sample was found to be positive in 12 pools and one pool had two positive samples, hence 14 (4.3%) samples were identified as positive by NAT. We also tested all blood samples individually with a viral load and found that all the 14 samples identified positive by NAT were identified as positive by the individual viral load, and all the samples identified negative by NAT were found to be negative by viral load testing the blood sample individually. As a part of NAT + DR platform, DR testing was next performed from the nested amplification product of deconvoluted individual testing. The DRM patterns and demographic characteristics of samples with VTF is shown in Table 2.

Table 1
Demographic characteristic of study participants.

Particulars	Participants on first line ART(n = 559)
Age (median)	39 (IQR 33 - 44)
Female (%)	313 (55.9%)
Male (%)	246 (44.6%)
Duration of first-line HAART, in months (median)	8 (IQR 6-12)
ART Details	
TDF/3TC/EFV	531(95 %)
AZT/3TC/NVP	5 (0.9%)
AZT/3TC/EFV	5 (0.9%)
ABC/3TC/EFV	7 (1.2%)
FTC/TDF/EFV	11 (2%)
Median CD4 Count (Cells/mm^3)	290 (IQR 153-422)
Median PVL (Copies/mL)	4369 (IQR 179 -67155)

TDF - Tenovofivr disoproxil fumarate, 3TC - Lamivudine, FTC, - emtricitabine, ABC - Abacavir, AZT - Azidothymidine, EFV - Efavirenz, NVP – Nevirapine.

Median age of the participants TF was 42 years (IQR 37–44), majority were female 8 (57%), edian viral load was 29,184 copies/mL (IQR 4079 - 74,233) and median duration on ART was 17 months (IQR 13–22).

4.1. Performance characteristics

The sensitivity, specificity, PPV and NPV of the NAT + DR assay in identifying TF was 100%. The relative efficiency of the NAT + DR in identifying VTF was 59%. In other words, 41% less testing was performed using pooled NAT in comparison to individual viral load testing.

4.2. Cost saving

For cost saving calculations, following prices were assumed based on the kit cost. Cost/test for performing HIV-1 VL assay by Abbott HIV-1 RealTime PCR assay was 36$ and cost/test for performing DR assay by conventional method was 30$. The cost/test for performing NAT assay was 12$ and the cost/test for performing DR assay, from NAT product (NAT + DR) was 17$.

By conventional individual viral load testing, 325 samples were tested, so the total cost was 325 × 36$ = 11,700$ and by NAT, 130 (total tests performed) X 12$ = 1560$. Hence, by the pooled NAT assay 87% cost could be saved. By individual viral load testing, 11,700$ would be required and 420$ would be required for DR testing of the 14 VTF cases in our study. Hence, 12,120$ would be required for identifying VTF and resistance by conventional testing. By NAT + DR assay, as explained above, 1560$ would be required for NAT and 14 × 17$ = 238$ would be required for DR assay, thus, 1798$ would be required for NAT + DR assay. Hence, 85% cost could be saved using the NAT + DR platform versus standard individual vial load and DR testing.

5. Discussion

Monitoring of TF is commonly performed clinically, immunologically and virologically [12]. Since, virological monitoring (VM) identifies VTF early, followed by immunological monitoring and clinical monitoring; VM is the preferred choice in developed countries [13]. Since 2013, WHO has recommended VM as the preferred method to identify VTF [14]. However due to the requirements such as shipping of plasma specimens under cold storage condition, limited availability of infrastructure and cost of the assays, viral load test was not performed in many resource limited settings. Many methods have been developed such as dried blood spot (DBS) VL testing [15], reducing the reagent volume [16] and point of care testing to reduce the cost as well as to increase the ease of VL testing.

In addition to VL testing, DR testing is also important as it determines the selection of next sequence of drug after treatment failure. Studies conducted in HIV infected adult and children shows, substantial proportion of patients who receive NNRTI based first-line therapy fail virologically during first 5 years [17,18]. Of these participants, 70–90% develops HIV DR, which limits the future treatment options; without DR testing, it is hard to select the appropriate therapy for next line of therapy [19–22]. This shows, VL testing cannot be viewed in isolation and it has to be tested along with DR testing.

In this study we developed a pooling PCR based combined VL and DR testing to identify TF and HIV drug resistance. The NAT + DR algorithm identified all 14 patients failing first-line ART and provided DR data for them. Pooled NAT + DR assay will be cost effective, only if, qualitative rather than quantitative based method is employed [23], prevalence of TF is low [24] and sequencing of short region rather than long region is employed [25]. In our study, the prevalence of virological failure was 4%, qualitative method was employed and short region of reverse transcriptase spanning 18–230 amino acids. Hence we achieved 87% cost saving for identifying VTF and 85% cost saving for combined identification of TF and DR. The rate of VTF is low in our setting

Table 2
Demographic characteristics and DRM pattern identified among failures.

