Original article

HIV type-1 clade C resistance genotypes in treatment-naive patients and after first virological failure in a large community antiretroviral therapy programme

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Background: This study aimed to evaluate HIV type-1 (HIV-1) drug resistance pretreatment and in those failing first-line non-nucleoside reverse transcriptase inhibitor (NNRTI)-based antiretroviral therapy (ART) in South Africa.
Methods: This was an observational cohort. Genotypic resistance testing was performed on treatment-naive individuals and those failing first-line ART (confirmed HIV-1 RNA>1,000 copies/ml) from public sector clinics in Cape Town (2002–2007). Resistance profiles and mutations relative to timing of known virological failure were examined.
Results: In total, 230 patients (120 treatment-naive and 110 with virological failure) were included: 98% had clade C virus. Among treatment-naive patients, prevalence of primary resistance was 2.5% (95% confidence interval 0.0–5.3). Three patients had one significant reverse transcriptase mutation: K65R, Y181C and G190A. Among treatment-experienced patients, 95 (86%) individuals had therapy-limiting NNRTI mutations, including K103N (55%), V106M (31%) and Y181C (9%). The M184V mutation was the most common mutation, found in 86 (78%) patients. In total, 10 (9%) patients had the K65R mutation. More individuals tended to develop thymidine analogue mutations when sampling occurred after 6 months of detected therapy failure (10/31 [32%] individuals) compared with those who had genotyping before 6 months (15/79 [19%] patients; *P*=0.246).
Conclusions: Prevalence of primary resistance in a sample of ART-naive clade C HIV-1-infected individuals in South Africa was low during the study period. Patients failing first-line ART most often developed resistance to NNRTIs and nucleoside reverse transcriptase inhibitors, the two drug classes used in first-line therapy. Viral load monitoring in this setting is crucial and individual genotypes in those failing first-line therapy should be considered.

Introduction

More than 3 million individuals now have access to antiretroviral therapy (ART) in low- and middle-income countries (LMIC) [1]. Delivery of ART on this scale has required utilization of a public health approach in which standardized, rather than individualized, regimens are prescribed to very large numbers of HIV type-1 (HIV-1)-infected individuals [2]. At present, the majority of individuals in these countries are initiating first-line therapy with a non-nucleoside reverse transcriptase inhibitor (NNRTI) and two nucleoside reverse transcriptase inhibitors (NRTIs) [3]. In addition to those receiving ART for treatment, many women receive nevirapine (NVP) and/or zidovudine (AZT) for prevention of mother-to-child HIV-1 transmission (PMTCT) [4].

Second-line ART, based on a boosted protease inhibitor with two NRTIs, is several-fold more expensive than the first-line regimens [1]. Although the proportion of patients receiving second-line therapy is

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presently estimated to be 4%, this is increasing by 3% per annum [5]. In LMIC the decision of when to change to a second-line regimen is frequently delayed, as it is often based on clinical or immunological criteria in the absence of viral load measurement [3,6]. Rational choice of the NRTI component of second-line therapy should be based on patterns of resistance developed during first-line therapy [7].

Much concern was expressed during the initial phase of expanded access to ART that 'antiretroviral anarchy and viral mayhem' might follow the widespread use of ART in LMIC [8]. However, despite the large scale of PMTCT and ART rollout, there has been little published data detailing resistance either prior to or within large scale ART programmes. The effect of the widespread use of single-dose NVP for PMTCT on primary resistance patterns of those entering ART programmes has not yet been widely characterized [9].

Furthermore, most data on viral mutations developing in patients on ART are from industrialized countries where HIV-1 subtype B is prevalent, whereas viral subtypes in LMIC are frequently non-B, and non-B subtypes might have different pathways to viral resistance [10–12]. Data on resistance patterns in both treatment-naive and treatment-exposed clade C subtype are limited [13–21]. Our objective was to describe the resistance genotype patterns in both ART-naive individuals and in those with first virological breakthrough while on first-line NNRTI therapy in the public sector ART programme in South Africa.

Methods

Study samples

Treatment-naive samples

Staff at the Desmond Tutu HIV Centre in Cape Town, South Africa, drew 30 samples per annum for genotyping from treatment-naive HIV-1-positive individuals between 2003 and 2006, resulting in 120 samples available for the current analysis. These individuals were from two periurban resource-poor communities in Cape Town. HIV-1-infected individuals attending HIV clinics at either of the two sites were asked to donate a sample on a first-come first-serve basis beginning in April each year until 30 samples had been collected. None of these individuals had been exposed to any ART, including PMTCT, at the time of sampling.

