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Level of viral load and antiretroviral resistance after 6 months of non-nucleoside reverse transcriptase inhibitor first-line treatment in HIV-1-infected children in Mali

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Objectives: To evaluate the virological response and to describe the resistance profiles in the case of failure after 6 months of first-line highly active antiretroviral therapy (HAART) in HIV-1-infected children living in resource-limited settings.

Patients and methods: Ninety-seven HIV-1-infected children who started two nucleoside reverse transcriptase inhibitors (NRTIs) and one non-nucleoside reverse transcriptase inhibitor (NNRTI) (mainly zidovudine/lamivudine/nevirapine) in Mali were prospectively studied. Virological failure (VF) was defined as loss to follow-up, death or HIV-1 RNA viral load (VL) of >400 copies/mL at 6 months. When VL was >50 copies/mL, a genotypic resistance test was performed.

Results: Among the 97 children, median age at antiretroviral initiation was 31 months and the majority were in WHO clinical (77.3%) and immunological (70.1%) stage III or IV. At month 6, 44% of children had VL >400 copies/mL (61% VF). Among the children with detectable VL, 30/37 genotypic resistance tests were available, 8 with wild-type viruses and 22 with resistance mutations (73%): 19 M184V/I, 21 NNRTI mutations and only 3 thymidine analogue mutations (TAMs) (K70R, D67N and L210W in three distinct viruses). At failure, 6/8 children with wild-type viruses had a VL of <1000 copies/mL whereas 21/22 with resistant viruses had a VL of >1000 copies/mL.

Conclusions: Under NNRTI-based regimens, early detection of VF could allow the reinforcement of adherence when VL was <1000 copies/mL, because in most of these cases no resistance mutations were detected, or a change to a protease inhibitor-based regimen if VL was >1000 copies/mL. The low frequency of TAMs suggests that most NRTIs can be used in a second-line regimen after early failure.

Keywords: Africa, mutations, failure

Introduction

In 2008, 2.5 million children were living with HIV/AIDS, of whom 90% were in Africa. Every day, 1000 children are infected. Without treatment, half of them will die before the age of 2 years, and all of them before adolescence. However, access to highly active antiretroviral therapy (HAART) for HIV-infected children in resource-limited settings has significantly improved during the past few years, taking advantage of international mobilization and national initiatives (www.who.org). Although the main issue remains scaling-up and recruitment of infected children into HAART programmes, new challenges have arisen particularly concerning sustainability of treatment efficacy and therapeutic failure. Many studies, mainly in adults, have shown the need for total and sustained viral load (VL) suppression to prevent the emergence of resistance mutations and stave off clinical therapeutic failure.[1,2] This is also certainly the main determinant of sustainable efficacy of HAART in children.

Residual replication under antiretroviral pressure leads to an accumulation of resistance mutations, limiting future therapeutic choices. It is thus clear that virological failure must be detected and managed early. In resource-limited settings,

where access to virological measurements remains scarce, it is essential for treatment management to have epidemiological data about early virological failure and resistance mutations present at this time. Unfortunately, there are few data on the subject, especially for children living in resource-limited settings.[3,4] During the past 5 years, reports of HAART experience with children in resource-limited settings, including sub-Saharan Africa, have demonstrated the feasibility and short- to medium-term efficacy of these treatments in different environments.[5–10] Morbidity and mortality reduction, growth, CD4 cell gains and good tolerance have been described, confirming European and North American experiences. However, there are still very few data describing the rate of virological failure (especially early in the course of treatment when clinical or immunological efficacy evaluations are often carried out[11–14]) and almost nothing is known about its link with resistance development.[15] Thus, evidence-based debate on the content and timing of second-line regimens remains difficult.

From 2001, through the Initiative Malienne d'Accès aux Anti-RétroViraux (IMAARV), HIV care has been provided free of charge to infected children (≤15 years old) at the Gabriel Touré Hospital, a tertiary paediatric centre in Bamako (Mali). As of December 2008, nearly 1000 HIV-infected children have been taken care of in this hospital. This study aimed to evaluate their virological response and to describe the resistance profiles in the case of virological failure at 6 months of a homogeneous first-line HAART in the field conditions of HIV-1-infected children followed at the Gabriel Touré Hospital. Identification of factors associated with virological failure has also been attempted.

