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## Viraemia and HIV-1 drug resistance mutations among patients receiving antiretroviral treatment in Mozambique

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**Summary** This study was conducted among individuals taking first-line antiretroviral treatment (ART) for at least 12 months under programme conditions in Maputo, Mozambique in order to report on the level of detectable viraemia and the proportion and types of drug resistance mutations among those with detectable viral loads. HIV-1 RNA viral load levels (lower detection limit <50 copies/ml) were measured, and resistance mutations were sequenced. One hundred and forty-nine consecutive patients (69% females, median age 36 years) were included after a mean follow-up time of 23 months. One hundred and seven (72%; 95% CI 64–79) had undetectable viral load, while in 42 (28%, 95% CI 21–36) viral load was detectable (range 50–58 884 copies/ml). From 15 patients with viral load >1000 copies/ml, 12 viruses were sequenced: eight were C subtypes and four were circulating recombinant forms (CRF08). Eight (5%; 95% CI 2–9) patients with detectable viral load had one or more major resistance mutations. Nucleoside reverse transcriptase inhibitor (NRTI) and non-NRTI mutations were observed. There were no major mutations for resistance to protease inhibitors. In Maputo, the level of detectable viraemia is reassuringly low. While embarking on ART scale-up, wider surveillance is warranted to monitor programme quality and limit the development of drug resistance, which remains a major potential challenge for the future of ART in Africa.

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## 1. Introduction

Mozambique, a resource-poor country in sub-Saharan Africa, faces a serious HIV epidemic with a prevalence of 16% in adults aged 15–49 years. In 2005, an estimated 140 000 deaths in adults and children were attributed to HIV/AIDS.<sup>1</sup>

Antiretroviral treatment (ART) can dramatically improve the survival of people living with HIV/AIDS<sup>2</sup> and Mozambique embarked on a national ART scale-up plan in 2005.<sup>3</sup> Effective ART achieves persistent viral suppression (the virus stays below the detection limit of common assays in blood) and immunological recovery. If viral suppression is not achieved for whatever reason, the chronology of events is that the viral load increases to detectable levels, leading in a second step to a significant drop in CD4 count (immunological failure) and the development of new or recurrent opportunistic infections (clinical failure).<sup>4</sup> A detectable viral load implies ongoing viral replication, which fosters the development of drug-resistant mutations and constitutes a major potential problem for the future of ART in Africa.<sup>4,5</sup>

Viral load monitoring, which typically measures HIV-1 RNA concentrations in plasma, is the most reliable method for detecting adherence problems and early treatment failure. In industrialized countries, individuals on ART have both viral load and CD4 levels measured regularly as part of routine follow-up. In Mozambique, as in many other low-resource countries, routine viral load monitoring is not yet feasible as this depends on the availability of sophisticated laboratory facilities. Patient monitoring thus relies on clinical assessment and, when available, CD4 monitoring. Consequently, virological failure is considered only in the event of clinical deterioration and/or decrease of CD4 count.<sup>4</sup> The unavoidable lag period, of up to two years, between virological failure and these two events is associated with the development of drug resistance.

Since 2002, Médecins sans Frontières (MSF) has been supporting the Mozambican Ministry of Health (MoH) to offer ART under routine programme conditions in Mavalane district, Maputo. Within a sample of individuals placed on first-line ART for at least one year, we report on the level of detectable viraemia and the proportion and types of drug resistance mutations among those with detectable viral loads.

## 2. Methods

### 2.1. Study setting and population

The study was conducted between June and December 2006 in one of the main public day clinics (Primeiro de Maio) that offer ART in Maputo. The study population included consecutive ARV-naïve individuals who had completed at least 12 months first-line ARV treatment, which in Mozambique comprises a fixed-dose combination of stavudine (d4T), lamivudine (3TC) and nevirapine (NVP) (Triomune), with zidovudine (AZT) and efavirenz (EFV) in association with lamivudine as alternatives in case of side-effects. All HIV-related medication and consultation is provided free of charge.