Treatment-experienced samples

All samples were from individuals failing first-line therapy in public sector ART clinics in the greater Cape Town area between 2002 and 2007. Eight clinics provided samples. HIV-1-positive individuals in these clinics could access ART if they had a CD4$^+$ T-cell count <200 cells/µl or with WHO stage 4 clinical disease. First-line ART consisted of stavudine (d4T) and lamivudine (3TC), with an NNRTI (efavirenz or NVP) [6]. Pregnant women who did not yet qualify for ART were commenced on AZT at 34 weeks and given a single dose of NVP in early labour [4]. For this study, virological breakthrough was defined as the first time a viral load was noted to be >1,000 copies/ml while on ART, and virological failure was defined as two consecutive viral loads >1,000 copies/ml.

Laboratory testing

Viral load and CD4$^+$ T-cell counts were monitored 4- to 6-monthly according to local protocol [4,6]. Viral load assays were done using the branch DNA hybridization technique (Bayer HIV-1 RNA 3.0 assay). Genotypic analyses of the reverse transcriptase and protease sequences of HIV-1 DNA were completed using either the Trugene® HIV-1 (Bayer Healthcare, Leverkusen, Germany) or GeneSeq™ (Monogram Biosciences, South San Francisco, CA, USA). The International AIDS Society (IAS)–USA list of mutations was used to determine which mutations might be related to drug resistance [22]. Mutations noted in the IAS–USA listing that were not noted in this data group were not listed in the results. The Stanford University HIV Resistance Database Genotypic Resistance Interpretation Algorithm was used to determine the possible drug resistance patterns per genotype [23].

The majority of samples were sourced from the Hannan Crusaid Treatment Centre (Cape Town, South Africa); this cohort has been described previously [13–15]. Excess plasma from the 4-monthly scheduled visits was frozen and stored. All individuals who reached a viral load of >1,000 copies/ml and who had an available stored plasma sample at the time of confirmation of virological failure were analysed for HIV-1 genotype. The dates of treatment initiation, first observed virological breakthrough (>1,000 copies/ml) and confirmation of virological breakthrough (the date of the sample used for genotype analysis) were recorded.

Other samples were sourced from other ART sites in the Western Cape. Clinicians were asked to refer patients with a previously noted viral load of >1,000 copies/ml on first-line therapy to the study site for one-off genotype sampling. The dates of treatment initiation, first virological breakthrough (>1,000 copies/ml) and confirmation of virological breakthrough were recorded.

Demographic data (age, gender and disease stage) were recorded for all individuals with genotype results, as was viral load and CD4$^+$ T-cell count at the time of genotypic sampling. Mutations considered related to the function of the HIV-1 reverse transcriptase and protease enzymes were recorded.

Statistical analyses

Demographic and baseline data were described using medians and proportions as appropriate. Baseline

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characteristics described by interval data were compared using non-parametric statistics for data not normally distributed. The 95% confidence intervals (CIs) around resistance mutation prevalence were constructed using the normal approximation of the binomial distribution. Among patients failing ART, we examined the association between timing of genotyping and resistance profile.

Results

Treatment-naive patient samples

Samples from 120 ART-naive HIV-1-infected individuals were included in the current analysis (Table 1). The median age of the cohort was 31 years (interquartile range [IQR] 25–38) and 63% were women. The median CD4+ T-cell count at the time of sampling was 262 cells/µl (IQR 149–405) and the median viral load was 4.88 $\log_{10}$ copies/ml (IQR 4.29– 5.23). In total, 117 (98%) samples were clade C; the other 3 samples were clade B and were known to be collected from men who have sex with men [24].

The individual genotype results for the treatment-naive sample group are shown in Table 2 (Figure S1 in the Additional file). There was very little variation in the reverse transcriptase gene. One individual (0.8%) had a K65R mutation, denoting probable reduced sensitivity to tenofovir and abacavir, and three (2.5%) had the V118I mutation. Despite this cohort having no prior NNRTI exposure, there were two (1.7%) individuals with single NNRTI mutations, one Y181C and one G190A. In contrast to the reverse transcriptase, in the protease inhibitor region there were a number of mutations that occurred frequently, although these were not expected to cause drug resistance. The most frequent protease mutations were L89I/M (89%), H69K (88%), L63P (52%) and M36I (87%). In addition, >10% had mutations at loci 20 (17%), 74 (10%) and 77 (18%).