Patients and methods

Study population

Following national and WHO recommendations at the time of the study, the first-line regimen for children of all ages was an association of two nucleoside reverse transcriptase inhibitors (NRTIs) and a non-nucleoside reverse transcriptase inhibitor (NNRTI): zidovudine or stavudine plus lamivudine plus nevirapine or efavirenz. According to local protocol, children were seen at 2 weeks, 1 month and then monthly for at least the first 6–12 months following HAART initiation.

All naive HIV-1-infected children who initiated a combination of two NRTIs and one NNRTI between September 2006 and October 2007 in one of the two HIV-dedicated clinics of the Gabriel Touré Hospital were enrolled in this study. This study was a retrospective analysis of genotypic resistance tests, HIV-1 plasma RNA and CD4 cell counts, which were performed as recommended by the IMAARV guidelines for the use of antiretroviral agents in HIV-1-infected patients in the context of routine clinical practice.

Data recording

For each child, data extracted from their medical file at HAART initiation were: gender, age, whether the mother received a prophylactic therapy for prevention of mother-to-child transmission (PMTCT) and identity of the caregiver. Data recorded both at HAART initiation and at 6 months of HAART were: height, weight, head circumference, WHO clinical stage, and immunological (CD4 and total lymphocyte counts) and virological measurements. HIV-1 VL was measured using an Abbott RealTime HIV-1 assay (Abbott, Wiesbaden, Germany), and CD4 count and total lymphocyte cell count were determined locally in a private laboratory agreed by the national health authority using the BD FACSCount Reagent Kit (BD Biosciences, USA).

Clinical and immunological staging criteria were those proposed by the 2006 WHO staging guidelines.

We also recorded, from the medical file, any report of treatment interruptions (>48 h) or frequent treatment omission (caregiver interview), without quantification of it, that occurred during the first 6 months under HAART. Although not measuring compliance, these data identified a category of obviously non-compliant children.

Resistance genotyping

Samples were divided into aliquots and frozen in the National Reference Laboratory in Mali and genotyping was performed for children with an HIV-1 RNA level of >50 copies/mL at 6 months, as previously described in the Pitié-Salpêtrière Virology Laboratory, Paris, France.[16] Resistance mutations were recorded according to the latest IAS-USA list of mutations (www.iasusa.org).

Phylogenetic analyses

To define the HIV-1 subtypes, phylogenetic analyses were performed by estimating the relatedness of *pol* sequences and reference sequences of HIV-1 genetic subtypes and circulating recombinants obtained from the Los Alamos database (http://hiv-web.lanl.gov). Nucleotide sequences were aligned using the CLUSTAL W program. Phylogenetic reconstruction was performed using a Kimura 2-parameter model and the neighbour-joining method.

Statistical analyses

Virological failure was defined as an HIV-1 VL measurement above the threshold of 400 copies/mL. The 400 copies/mL threshold was chosen here because it is one of the most commonly used in similar studies conducted in Africa. Deaths occurring before 6 months and loss to follow-up before 6 months were also considered as virological failure. In an attempt to do a sensitivity analysis, we processed analyses with a variety of VL thresholds (50–400 and 1000 copies/mL) or excluding those patients who had died or were lost to follow-up.

Factors associated with virological failure at 6 months were identified using a logistic regression model. Factors investigated, measured at HAART initiation, were: gender, age, immunological and clinical WHO stages, HIV VL (categorized as <5, 5–6 and >6 $\log_{10}$ copies/mL) and *z*-score for weight, height and cranial perimeter. Continuous *z*-scores were calculated based on the tables provided by WHO in children ≤5 years of age, and by using WHO or CDC charts in children >5 years. Then, *z*-scores were categorized as less than or equal to −3, −2 and −1/0 standard deviation (SD) from the mean. We also investigated changes in *z*-scores and CD4 counts measured at HAART initiation and 6 months later as factors associated with virological failure, this analysis being restricted to children still alive and followed under HAART at 6 months.

