

Genetic analyses of HIV-1 *pol* sequences from Zimbabwean patients

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SUMMARY

This study analysed the sequence of HIV-1 *pol* gene derived from Zimbabwean infected patients. Sequence analysis, performed on 8 samples, revealed that sequences were classified as subtype C (n=5), subtype B (n=2) and CRF01_AE (n=1). Two patients, treated with a therapeutic regimen containing NRTI/NNRTI, harboured drug resistance mutations in HIV-1 DNA. Phylogenetic analysis performed on subtype C sequences showed that our strains were aggregated in different clusters depending on the country of origin.

KEY WORDS: *Pol* sequence, Drug resistance, Phylogeny, Zimbabwe.

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At the end of 2011, UNAIDS estimated that 34 million people were infected by HIV (http://www.unaids.org/en/resources/campaigns/20121120_globalreport_2012/) and countries with the largest epidemics are in southern Africa (Lihana *et al.*, 2012). Zimbabwe is one of the five countries worst affected by the HIV/AIDS pandemic in the world. According to the Zimbabwe Demographic Health Survey (ZHDS) 2010/11, the national adult HIV prevalence is 15% with 17% and 15% in urban and rural areas respectively. The HIV prevalence is highest among women aged 30-39 and men aged 45-49 and higher in females (18%) than in males (12%) (ZIMSTAT 2010-2011). HIV subtype C has begun to spread in some areas of East Africa (Congo, Tanzania,

Burundi and Kenya) as predominant ones (Bredell *et al.*, 2007; Bessong, 2008; Lihana *et al.*, 2012). The recent consistent flow of subtype C suggests that transmission and replication in the populations living in these regions is apparently easy.

In the framework of a volunteer experience in Saint Albert Hospital, a Zimbabwean hospital in the north of the Country, with 5000 admissions per year and HIV therapy administration facilities, we collected dried blood spot (DBS) samples from HIV-1 positive patients.

The main HIV-1 strain circulating in this area of Zimbabwe and drug-resistance mutations and/or polymorphisms eventually present in the *pol* gene were investigated.

DBS were obtained from finger-prick, 1 spot per card. The samples were collected from 44 HIV-1 Zimbabwean patients of whom 13 were men (29.5%) and 31 women (70.5%). The median age was 35 years for women (range 5-64 years) and 40 years for men (range 15-68 years). The median value for CD4+ count was of 200 cells/ μ l (range 15-1114/ μ l), all patients

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were without opportunistic infections. The viral load values were unknown. Thirty-two per cent of patients (14/44) were treated with antiretroviral therapy (ART) and were taking a co-formulation regimen based on fixed doses of stavudine, lamivudine and nevirapine.

DBS were extracted by the DNeasy Blood & Tissue Kit (Qiagen, Milan, Italy) in accordance with the manufacturer's instructions. The DNA content of each sample was determined by spectrophotometric analysis at 260/280 nm. The quantitative determination of HIV-1 DNA load was performed by home-made real time PCR able to amplify a highly conserved HIV-1 *pol* region. The reference standard cure was de-

termined using scalar DNA dilutions achieved from 8E5 lymphoblastoid cell, a cell line carrying one copy of integrated HIV-1 per cell. HIV-1 DNA load (median value 472 copies/ μ g DNA; range 145-18500 copies/ μ g DNA) was detected in 15 out of 44 (34%) patients. Viral sequences were obtained in only 8 out of 15 samples with detectable HIV-1 DNA load (53%). Specifically, HIV-1 RT and PR regions were amplified and sequenced using a TruGene HIV-1 Genotyping kit (Siemens Healthcare Diagnostics, Deerfield, IL, USA). Resistance mutations were identified by using the Guidelines[®] Rules 16.0, within the Gene Objects 3.2 software.