Treatment-experienced patient samples

In total, 119 individuals attending the Hannan Crusaid Treatment Centre between September 2002 and December 2007, who were taking first-line therapy, experienced virological breakthrough. Six individuals who had failed a protease-inhibitor-based first-line regimen were excluded from the analysis. Stored samples were not available for 34 individuals and genotype results were obtained for the remaining 79 individuals. Samples from an additional 31 individuals with the same failure criteria were received from seven other public sector antiretroviral clinics bringing the total number of genotypes available from individuals failing first-line therapy to 110.

The demographics of the treatment-experienced group were similar to that of the treatment-naive cohort. Their median age was 32 years (IQR 28–35) and 70% were women. The median CD4+ T-cell count was significantly lower than in the treatment-naive group, at 192 cells/µl (IQR 128–288; P=0.003) and the median log viral load at time of sampling was significantly lower at 4.02 $\log_{10}$ copies/ml (IQR 3.61–4.76; P<0.001). The median time from treatment start date to initial detected virological breakthrough (>1,000 copies/ml) was 271 days (IQR 177–525), and that from first detected virological breakthrough to the time of confirmation and sampling for genotype was 97 days (IQR 31–195). Overall, 79 (72%) people had their repeat sample within 180 days of their initial raised viral load and 39 (28%) people after 180 days.

There were many more reverse transcriptase mutations in the treatment-experienced samples than in the

Table 1. Demographic characteristics and laboratory results for treatment-naive and first virological failure groups in a sample from Cape Town, South Africa

Characteristic	Treatment-naive	Virological failure	*P*-value
Total samples, *n* (%)	120 (100)	110 (100)	–
Mean age, years (IQR)	31 (25–38)	32 (28–35)	0.535
Female gender, *n* (%)	75 (63)	77 (70)	0.588
Median CD4+ T-cell count, cells/µl (IQR)	262 (149–405)	192 (122–283)	0.003
Median viral load, $\log_{10}$ copies/ml (IQR)	4.88 (4.29–5.43)	4.02 (3.61–4.76)	<0.001
HIV-1 clade C, *n* (%)	117 (98)	56 (97)[a]	0.966
HIV-1 clade B, *n* (%)	3 (2)	2 (3)[a]	1.00
Treatment regimen			
d4T+3TC+EFV, *n* (%)	–	78 (67.2)	–
d4T+3TC+NVP, *n* (%)	–	18 (15.5)	–
AZT+3TC+EFV, *n* (%)	–	7 (6.0)	–
AZT+3TC+NVP, *n* (%)	–	6 (5.2)	–

The viral load and CD4+ T-cell count presented for the first-time virological failure group are those at the time of second consecutive viral load >1,000 copies/ml, a median of 97 days from initial viral load >1,000 copies/ml. [a]Of 58 available. AZT, zidovudine; EFV, efavirenz; HIV-1, HIV type-1; IQR, interquartile range; d4T, stavudine; NVP, nevirapine; 3TC, lamivudine.

Table 2. Genotype results in a sample of ART-naive patients in Cape Town, South Africa

NRTI mutations				NNRTI mutations				PI mutations			
Ref	Loci	AA	*n* (%)	Ref	Loci	AA	*n* (%)	Ref	Loci	AA	*n* (%)
K	65	R	1 (0.8)	Y	181	CY	1 (0.8)	L	10	IVF	6 (5.0)
V	118	I	3 (2.5)	G	190	ACE	1 (0.8)	G	16	E	2 (1.6)
–	–	–	–	–	–	–	–	K	20	RT	20 (16.6)
–	–	–	–	–	–	–	–	M	36	I	104 (86.7)
–	–	–	–	–	–	–	–	M	46	I	1 (0.8)
–	–	–	–	–	–	–	–	I	47	IV	1 (0.8)
–	–	–	–	–	–	–	–	I	50	V	1 (0.8)
–	–	–	–	–	–	–	–	I	54	V	1 (0.8)
–	–	–	–	–	–	–	–	L	63	HLPSTV	62 (51.7)
–	–	–	–	–	–	–	–	H	69	K	108 (90)
–	–	–	–	–	–	–	–	A	71	ATV	3 (2.5)
–	–	–	–	–	–	–	–	G	73	S	3 (2.5)
–	–	–	–	–	–	–	–	T	74	S	12 (10)
–	–	–	–	–	–	–	–	V	77	I	22 (18.3)
–	–	–	–	–	–	–	–	LV	82	AF	1 (0.8)
–	–	–	–	–	–	–	–	L	89	IM	107 (89.2)

Overall *n*=120. Prevalence of wild-type virus was 114 (95%). Mutations noted in the International AIDS Society–USA listing that were not noted in this group are not listed here. AA, amino acid; ART, antiretroviral therapy; NNRTI, non-nucleoside reverse transcriptase inhibitors; NRTI, nucleoside reverse transcriptase inhibitors; PI, protease inhibitor; Ref, reference.