Results

Among the 105 children included, 8 were not retained for analysis: 2 missed their month 6 VL sampling, 1 had a VL assessment at month 5 followed by therapeutic adaptation and 5 were transferred to another hospital before month 6 because of decentralization of HIV care in Mali. Of the remaining 97 children, 20 were lost to follow-up and 9 died within the 6 months. Finally, 68 children had a VL measurement 6 months after HAART initiation.

Characteristics at baseline (Table 1)

Among the 97 children included in the analysis, 53.6% were male. The median age at HAART initiation was 31 months [interquartile range (IQR) 20–69] and 24.7% were ≤18 months. The main therapeutic combination was zidovudine/lamivudine/nevirapine (compared with 4% stavudine/lamivudine/nevirapine). Seven children were born to mothers who received antiretroviral treatment during pregnancy. The majority of children were in WHO clinical (77.3%) and immunological (70.1%) stage III or IV. The median VL was 5.94 $\log_{10}$ copies/mL (IQR 5.41–6.46) with a trend of lower VL with age. The median *z*-score for weight and height were, respectively, −3.0 (IQR −4.7 to −2.2) and −2.6 (IQR −3.5 to −1.8). In children ≤5 years, 23.2% children had a *z*-score for head circumference of <3 SD from the mean.

Characteristics 6 months after treatment initiation

At the month 6 milestone for treatment evaluation, clinical and immunological status had improved with 23.5% of children in stage III or IV. CD4 median gain was 777 cells/mm^3 (IQR 810–1943) for children ≤18 months, 450 cells/mm^3(IQR 187–540) for those aged 19–60 months and 219 cells/mm^3 (IQR 69–443) for children >60 months. Regarding VL, 31 children had a VL of <50 copies/mL, 38 had a VL of <400 copies/mL and 43 had a VL of <1000 copies/mL (7 children had a VL of between 50 and 400 copies/mL and 5 children had a VL of between 400 and 1000 copies/mL). Considering virological failure (when children had a VL of >400 copies/mL, had been lost to follow-up or had died) 59 children were in virological failure at month 6, a failure rate of 61% (95% confidence interval 51%–71%). Finally, on the anthropometric data, the median *z*-scores for weight and height were, respectively, −1.84 (IQR −2.65 to −0.68) and −1.67 (IQR −2.65 to −0.55) and the evolution medians for these two parameters were, respectively, 1.16 (IQR 0.30–2.12) and 0.74 (IQR 0.16–1.46).

Factors predictive of failure

The failure proportion was not higher among children ≤18 months compared with children >18 months (70.8% versus 57.5%, $P=0.25$). Among children ≤18 months, CD4 gain over 6 months was statistically higher in virological success (+253 compared with +790 cells/mm^3, $P=0.08$), but among older groups, there were no significant differences. A marked improvement in clinical stage was not predictive of virological success ($P=0.15$). Antiretroviral therapy during pregnancy was not a predictive factor of failure. Univariate logistic regression analysis, taking into account all the baseline parameters, anthropometric *z*-score and CD4 range of progression and report of obvious non-compliance, showed only three associated factors: CD4 gain <median gain for age, obvious non-compliance and, unexpectedly, low initial *z*-score for height (Table 2). Similar results were found when the 50 copies/mL threshold was used to define virological failure.

Resistance mutations

Among the 37 children with a detectable VL (>50 copies/mL), 33 were sampled for genotypic resistance testing. For three of them, we did not achieve viral genome amplification. The major HIV subtype in this study was CRF02_AG (83%), the others being

Table 1. Baseline characteristics

Characteristics of children	Value
Gender	
male	52/97
Age (months)	
median (IQR)	31 (20–69)
≤18	24
19–59	43
60–120	22
>120	8
Therapeutic regimen	
AZT/3TC/NVP	93
d4T/3TC/NVP	4
PMTCT	
yes	7
WHO clinical stage	
I	14
II	8
III	35
IV	40
Immunological stage	
I	13
II	16
III	10
IV	58
CD4 (cells/mm^3), median (IQR)	
all ages	491 (207–806)
≤18 months	870 (468–1225)
19–60 months	630 (359–833)
>60 months	183 (27–400)
HIV VL ($\log_{10}$ copies/mL), median (IQR)	
all ages	5.94 (5.41–6.46)
≤18 months	6.32 (5.59–6.97)
19–60 months	5.98 (5.32–6.47)
>60 months	5.62 (5.26–6.12)
Weight-for-age *z*-score	
median (IQR)	−3.0 (−4.7 to −2.2)
−4 SD	34
−3 SD	15
−2 SD	27
−1 SD	11
>0 SD	10
Height-for-age *z*-score	
median (IQR)	−2.6 (−3.5 to −1.8)
−4 SD	12
−3 SD	23
−2 SD	31
−1 SD	20
>0 SD	11