Subtype assignment was performed uploading

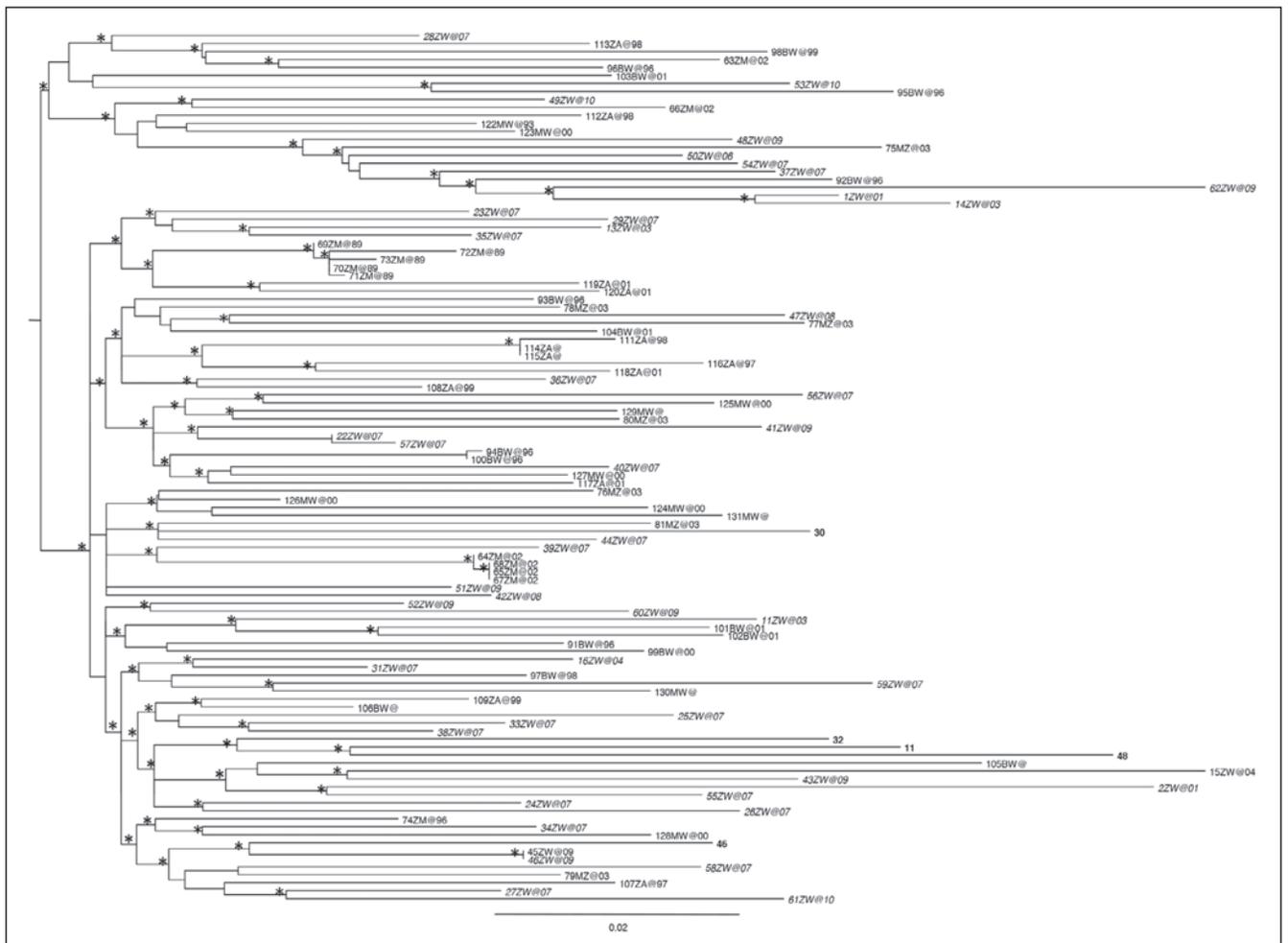


FIGURE 1 - Bayesian tree of the 109 *pol* gene sequences classified as HIV-1 C subtype. The asterisks (*) along a branch represent significant statistical support for the clade subtending that branch (posterior probability >98%). The five Zimbabwean infected patients are in bold. The reference Zimbabwean sequences downloaded from Genbank are in italics. The line at the bottom represents the number of substitutions per site.

sequences individually into the REGA HIV-1 automated Subtyping Tool v2.011 (<http://www.bioafrica.net>). This analysis showed that HIV-1 patient sequences were classified as subtype C (n=5) followed by subtype B (n=2) and CRF01_AE (n=1). The data set for phylogenetic analysis contained 109 sequences classified as HIV-1 C subtype, of which 104 were downloaded from the HIV-1 Los Alamos database (www.hiv.lanl.gov/content/index) and used as reference sequences.

These last sequences were selected by a randomization process from neighbouring countries, 46 from Zimbabwe, 12 from Zambia, 7 from Mozambique, 16 from Botswana, 10 from Malawi and 13 from South Africa. Sequence alignments were obtained using the Clustal algorithm followed by manual editing with BioEdit software to remove gaps and identical sequences as already described (Ciccozzi *et al.*, 2011, Ciccozzi *et al.* 2012). To investigate whether the sequences formed a monophyletic cluster or were interspersed, a Bayesian phylogenetic tree was reconstructed by means of Mr Bayes using a general-time-reversible model of nucleotide substitution, a proportion of invariant sites, and gamma-distributed rates among sites GTR + I + G. A Markov Chain Monte Carlo search was made for 10×10^6 generations using tree sampling every 100^{th} generation and a burn-in fraction of 25%. Statistical support for specific clades was obtained by calculating the posterior probability of each monophyletic clade, and a posterior consensus tree was generated after a 25% burn-in. The posterior probability was used as a statistical support for each cluster.