Table 3. Genotype results in a sample of patients with virological failure to first-line ART in Cape Town, South Africa

NRTI mutations				NNRTI mutations				PI mutations			
Ref	Loci	AA	*n* (%)	Ref	Loci	AA	*n* (%)	Ref	Loci	AA	*n* (%)
M	**41**	**ML**	**1 (0.9)**	A	98	G	4 (3.6)	L	10	IVF	7 (6.4)
K	65	R	10 (9.0)	L	100	I	2 (1.8)	I	13	V	8 (7.3)
D	**67**	**N**	**14 (13)**	K	101	EP	18 (16)	G	16	E	9 (8.1)
T	69	DN	2 (1.8)	K	103	N	60 (55)	K	20	RT	30 (27)
K	**70**	**KR**	**4 (3.6)**	V	106	M	34 (31)	D	30	N	1 (0.9)
L	74	L/I/V	1 (0.9)	V	108	IV	13 (12)	L	33	FV	2 (1.8)
V	75	IM	3 (2.7)	E	138	A	2 (1.8)	M	36	I	95 (86)
V	118	I	2 (2.7)	V	179	DV	6 (5.5)	L	63	HLPSTV	66 (60)
M	184	V	86 (78)	Y	181	CY	11 (10)	H	69	K	103 (94)
T	**215**	**FSY**	**9 (8.1)**	Y	188	HL	9 (8.1)	G	73	S	1 (0.9)
K	**219**	**EQ**	**5 (4.5)**	G	190	ACE	22 (20)	T	74	S	14 (13)
K	238	T	2 (1.8)	P	225	H	15 (14)	V	77	I	17 (15)
–	–	–	–	F	227	L	7 (6.4)	LV	82	AF	1 (0.9)
–	–	M	230	L	8 (7.3)	L	89	IM	90 (82)	–	–

Overall *n*=110. Mutations noted in the International AIDS Society-USA listing that were not noted in this group are not listed here. Text in bold indicates thymidine analogue mutations (TAMs). Prevalence of wild-type virus was 7 (6.4%). AA, amino acid; ART, antiretroviral therapy; NNRTI, non-nucleoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; PI, protease inhibitor; Ref, reference.

treatment-naive samples (Table 3 and Figure S2 in the Additional file): 91 (83%) individuals had ≥1 mutations limiting susceptibility of NRTIs and 97 (88%) individuals had ≥1 therapy-limiting NNRTI mutations. The M184V, conferring resistance to 3TC and emtricitabine (FTC), was the most common single mutation (*n*=86 [78%]) and emerged rapidly in failure (Figure 1A). According to the genotypic resistance interpretation algorithm four (4.0%) more people were likely to have intermediate resistance to 3TC and FTC because of the presence of K65R (Table 4) [23]. Seven (6.4%) of the samples had no reverse transcriptase mutations.

A total of 10 (9.0%) individuals, all taking d4T, had developed the K65R mutation, limiting future use of didanosine (ddI), tenofovir and abacavir, without having had exposure to any of these medications (Table 4). In this group, those with the K65R did not have a significantly higher mean viral load than those without [25]. Six (5.5%) individuals had both the K65R and the M184V mutations.