IQR, interquartile range; AZT, zidovudine, d4T, stavudine; 3TC, lamivudine; NVP, nevirapine; PMTCT, prevention of mother-to-child transmission; SD, standard deviation.

Table 2. Factors associated with virological failure (HIV-1 RNA VL >400 copies/mL, death or loss to follow-up) at 6 months

	n	Virological failure, *n* (%)	Crude OR (95% CI)	*P*
Gender				0.57
male	52	33 (63.5)	1	
female	45	26 (57.8)	0.8 (0.3–1.8)	
Age (months)				0.47
≤18	24	17 (70.8)	1	
19–59	43	24 (55.8)	0.5 (0.2–1.5)	
≥60	30	18 (60.0)	0.6 (0.2–1.9)	
WHO clinical stage				0.26
I–II	22	11 (50.0)	1	
III	35	20 (57.1)	1.3 (0.5–3.9)	
IV	40	28 (70.0)	2.3 (0.8–6.8)	
Immunological stage				0.25
I–II	29	14 (48.3)	1	
III	10	7 (70.0)	2.5 (0.5–11.6)	
IV	58	28 (48.3)	2.0 (0.8–5.0)	
HIV VL (log_{10} copies/mL)				0.30
<5	14	8 (57.1)	1	
5–6	38	20 (52.6)	0.8 (0.2–2.9)	
>6	45	31 (68.9)	1.7 (0.5–5.7)	
Height *z*-score				0.01
−4/−3 SD	35	22 (62.9)	0.5 (0.2–1.5)	
−2 SD	31	24 (77.4)	1	
−1/0 SD	31	13 (41.9)	**0.2 (0.1–0.6)**	
Weight *z*-score				0.65
−4/−3 SD	49	32 (65.3)	1.5 (0.6–3.9)	
−2 SD	27	15 (55.6)	1	
−1/0 SD	21	12 (57.1)	1.1 (0.3–3.4)	
Cranial perimeter *z*-score[a]				0.78
−4/−3 SD	10	6 (60.0)	0.5 (0.13.8)	
−2 SD	8	6 (75.0)	1	
−1/0 SD	25	16 (64.0)	0.6 (0.1–3.6)	
Adherence				0.01
yes	85	48 (56.5)	1	
no	12	11 (91.7)	**8.5 (1.1–68.7)**	

OR, odds ratio; CI, confidence interval; SD, standard deviation.
Bold text indicates significant *P* values ($P<0.05$).
[a]In 43 children aged ≤60 months.

complex recombinants (data not shown). The different profiles of resistance and their distribution by range of VL are reported in Table 3. Eight children showed no resistance mutations in the reverse transcriptase (RT) gene of their viruses and 22 children (73%) showed viruses harbouring resistance mutations. A relationship between the VL and the selection of resistance mutations was observed: most of the children with a VL of >1000 copies/mL harboured viruses with resistance mutations whereas most children with a VL of <1000 copies/mL had wild-type viruses. Twenty-one children (70%) showed viruses harbouring various NNRTI resistance mutations. The major mutations were K103N and Y181C, followed by G190A, Y188L and V106A/M. Considering the observed genotypic profiles at failure, none of the viruses was considered resistant to the second-generation NNRTI etravirine. The M184V/I mutation, associated with lamivudine resistance, was present in 19 cases (63%). Eighteen children (60%) were infected with viruses harbouring both lamivudine and NNRTI resistance mutations. Finally, there were only three children with viruses harbouring thymidine analogue mutations (TAMs), associated with zidovudine/stavudine resistance: one D67N, one K70R and one L210W. None of those viruses achieved full thymidine analogue

Table 3. Levels of HIV VL and profiles of resistance mutations in children with VL >50 copies/mL at 6 months