All HIV-positive individuals who present at the clinic undergo a complete medical assessment for HIV-related

diseases. ART eligibility is guided by CD4 count and in accordance with WHO guidelines.<sup>2</sup> Prior to ART initiation patients receive at least four counselling/educational sessions (individual and group-based), and are encouraged to choose a 'treatment buddy', who will be the cornerstone of adherence and support at home. After initiation, patients are individually followed monthly (clinical check and adherence counselling) and invited to join support group activities. All counselling/educational activities are provided by trained lay personnel. Immunological monitoring (CD4 count) is done on a six monthly basis. Adherence to ART is graded according to both pill count and punctuality of attendance at scheduled clinical consultations.

At the time of the study, the HIV clinic had 2139 patients on highly active antiretroviral therapy and was initiating ART at a rate of 100 new patients per month. At the end of 2006, standardized treatment outcomes for the entire cohort included 81% alive and on ART, 8.5% died, 8.5% lost to follow up, 0% stopped and 2% transferred out.

### 2.2. Specimens and laboratory procedures

Viral loads and genotyping were performed on specimens transported to the Retrovirology Laboratory, Luxembourg. Plasma was separated from 5 ml of drawn blood and 100  $\mu$ l was placed on two separate points on a filter paper (Whatman Schleicher & Schuell FTA Mini Cards, Kent, UK) and then dried to obtain dried plasma spots (DPS). The cards were packed individually in plastic zip-log sachets with desiccant packs and stored on site at 4 °C until transport. The remaining plasma was stored at –20 °C. Local laboratory workers found the DPS specimens easy to use, store and transport.

Viral load detection was by quantitative RNA-PCR using DPS; the lower detection limit of the assay was <50 copies/ml. Results obtained from DPS were subjected to a standardized correction factor based on average known differences in viral load levels obtained from plasma (the gold standard) and DPS stored at 4 °C for a period of 1–10 weeks. Drug resistance genotyping was performed on plasma stored at –20 °C and transported on dry ice. Genotyping was performed on specimens having a viral load of  $\geq$  1000 copies/ml and drug-resistant mutations compared with the HXB2 wild type strain were listed. For the purpose of this study, virological failure was defined as a viral load of  $\geq$  50 copies/ml, which is considered the gold standard threshold.

### 2.3. Data collection, sample size and statistical analysis

A structured record form and patient cards were used to gather information. These were entered into standardized monitoring software (FUCHIA, Epicentre, Paris, France). The main outcome measure of this study was the level of detectable viraemia from 12 months after starting first-line ART (virological failure) and the proportion and types of drug resistance among those with detectable viraemia. A previous MSF study conducted in South Africa<sup>6</sup> showed a virological failure rate of 15% at 12 months. We assumed a similar scenario and aimed to detect up to 21% virological failure with 95% confidence and 80% power. This required at least 136 individuals and was the basis of the sample size.

**Table 1** Characteristics of patients with and without detectable ( $\geq 50$  copies/ml) viral loads

	Viral load < 50 copies/ml	Viral load $\geq 50$ copies/ml	P-value <sup>a</sup>
Total (n = 149)	107 (72) [95% CI 64–79]	42 (28) [95% CI 21–36]	–
Gender (n) (%)			
Male	34 (32)	13 (31)	0.92
Female	73 (68)	29 (69)	
Mean age (years)	36.9	34.9	0.57
Baseline WHO clinical stage (n) (%)			
Stage 1	12 (11)	5 (12)	0.86
Stage 2	14 (13)	7 (17)	0.57
Stage 3	58 (54)	21 (50)	0.64
Stage 4	23 (21)	9 (21)	0.99
Baseline body mass index (kg/m <sup>2</sup> )	21.3	20.6	0.54
Baseline CD4 count (cells/ $\mu$ l) (mean)	178	103	0.65
Substitution of at least one ARV drug <sup>b</sup> (n) (%)	19 (18)	13 (31)	0.07
Interrupted <sup>c</sup> ART for at least one month (n) (%)	1 (0.9)	3 (7)	0.12
Delay in attending scheduled appointments <sup>d</sup> (n) (%)	15 (14)	6 (14)	0.57
Duration on ART $\geq 24$ months (n) (%)	44 (41)	22 (52)	0.21

ARV: antiretroviral; ART: antiretroviral treatment.

<sup>a</sup>  $\chi^2$  test for categorical variables and the Mann-Whitney *U* test for continuous variables.

<sup>b</sup> Antiretroviral drug substitution by alternative drug due to specific toxicity.

<sup>c</sup> ART interruption due to initiation of tuberculosis treatment (1), severe skin reaction (1), suspected lactic acidosis (1), unknown reason (1).