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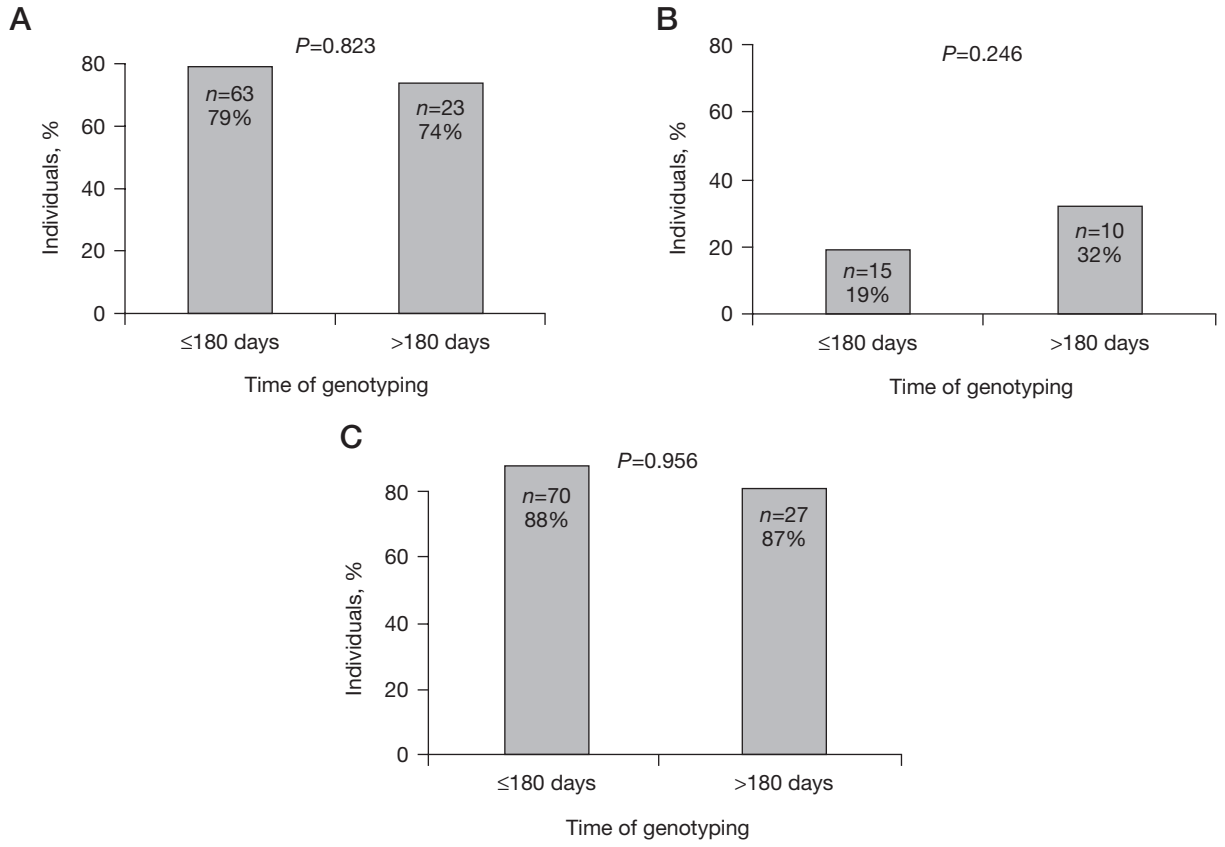
Figure 1. Prevalence of M184V, thymidine analogue and NNRTI mutations in plasma samples taken ≤180 days and >180 days following initial viral failure



The proportion of individuals with either **(A)** the M184V mutation **(B)** thymidine analogue mutations (TAMs) or **(C)** non-nucleoside reverse transcriptase inhibitor (NNRTI) mutations by time between initial virological breakthrough (first viral load >1,000 copies/ml) and failure (at the time of the second consecutive viral load >1,000 copies/ml). Individuals were divided as those whose genotype (taken at the time of virological failure) was completed on or before 180 days (*n*=79) from breakthrough or after 180 days (*n*=31). None of the differences were significant, although a trend toward more TAMs with more time on failing therapy was noted.

Overall, 25 (23%) individuals had a total of 33 thymidine analogue mutations (TAMs). Those with TAMs had a median viral load of 4.32 $\log_{10}$ copies/ml (IQR 3.47–4.71) compared with a median of 4.01 $\log_{10}$ copies/ml (IQR 3.47–4.71) in those without TAMS at the time of genotype sampling (*P*=0.896) [26]. Only five (4.5%) individuals had ≥1 (TAM). Figure 2 describes the proportion of those with non-TAM resistance that had also developed TAMs in the reverse transcriptase gene. There were relatively few individuals with resistance to either 3TC, that is, individuals presenting with the M184V mutation (*n*=6 [5.4%]), or NNRTIs (*n*=20 [18%]) alone. These individuals also had few TAMs, and no sample had ≥1 TAM. Over two-thirds (*n*=75 [68%]) of individuals had a combination of 3TC and NNRTI resistance. Of these, 21 (28%) had TAMs and it was only in these samples that 2 or 3 TAMs were noted.