Child no.	HIV-1 VL (copies/mL)	RT mutations	PMTCT
5499	86	NA	−
5630	96	no mutation	−
5546	100	ND	−
5674	120	no mutation	−
5534	140	NA	−
5578	150	no mutation	−
5590	360	no mutation	−
5597	450	no mutation	−
5536	530	ND	−
5653	580	M184I, K103N	−
5627	640	NA	−
5599	880	no mutation	+
5593	2200	M184V, K103N, Y181C	−
5588	2700	M184V, V106A	−
5519	2900	M184V, K103N	−
5628	3000	no mutation	+
5494	4600	M184V, Y181C	−
5533	5100	M184V, K103N	−
5574	7600	M184V, G190A	−
5531	7700	K70R, M184V, Y188L	−
5568	17000	M184V, K101E/Q, Y181C	−
5676	31000	M184V, Y188L	−
5582	37000	M184V, K103N, G190A	−
5609	51000	D67N, Y181C	−
5636	58000	M184V, V106A	−
5621	63000	L210W, K103N	−
5492	71000	M184V, Y188L	+
5505	100000	M184V, K103N	−
5665	150000	M184V, V106A, G190A	−
5518	170000	M184V, K103N	−
5513	260000	no mutation	−
5632	270000	M184V, K103N	−
5516	280000	ND	−
5495	460000	M184V	−
5626	1300000	K103N	−
5600	2500000	M184V, K103N, Y181C, G190A	+
5538	2600000	ND	+

VL, viral load; RT, reverse transcriptase; PMTCT, prevention of mother-to-child transmission; NA, no amplification; ND, not done.
Thymidine analogue mutations are underlined.

resistance. Seven children were born to mothers who received PMTCT therapy: two were virological successes and five were virological failures (two with wild-type viruses and two with resistance mutations).

Discussion

The population of our study is probably quite representative of the global population of infected children in terms of age, sex and severity since each category seems significantly represented. The tertiary paediatric centre location of the clinic explains this wide recruitment: some of the children, mostly infants, were referred by the PMTCT consultation, some from the paediatric clinic, and the others by lower level medical centres. In particular, compared with other published series, there seems to be no bias either in the severity of the infection or in treatment initiation criteria that respected strictly the WHO criteria.

However, a 61% failure rate as defined is puzzling in many ways. First, it should be noted that we included children who had died or were lost to follow-up, which is unusual in this type of virological study. Excluding those lost to follow-up, the rate of failure falls to 51% and taking only children with a VL

of >400 copies/mL, it is 44%. With these corrections, our results are roughly comparable to those of the few published series, despite the fact that different VL thresholds (50–1000 copies/mL) defining virological failure were used in these studies. In Kenya, at 6 months of a first-line HAART containing an NNRTI, Wamalwa *et al.*[14] reported a 33% rate of virological failure. In Côte d'Ivoire, Fassinou *et al.*[11] showed a 50.5% rate of virological failure after 11 months of various first-line treatments, and Adje-Toure *et al.*[15] reported a 46% rate of never achieving undetectable VL during a follow-up from 4 to 24 months after NNRTI first-line initiation. These virological failure rates are consistent with experience in Europe or North America where virological success has long been much harder to achieve with children than adults. Thus, it is around twice the 24% rate observed in a comparable adult population in Mali by Marcelin *et al.*[3] More surprisingly, it is also a much higher rate than reported by Reddi *et al.*[12] with South African children, where a first-line NNRTI regimen reached the very low failure rate of 14%, increasing to 26% taking into account children who had died, without any information about those lost to follow-up. Local setting specificity and a very selective control of treatment administration probably account for part of such a good but unusual result.[17] Indeed, the powerfulness of HAART is probably not the main driver of this relative paediatric lack of success rate, since proposed HAART combinations have been proved efficient in children, in spite of very high VL and probable immunological immaturity.[18,19] The quality of administration and effective drug dosage is more probably the central issue, mainly with compliance deficiency and caregiver failures, even if child-specific malabsorption or pharmacokinetic problems are not to be totally excluded. It is of peculiar interest on that matter to see that obvious non-compliance is one of the few predictive factors of virological failure in our study.