<sup>d</sup> Patients arriving with delay for at least one drug refill in the six months prior to study enrolment.

This sample size also exceeded the number of consecutive specimens recommended by the WHO to monitor HIV drug resistance prevention in sentinel ART sites.<sup>7,8</sup> Differences between groups were compared using the  $\chi^2$  test for categorical variables and the Mann-Whitney *U* test for continuous variables. The level of significance was set at  $P = 0.05$  or less and 95% CIs were used throughout. Analyses were performed using SPSS version 10.1 (SPSS Inc., Chicago, IL, USA).

### 3. Results

#### 3.1. Characteristics of the study population

At the time of the study there were 1314 ART-naive patients attending Primeiro de Maio Health Centre who had completed at least 12 months of first-line ART. From these, a total of 149 consecutive patients (69% women) on follow-up visits were included in the study after a mean time of 23 months [interquartile range (IQR) 17–28] on ART.

Median age at the start of ART (baseline) was 36.4 years (IQR 29–42); 75% were in WHO clinical stage 3 or 4; median CD4 count was 115 cells/ $\mu$ l (IQR 58–204) and 23% had a body mass index under 18.5 kg/m<sup>2</sup>. ART regimens included d4T/3TC/NVP in 132 (89%) patients, AZT/3TC/NVP in 11 (7%) patients and d4T/3TC/EFV in 6 (4%) patients. Median CD4 count at the time of our study was 381 cells/ $\mu$ l (IQR 252–529; median gain compared to baseline 266 cells/ $\mu$ l)

#### 3.2. Viral loads and drug resistance

Of 149 patients who had their viral load measured using DPS, 107 (72%, 95% CI 64–79) had an undetectable viral load (<50 copies/ml), while 42 (28%, 95% CI 21–36) had a detectable

viral load (range 50–58 884 copies/ml). The latter included 10 (7% 95% CI 3–11) with a viral load of 50–399 copies/ml, 17 (11%, 95% CI 7–18) with a viral load 400–999 copies/ml, 10 (7%, 95% CI 3–11) with 1000–9999 copies/ml and 5 (3%, 95% CI 0.4–5) with  $\geq 10\,000$  copies/ml. The characteristics of patients with and without a detectable viral load are shown in Table 1.

Of 15 (10%) specimens with viral load  $\geq 1000$  copies/ml, three were not amplifiable. Of the twelve sequenced specimens, eight belonged to subtype C and four were circulating recombinant forms (CRF08). Eight (5%, 95% CI 2–9) had one or more major mutations. The major nucleoside reverse transcriptase inhibitor mutations (NRTI) mutations were M184V ( $n = 7$ ), T215F ( $n = 4$ ) and D67N ( $n = 1$ ) while non-NRTI (NNRTI) mutations included K103N ( $n = 3$ ), M230L ( $n = 1$ ), K101E ( $n = 1$ ), V1081 ( $n = 2$ ), Y181C ( $n = 4$ ), V106M ( $n = 3$ ), F227L ( $n = 1$ ) and G190A ( $n = 1$ ) (Table 2). Five patients were infected with viruses exhibiting a thymidine analogue mutation (TAM, D67N, T215F). All sequenced isolates displayed a series of polymorphisms at residue positions that have been associated with protease resistance in subtype B HIV-1. These substitutions are natural polymorphisms in non-B subtypes and thus were not interpreted as resistance-coding mutations.

Clinical criteria for suspicion of virological failure (>50 copies/ml) were satisfied in only four (9.5%) patients; three had apparent unspecified skin conditions and/or weight loss of >10% and one had developed an episode of extra-pulmonary tuberculosis (EPTB). The WHO immunological criterion for suspicion of virological failure ( $\geq 50\%$  drop in CD4 counts from the on-treatment peak<sup>2,9</sup>) was satisfied in only three (9%) patients with a detectable viral load. All three had viral load titres  $\geq 10\,000$  virus copies/ml (two had mutations and one was the EPTB case). Five patients had