More TAMs were noted in those individuals who had failure confirmed >180 days after initial virological breakthrough (Figure 1B). A total of 17 TAMs were noted in 15/79 (19%) individuals whose genotype was completed within 6 months, compared with 16 TAMs noted in 10/31 (32%) individuals whose genotype was completed after 6 months (*P*=0.246). Multivariate logistic regression modelling of factors associated with acquiring a TAM demonstrated that for every 20 unit increase in $CD4^+$ T-cell count at time of genotyping, the reduction in risk of developing TAMs was 9% (odds ratio 0.91, CI 0.83–0.99; *P*=0.035). Age, gender, time from failure to sample, viral load and NRTI used did not affect the acquisition of TAMs. There was no significant difference in the number of TAMs generated by the specific thymidine analogue taken, whether AZT (4/13 [31%] individuals) or d4T (28/97 [29%] individuals; *P*=0.917). Susceptibility to AZT and d4T remained high in this group (Table 4).

Development of NNRTI resistance occurred rapidly and these mutations were the most common noted in this group (Figure 1C). A total of 70/79 (88%)

Table 4. Expected resistance patterns according to the Stanford University HIV Resistance Database genotypic resistance interpretation algorithm in a sample of patients with virological failure to first-line ART in Cape Town, South Africa

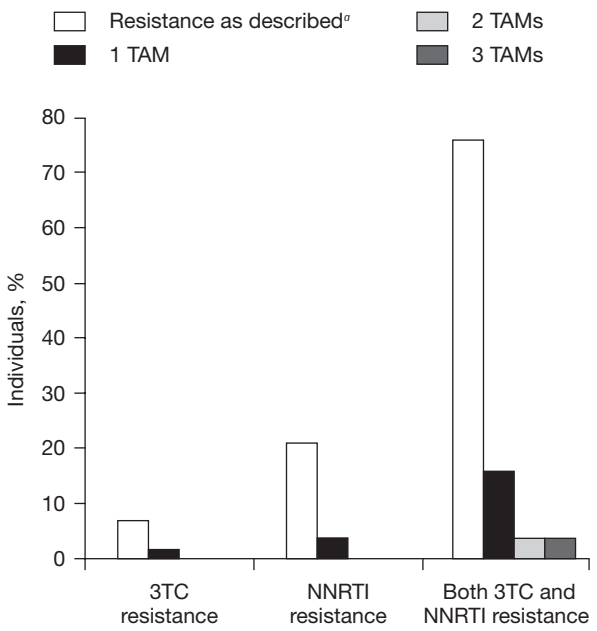
ART	Susceptible, *n* (%)	Possible low-level resistance, *n* (%)	Low-level resistance, *n* (%)	Intermediate resistance, *n* (%)	High resistance, *n* (%)
Lamivudine/emtricitabine	22 (20)	–	–	4 (4.0)	86 (78)
Abacavir	20 (18)	55 (50)	15 (14)	20 (18)	–
Zidovudine	98 (89)	1 (1.0)	6 (5.5)	3 (2.7)	2 (1.8)
Stavudine	87 (79)	6 (5.5)	12 (11)	5 (4.5)	–
Didanosine	76 (69)	9 (8.1)	9 (8.1)	14 (13)	2 (1.8)
Tenofovir	97 (88)	1 (1.0)	4 (3.6)	8 (7.3)	–
Efavirenz	10 (9.0)	2 (1.8)	–	13 (12)	85 (77)
Nevirapine	10 (9.0)	2 (1.8)	1 (1.0)	4 (4.0)	93 (85)
Etravirine	10 (9.0)	15 (14)	49 (45)	32 (29)	4 (4.0)
Protease inhibitors	93 (84)	16 (15)[a]	1 (1.0)[b]	–	–

Overall *n*=110. [a]Fourteen individuals with possible low-level resistance to nelfinavir alone; two with possible low-level resistance to fosamprenavir and tipranavir. [b]One individual with low-level resistance to atazanavir, fosamprenavir, lopinavir and saquinavir and intermediate resistance to indinavir and nelfinavir. ART, antiretroviral therapy.

individuals whose genotype was completed within 6 months had NNRTI mutations, compared with 27/31 (87%) individuals whose genotype was completed after 6 months (*P*=0.956). In total, 97 (88%) individuals had ≥1 therapy-limiting NNRTI mutations (Table 3), including K103N (55%), V106M (31%) and Y181C (10%), and probable drug susceptibility according to the genotypic interpretation algorithm was poor (Table 4) [23]. In total, 31 (28%) individuals had 1 NNRTI mutation, 46 (42%) had 2 NNRTI mutations, 16 (15%) had 3 NNRTI mutations and 3 (3%) samples had as many as 4 NNRTI mutations. One (0.9%) individual had 6 NNRTI mutations. The Y181C mutation emerged more frequently in those failing NVP (8/25 [32%]) than efavirenz (3/85 [3.5%]; *P*=0.0004). There was no significant difference in the emergence of K103N whether NVP (9/25 [36%]) or efavirenz (51/85 [60%]) was taken (*P*=0.229) [27]. There was no significant difference in the emergence of V106M by drug. This mutation was seen in 8/25 (32%) people on NVP and 26/85 (31%) people on efavirenz (*P*=0.923). The ratio of V106M/K103N in patients failing EFV therapy was 0.5.