The implications of virological failure after 6 months of a first-line regimen are determined by the probability and nature of underlying resistance. If none or very little resistance is expected, incentive and educational action would be able to correct the negative evolution. On the contrary, a very high level and rate of resistance will make those actions ineffective by themselves and indicate drug modification, even at 6 months. Our results on that matter are rather instructive since they allow a 1000 copies/mL threshold of HIV VL to be defined above which the majority (~90%) of the children carry virus with major resistance mutations almost for lamivudine and/or NNRTI. Moreover, they indicate that after 6 months, <1000 copies/mL, almost no resistance is to be expected, and at any level of viral load almost no TAMs have to be feared. The near absence of TAMs is in accordance with previous results showing a low rate of TAM selection when lamivudine is combined with zidovudine or stavudine, probably in relation to the interaction between M184V and T215Y/F mutations.[20] The presence of the M184V mutation in most cases, which appears to delay or prevent the emergence of TAMs, combined with the fact that sequences were obtained early, probably explains this result. The absence of TAMs suggests that most NRTIs can be used in a second-line regimen after early failure on zidovudine or stavudine/lamivudine/nevirapine. Thus, Marcelin *et al.*[3] in Malian adults showed 45% NNRTI resistance and 41% lamivudine- and NNRTI-associated resistance and no TAMs. Adje-Toure *et al.*,[15] in Ivorian children, showed NNRTI resistance in approximately two-thirds of cases of virological failure. These data are scarce but precious as they give epidemiological evidence allowing the design of early second-line regimens in areas where genotyping is not available. In addition, our results showed that in the case of early virological failure detection under an NNRTI regimen, none of the viruses was considered as resistant to the second-generation NNRTI, etravirine. It is noteworthy that only 7% of the children of our study were born to mothers who received PMTCT therapy, with no significant difference between success and failure. This, together with the low prevalence of primary resistance in the Malian HIV-infected population, suggests that the resistance observed is probably acquired during the 6 months of treatment.[21,22]

Both the rate and type of associated resistance point out the inescapable necessity of making the diagnosis of early virological failure. However, as VL assessment tools are not yet widely available in resource-limited settings, the search for clinical or other biological predictive factors for virological failure is of interest. However, we found very few markers or predictors, none being sensitive enough to substitute for VL measurement. The only baseline characteristic associated with failure is a size *z*-score of less than −3 SD for age. Classical severity markers such as clinical WHO stage (cachexia, microcephalia…) or very low CD4 percentage did not reach significance. Apart from a sensitivity problem, they are probably truly better predictors of clinical failure and death than early virological failure as we defined it here. We tried to determine whether the young (infants ≤18 months of age) were a more difficult population to treat. Here again, the trend observed did not reach significance, a 4-fold larger population being needed to prove the tendency. Dynamic predictors based on growth or CD4 evolution over the 6 months are disappointing. Indeed, a too small increase in CD4 count is associated with virological failure but too inconsistently and mostly in infants. Situations of virological/immunological discrepancy are numerous and illustrate this too loose correlation. More surprisingly, given that persistent viral replication is clinically known to be associated with long-term failure to thrive, growth parameters are of no help in predicting early virological failure. Finally, and even with our very coarse evaluation of it, obvious non-compliance appears as the only strong predictor. After all, even if a bigger series would probably be able to bring to statistical significance some of the empirically presumed predictive factors, our results emphasize the fact that VL is the only sensitive marker of early treatment failure, which is all the more relevant since we showed a very high rate of both failure and resistance at this stage.

In conclusion, 6 months after initiation of a zidovudine or stavudine/lamivudine/nevirapine regimen, in real field conditions, 44% of the children still followed showed virological failure, of whom 73% harboured viruses resistant to both lamivudine and NNRTI, but none to thymidine analogues or etravirine. These results reinforce the importance of the accessibility of HIV RNA assays for monitoring treated patients in resource-poor countries, where resistance testing is not available, to detect early virological failure in order to preserve future therapeutic options. Indeed, in the case of prolonged viral replication under treatment, resistance mutations would emerge, and late detection of virological failure could limit the use of NRTIs in second-line regimens. These results also strongly suggest the need for compliance improvement in the paediatric population,

based on intervention that should not wait until the month 6 evaluation because then failure is already associated with high resistance levels.

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