**Table 2** HIV-1 subtypes and profile of drug resistance mutations, Maputo, Mozambique

Patient No.	HIV-1 subtype	HIV-1 RNA (copies/ml)	Resistance mutations to NNRTI (RT mutations)	Mutations associated with resistance to:	Resistance mutations to NNRTI (RT mutations)	Mutations associated with resistance to:	Resistance mutations to protease inhibitors (P mutations)
1	C	58 884	—	—	—	—	—
2	C	27 542	T215F + M184V	d4T, ZDV, FTC, 3TC	K103N/M230L	NVP, EFV	—
3	C	1308	—	—	—	—	—
4	C	19 498	M184V	3TC, FTC	K101E/V1081/Y181C K103N	NVP, EFV NVP, EFV	—
5	CRF08	4786	—	—	V106M/Y181C	NVP, EFV	—
6	CRF08	10 233	T215F + M184V	d4T, ZDV, FTC, 3TC	—	—	—
7	CRF08	2089	—	—	—	—	—
8	C	33 884	T215F + M184V	d4T, ZDV, FTC, 3TC	K103N/Y181C	NVP, EFV	—
9	C	1096	M184V	3TC, FTC	V106M/F227L	NVP, EFV	—
10	C	6607	—	—	—	—	—
11	CRF08	5623	T215F + M184V	d4T, ZDV, FTC, 3TC	V106M/G190A	NVP, EFV	—
12	C	3631	D67N + M184V	d4T, ZDV, FTC, 3TC	V1081/Y181C	NVP, EFV	—

NNRTI: nucleoside reverse transcriptase inhibitor; NNRTI: non-nucleoside reverse transcriptase inhibitor; RT: reverse transcriptase (major mutations); d4T: stavudine; ZDV: zidovudine; FTC: emtricitabine; 3TC: lamivudine; NVP: nevirapine; EFV: efavirenz.

crossed the WHO threshold for virological failure (defined as  $\geq 10\,000$  copies/ml), of which four (with the exception of one case of EPTB) exhibited major resistance mutations.

Six cases with resistance mutations in this study were switched to appropriate second-line ART regimens, while two cases remained on first-line therapy because of a viral load  $< 10\,000$  copies/ml. All eight patients received intensified adherence support and close monitoring. Eighteen months after the study, four cases on second-line and one case on first-line therapy still presented undetectable viral load, two cases on second line were failing virologically and one case on first-line therapy had a detectable viral load associated with an episode of PTB. The patient with EPTB had an undetectable viral load on completion of EPTB treatment.

#### 4. Discussion

Among individuals placed on first-line ART regimens under routine programme conditions in Maputo, the level of detectable viraemia and drug resistance after a mean follow-up time of 23 months was reassuringly low. The fact that 72% of patients had an undetectable viral load compares well with that reported from both developed countries<sup>9</sup> and other resource-limited settings in Africa.<sup>10</sup> Reports from South Africa, Cote d'Ivoire and Kenya showed the proportion of patients with undetectable viraemia to be 51% (95% CI 26–75) at 24 months.<sup>10</sup> Our figure also exceeds the 70% recommended by WHO for drug resistance prevention at 12 months on treatment.<sup>7</sup>

The low level of drug resistance is encouraging in this setting with restricted access to second-line ART as it shows that implementation of ART can be achieved with limited emergence of resistant strains. Another study from Mozambique similarly showed a limited presence of resistant HIV-1 strains.<sup>11</sup> We attribute these acceptable results to a number of factors, including standardized ART regimens and fixed-dose combinations, clear and simple management protocols, uninterrupted drug procurement and supply, ART offered free-of-charge, and a standardized monitoring system. We are of the opinion that lay counsellors have played a critical role in supporting treatment adherence in our setting as they are likely to have positively influenced patient empowerment,<sup>12,13</sup> which in turn is likely to have favourably influenced the level of viral suppression. Lay counsellors are not recognized within Ministry of Health structures in Mozambique but our finding of very low resistance is, in our opinion, an important tribute to their work that should be officially recognised. Specific studies are, however, required to establish the relationship between lay counsellors and viral suppression. In the meantime, Mozambique has a tremendous shortage of skilled health workers, and trained lay counsellors could be actively involved within the scale-up efforts.

Sub-Saharan African countries, including Mozambique, are rolling out ART to thousands of patients countrywide. Even in a best-case scenario of a 5% (95% CI 2–9) resistance level, clinics which initiate about 100 patients per month could have between two and nine patients of this cohort developing resistance mutations in the medium term.