The protease gene had similar mutations to those noted in the treatment-naive population. The median number of mutations was four (IQR 3–5). The most frequent protease mutations remained M36I (86%), L63P (60%), H69K (94%) and L89I/M (82%), similar to the consensus sequence noted for clade C (differing amino acids compared with clade B subtypes at positions M361, R41K, H69K and L89M) [26]. As in the treatment-naive cohort, >10% had mutations at loci 20 (27%), 74 (13%) and 77 (15%). According to the genotypic resistance algorithm, those with mutations at point 74 (*n*=14 [13%]) had possible low-level resistance to nelfinavir (Table 4) [23]. Two (1.8%) individuals with mutations at point 33 had possible low-level resistance to fosamprenavir and tipranavir, and one (0.9%) individual had multiple protease inhibitor resistance because of mutations at positions 73 and 82 (Table 4).

Figure 2. Association between non-TAM resistance and development of TAMs in the reverse transcriptase gene



[a]Lamivudine (3TC) resistance, non-nucleoside reverse transcriptase inhibitor (NNRTI) resistance or both. TAM, thymidine analogue mutation.

Discussion

With increasing access to ART in LMIC, increased numbers of patients are failing first-line therapy and

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thus being switched to second-line therapy [15]. In higher income countries, both the choice of initial therapy and switch to second-line therapy are done with the use of individual genotypes [7]. In LMIC, a public health approach with more limited first- and second-line treatment options has been used. In this setting, it is crucial to understand the evolution of resistance patterns, and whether the current treatment regimens are adequate.

The majority of virus in this South African sample, in both treatment-naive and treatment-experienced patients, was clade C [24]. In both the treatment-naive and treatment-experienced group there were a number of mutations in the protease enzyme, possibly indicating divergence from clade B virus. The effect of these mutations on viral drug susceptibility is uncertain, but many, including L10I/V, K20R, M36I, L63P, A71V/T and V77I, are not expected to cause major drug resistance [28]. These mutations are similar to those noted in other African clade C virus and clade C consensus sequences [16–18,29]. Other mutations that are more likely to affect future use of a protease inhibitor, including D30N, M46I, I47V, I50V and V82A/F, were present, but in a minority of individuals, with only a single mutation noted per individual. The effect of such single mutations on the use of lopinavir in second-line therapy is not clear in our population.

There were very few mutations noted in the reverse transcriptase enzyme in the treatment-naive group. The two mutations that are likely to reduce susceptibility to NRTIs if transmitted, T215C and M41L, were not seen in either the treatment-naive or treatment-experienced groups [28]. The multiplicity of NNRTI mutations seen in the treatment-experienced group make NNRTI resistance the most likely to be transmitted in our population. Although the K103N mutation, which is expected to have the greatest effect on the use of NNRTIs, was not seen in the treatment-naive samples, there were two (1.6%) individuals each with a single mutation (Y181C and G190E) that would have some effect on NNRTI susceptibility. Although the currently recommended treatment regimens for first-line therapy remain appropriate, ongoing surveillance of NNRTI-resistant virus remains important [3,6].

The samples in the treatment-experienced group were taken from 110 individuals failing initial NNRTI therapy in the South African public sector. Previous data have shown that the rate of confirmed virological failure in this cohort was 5.6% at 32 months [15]. Adherence is monitored by tablet count in all public sector clinics in South Africa and any viral load increase should initiate a stepped-up adherence package including counsellor-driven re-education sessions, more regular clinic visits with emphasis on the use of a pill box as a reminder system, as well as a home visit to assess living circumstances where the resources are available for this service [15]. Overall, 75% of those with an initial viral load breakthrough of >1,000 copies/ml again achieved suppression after structured adherence interventions [15]. For those in whom failure was confirmed with a second specimen >1,000 copies/ml, the median time from treatment commencement to noting initial virological breakthrough was 9 months.