The Bayesian tree showed that our strains were aggregated in different clusters depending on the country of origin (Figure 1). Three patients (labelled 32, 11 and 48) clustered together with sequences from Botswana and Zimbabwe, whereas the patient labelled 46 was closely related with sequences from Zimbabwe only. The last patient, labelled 30, was closely related with sequences from both Zimbabwe and Mozambique.

The analysis of *pol* sequence was obtained from 6 patients treated with stavudine, lamivudine and nevirapine combination therapy and from two untreated patients. RT region mutations

were found in 2 out of 6 treated patients. Specifically, V108I, Y181C, M184V were detected in a patient infected with subtype C, receiving therapy for 2 years; L74V and T215Y were found in HIV-1 subtype B infected individuals receiving ART for 6 months.

Analysis of the PR region found the predominant secondary mutation, M36I, in 5 out of 8 viral sample sequences. This mutation represents a natural polymorphism not forcedly related to previous treatment and as confirmed by Holguin and other authors (Holguin *et al.*, 2002; Papa *et al.*, 2003; Turriziani *et al.*, 2008), M36I may be considered a genetic marker for HIV-1 group M non-B subtypes. According to these data we found that all strains harbouring this polymorphism were non-B subtype (4 subtype C and 1 CRF01_AE). Other mutations detected in the PR region were A71T/V in both B subtype samples; L33F and M36V in one B subtype sample.

In all subtype C, polymorphisms or silent substitutions at drug resistance sites were detected. These polymorphisms are already known in the literature (Turner *et al.*, 2004; Grossman *et al.* 2004; van de Vijver *et al.*, 2006) but their identi-

TABLE 1 - Wild type codons in C subtype and their impact on number of transition (ts) and transversion (tv) required for evolution to a drug resistance associated mutations

Substitution	WT codon (subtype)	Resistant codon	Mutations
K65R	AAA (B)	AGA	1ts
	AAG (C)	AGG	1ts
K70R	AAA (B)	AGA	1ts
	AAG (C)	AGG	1ts
V106I	GTA (B)	ATG	1ts
	GTG (C)	ATG	2ts
V106M	GTA (B)	GGG	2ts
	GTG (C)	GGA	1ts
E138G	GAG (B)	CAG	1ts
	GAA (C)	CAA	1ts
E138Q	GAG (B)	AAG	1tv
	GAA (C)	AAA	1tv
E138K	GAG (B)	AGG	1ts
	GAA (C)	AGA	1ts

fication is of interest because polymorphisms at drug resistance positions may modify the propensity to acquire mutations associated with drug resistance and change the genetic barrier for non-B subtypes. In fact, it is known that different codons at these positions could modify the number of transitions (A<->G o C<->T) and transversions (A<->C, A<->T, C<->G, G<->T) required for evolution to drug resistance (van de Vijver *et al.*, 2006).

The polymorphisms detected in subtype C samples are reported in table 1. As described by other groups (Turnet *et al.*, 2004; Grossman *et al.*, 2004; van de Vijver *et al.*, 2006), subtype C had a GTG codon at position 106 while subtype B had a GTA codon. The presence of this codon decreases genetic barrier in this subtype to establish the V106M mutation. In the remaining polymorphisms analysed the substitutions in sites 65, 70 and 138 did not affect the genetic barrier.

The main limit of this study is the low number of samples successfully sequenced, probably due to the insufficient quantity of blood and/or poor quality of extracted DNA. The use of DBS is widely documented in the literature (Bertoglio *et al.*, 2007; Gibellini *et al.* 2012; Yapo *et al.*, 2013; Fajardo *et al.*, 2014; Seu *et al.*, 2014; Smith *et al.*, 2014) and several studies performed, using infant specimens, reported that DBS are a good alternative to plasma both for measuring viruses and genotyping test. However in these studies at least three spots of blood were used, while two spots for each patient were available for the present analysis.

Although this study was performed on a very small number of samples, it revealed that 2 patients, treated with a therapeutic regimen containing NRTI/NNRTI, harboured drug resistance mutations in HIV-1 DNA. The presence of drug-resistant strains might have a great impact on the control of HIV-1 infection in this area, since NNRTI and NRTI are the main drugs used.

Furthermore, this study used a phylogenetic method to better understand the nature of this HIV-1 subtype C epidemic as a pilot study to continue the investigation in other areas of Zimbabwe. Although few samples could be analyzed, phylogenetic methods are powerful tools that can yield important information for

subsequent analysis even if applied in a small series of patients.

Interestingly, we found that some of our sequences were closely related with Mozambique and Botswana, and this may represent an important starting point for studying the viral flow from neighbouring countries and eventually implementing a surveillance system to contain HIV diffusion.

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