If this is multiplied by the hundreds of ART initiation sites embarking on ART rollout countrywide, the overall impact on the transmission and prevalence of drug resistant mutations cannot be underestimated.

Of the 42 patients with a viral load  $\geq 50$  copies/ml only eight were found with drug resistance mutations. This suggests that in the 81% of patients with a detectable viral load but no apparent mutations, adherence was inadequate for some reason, leading to a real risk that these patients would develop drug resistance over time. Even if we assumed that viral loads of under 1000 copies/ml might be related to “blips” (transient viraemia that returns spontaneously to undetectable levels without apparent clinical consequences,<sup>14,15</sup>) there were only five (33%) of 15 patients with viral load titres  $\geq 1000$  copies/ml who had clinical and/or immunological signs suggesting failure. This implies that routine clinical examinations, pill-count monitoring, immunological and other existing forms of adherence monitoring would still miss the great majority of patients having inadequate viral suppression. Even when there is excellent adherence with pill counting, self-reporting or punctuality to clinic visits (measures most developing countries rely upon for monitoring adherence), these have been shown to correlate poorly and tend to overestimate adherence as has been demonstrated in Blantyre, Malawi.<sup>16</sup>

In Khayelitsha, South Africa, where routine viral load testing is performed, patients with detectable viral loads are offered intensive adherence support and counselling and the viral load is reassessed four weeks later. The ability of viral loads to return to an undetectable level correlated with the timing of detection and the subsequent adherence support intervention provided.<sup>17</sup> This emphasizes the important role of viral load measurement as a sensitive and useful operational support tool for adherence. The development of an easy-to-use, semi-quantitative and inexpensive rapid test to detect early virological failure is not a luxury and would play an important role in optimizing the response to both first- and second-line therapy in developing countries.<sup>17,18</sup>

One patient who developed EPTB and who was on both anti-TB treatment and ART was found to have a viral load of  $>10\,000$  copies/ml but with no mutations. Although we do not know the precise reasons for this high viral load, it is likely that this patient stopped ART completely, since in the absence of drug pressure, mutations do not occur. Another possibility might be the presence of active TB, which increases viral loads, although not usually to such an extent. In any case, the fact that the viral load subsequently fell to an undetectable level on completion of TB treatment is encouraging and suggests that in patients who develop an active opportunistic infection, particularly TB,<sup>19</sup> during the course of ART, further adherence support and viral load monitoring would be justified without rushing to a treatment switch.

The strengths of this study are that it was conducted under routine programme conditions and the findings are thus likely to reflect the operational reality. We also used an easy-to-use DPS technique to collect and store specimens and the fact that the yield was very satisfactory implies that this tool could facilitate wider surveillance.<sup>20–22</sup>

In terms of limitations, the sample was not subjected to drug resistance monitoring prior to ART initiation (base-

line resistance testing) and we thus do not know the level of primary resistance in the cohort. We primarily aimed to monitor viral suppression as a measure of HIV drug resistance prevention at a particular site. The mutations and resistance levels in this study thus only reflect a snapshot of the situation at that site. Similar studies will have to be conducted at other sentinel sites to give a truly representative view of the situation in Maputo. We have also reported a series of substitutions in the non-B subtypes but do not know the clinical significance of such mutations in the longer term. There has been discussion on the fact that such mutations might facilitate the development of virological failure during protease inhibitor therapy and further research in this domain would be justified. Finally, our conclusions are drawn on a sample of patients within the overall cohort as it was not feasible to offer viral load testing to the entire cohort, and the related limitations thus apply.

In Maputo, the level of detectable viraemia and the proportion of patients with drug resistance to first-line ART is reassuringly low. While countries such as Mozambique are embarking on ART scale-up, wider surveillance is warranted to monitor programme quality (particularly aspects of adherence and patient education) and limit the development of drug resistance and the need for second-line therapies. These remain major potential challenges for the future of ART in Africa.

**Authors' contributions:** MB, FR, CM, MA, RB, HCC, VA, JCS and RZ were involved with the study conception and design; FM, MB, FR, CM, MA, HCC and JCS were involved with the field implementation or laboratory analysis; FM, MB, FR, VA, JCS and RZ were involved with data analysis and interpretation; FM, MB and RZ drafted the first version of the manuscript and all co-authors were involved with critical revision. All authors read and approved the final manuscript. RZ is guarantor of the study.

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