The focus on adherence might explain the relatively small number of individuals with virological failure who did not have a significant drug-resistant mutation. Only seven (6.4%) individuals had wild-type virus at genotype, a smaller proportion than seen in the DART study (10%) [20]. Most of these individuals with confirmed failure had resistance mutations, which would exclude use of two of the antiretrovirals used in first-line regimens, that is, 3TC (83%; a similar proportion to that seen in the DART study of 70% [20], and the NNRTIs (86%). Resistance to both NNRTIs and 3TC (M184V) develops rapidly after initial virological breakthrough.

Resistance to the third drug in the regimen, the thymidine analogue, occurred more slowly. Although 23% of the group had ≥1 TAM, relatively few had two or three TAMs and the majority of individuals remained susceptible to both AZT and d4T. A trend towards TAM accumulation with prolonged time on failing therapy was noted, but was non-significant. People with lower $CD4^+$ T-cell counts at the time of genotyping were also more likely to have acquired a TAM, perhaps indicative of a longer time on failing therapy than noted here, because of the length of time between viral loads in this cohort. Thymidine analogues (AZT, ddI and lopinavir/ritonavir) are currently recycled in second-line therapy in South Africa, so TAM accumulation might reduce the efficacy of this therapy. However, if failure is identified before acquisition of TAMs, second-line therapy might remain more efficacious.

The increased presence (9%) of the K65R mutation in the treatment-experienced samples was unexpected, given the absence of abacavir or tenofovir in the South African treatment regimens. There is emerging evidence that non-subtype B virus might have a propensity to develop the K65R more readily compared with subtype B [10,25,26]. Doualla-Bell *et al.* [10] noted that d4T also selected for K65R in subtype C virus in Botswana, and that the mutation developed within 3 months of tenofovir therapy, unlike in subtype B where the K65R tends to emerge slowly in a small proportion of individuals on tenofovir. It is also possible that 3TC might select for the K65R mutation as recently described [22]. With the registration of tenofovir in South Africa in 2007, there is a push for the widespread use of this agent to replace d4T, initially in those experiencing adverse effects, but with the view to broad-spectrum first-line use. The likely rapid emergence of resistance

to tenofovir in clade C virus should be of concern for treatment programmes, as the presence of this mutation reduces susceptibility to all NRTIs except AZT, and thus would limit the choice of NRTIs for second-line therapy [30].

A limitation of this study is the 4–6 month window between viral load samples in the South African antiretroviral programme. Some individuals might have failed within weeks of their last suppressed viral load and others within days of their first raised viral load. Time from first virological breakthrough to time of genotype might therefore be an underestimate.

Resistance to the reverse transcriptase enzyme after exposure to NNRTI-containing first-line therapy follows a pattern that is predictable and similar to that of clade B: initial resistance to antiretrovirals that require a single point mutation, followed by slower development of resistance to drugs with a higher genetic barrier to resistance, such as the thymidine analogues.

Had second-line treatment been commenced within 6 months of initial virological breakthrough in the treatment-experienced group in this study, the likelihood of accumulating TAMs might have been reduced, with a potential increase in the efficacy of the recycled thymidine analogue in second-line therapy. Identification of and rapid response to virological failure is thus important to maintain the full benefit of second-line therapy. This would suggest clinical value to regular viral load testing to identify virological failure soon after it occurs, in contrast to a recently published model [31]. Because of the unexpected emergence of the K65R mutation in a substantial proportion of the cohort, tenofovir should be introduced cautiously with careful assessment of its effect on the emergence of resistance.

This study suggests that at present it is not crucial, in the context of the South African National ART programme, to have routine access to genotypes at baseline, as the vast majority of treatment-naive samples continue to be wild type. By contrast, the development of extensive resistance in those failing first-line therapy suggests that viral load monitoring is crucial and there could be a role for individual genotypes in those failing first-line therapy, particularly if second-line therapy is likely to be compromised by resistance to first-line therapy. Increased availability of low-cost assays for identifying resistance in patients in South Africa would be clinically valuable.

Acknowledgements

Many thanks go to Monica Vogt and Felicity Cope, who carefully stored and retrieved samples for us for many years. We would also like to thank the staff at Toga Laboratories that stored and retrieved the more recent samples.

This work was supported by the National Institute of Allergy and Infectious Diseases (R01 AI058736, K24 AI062476, P30 AI060354) and the Doris Duke Charitable Foundation Clinical Scientist Development Award.

Disclosure statement

CO and RW are partially supported through the NIH CIPRA-SA grant. In addition, CO receives partial funding from the PEPFAR grant. All other authors declare no competing interests.

Additional file

The additional file 'Supplementary figures' can be accessed at www.intmedpress.com

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Accepted for publication 30 January 2009