	Age	Sex	PVL (cps/mL)	ART Regimen	Duration on ART (months)	NRTI DRMs	NNRTI DRMs
PID 1	50	F	6602	TDF/3TC/EFV	13	K65R	V106M,Y188C
PID 2	42	M	3238	TDF/3TC/EFV	14	None	K103KN
PID 3	32	M	47587	TDF/3TC/EFV	17	None	K103KN
PID 4	42	M	1095	TDF/3TC/EFV	17	None	None
PID 5	51	F	9716	TDF/3TC/EFV	19	M41L	K103KN,V106VM
PID 6	41	F	10781	TDF/3TC/EFV	10	None	K103KN,V106VM
PID 7	38	F	77998	AZT/3TC/EFV	23	None	None
PID 8	42	F	1508	TDF/3TC/EFV	28	None	None
PID 9	37	M	140493	TDF/3TC/EFV	11	None	K103N
PID 10	37	F	72583	TDF/3TC/EFV	24	M184V	K103N,V108I,P225PH
PID 11	44	F	54679	TDF/3TC/EFV	34	None	K103N
PID 12	58	M	1525	TDF/3TC/EFV	15	D67G,K70E,M184V	K103N,V106M,Y181C
PID 13	34	M	553552	TDF/3TC/EFV	12	L74LI,Y115YF,M184V,K219KE	K103N,V106M
PID 14	28	F	74783	ABC/3TC/EFV	17	None	V106M

compared to results from resource constrained countries of Asia and Africa, where 11–28% prevalence was reported [26–30]. This assays will work in the settings where a maximum of, less than 30% TF is seen [31], since the pooling based testing is effective only if the prevalence is less than 30%. In view of these, in many resource limited settings the prevalence of TF is less than 30% and hence this algorithm can be adopted in resource limited settings. Pooling based algorithm can also be designed for a minipool comprising 10 pools per sample, which would be very cost-effective; however, the sensitivity of the pooling PCR in identifying one VF (with a VL close to 1000 copies/mL) in a pool of 10 samples will be very difficult.

The combined NAT + DR testing were earlier done in United States [32] and South Africa [33]. In the former, the NAT + DR assay was compared with the Roche ultra-sensitive Amplicor HIV-1 monitor viral load assay, and they reported the sensitivity of 100% and specificity of < 80% and 71% cost saving. However, due to limited sample availability, deconvolution was not performed in real time. In the latter, NAT + DR assay was compared with Abbott HIV-1 RealTime PCR assay as done in our setting, and they reported the sensitivity and specificity of 92% and 99% and cost saving of 27%. However, in our setting we achieved sensitivity, specificity, PPV, and NPV of 100% and 85% cost saving.

Concerning HIV-1 drug resistance, the NAT + DR assay amplified all the samples, and 21% of participants have not exhibited any mutation for both NRTI and NNRTI. 36% had NRTI resistance and 79% had NNRTI resistance. K103 N mutation causing resistance to EFV and NVP were the most common mutation seen.

In our study, cost effectiveness was high and was similar for both identifying VTF and DR, hence, this assay could be adopted only for identifying VTF. However, we strongly recommended that this assay should be used for identifying both VTF and DR. WHO has reported that among failures 70% exhibit at least one DRM and 30% exhibit no DRM [34]. In the setting where, drug is switched without DR testing, there is not only the risk of switching to wrong regimen but 30% of the patients would be unnecessarily switched to second-line regimen even in the absence of mutation.

In dealing with the test and treat strategy and increase in the rapid ART coverage, implementation of periodical VM and detection of DRM in TF is very important to reduce wrong switching and unnecessary switching to limited second line treatment options, especially in resource limited settings. Although the pooling PCR we developed is not pan genotypic, the method, which combines the identification of VF and DR can be used in resource limited settings where subtype C virus is prevalent. In future, pan genotypic pooling algorithm should be developed, which should target IN, PR and POL gene for the use in monitoring first line, second line and third line therapy.

Author contributions

J Boobalan, T.R. Dinesha: Methodology, validation, formal analysis, investigation & writing - original draft. **S. Gomathi, S. Sivamalar, E. Elakkiya:** Methodology, writing - original draft. **A Pradeep, D Chitra:** Formal analysis, resources, writing - review editing. **KG Murugavel, P Balakrishnan, S Shantha, SS Solomon:** Supervision, investigation, writing - review & editing. **N Kumarasamy, DM Smith, S Saravanan:** Conceptualization, funding acquisition, project administration, supervision and writing - review & editing

Conflict of interest

None declared.

CRediT authorship contribution statement

Methodology, Validation, Formal analysis, Investigation, Writing - original draft.Methodology, Validation, Formal analysis, Investigation, Writing - original draft.Methodology, Writing - original draft.Methodology, Writing - original draft.Formal analysis, Resources, Writing - review & editing.Formal analysis, Resources, Writing - review & editing.Supervision, Investigation, Writing - review & editing.Supervision, Investigation, Writing - review & editing.Supervision, Investigation, Writing - review & editing.Supervision, Investigation, Writing - review & editing.Conceptualization, Funding acquisition, Project administration, Supervision, Writing - review & editing.Conceptualization, Funding acquisition, Project administration, Supervision, Writing - review & editing.Conceptualization, Funding acquisition, Project administration, Supervision, Writing - review & editing.

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