Analysis of HIV-1 pol sequences from Panama: Identification of phylogenetic clusters within subtype B and detection of antiretroviral drug resistance mutations

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ARTICLE INFO

Article history:
Received 13 February 2009
Received in revised form 13 June 2009
Accepted 15 June 2009
Available online 24 June 2009

Keywords:
HIV-1
Panama
Subtype B
Phylogeny
Antiretroviral drug resistance

1. Introduction

The high genetic diversity of human immunodeficiency virus type 1 (HIV-1) derives from a highly error-prone viral reverse transcriptase (RT), frequent recombination events during reverse transcription, short replication times and large population sizes (Nájera et al., 2002; Kijak and McCutchan, 2005). By means of these mechanisms, HIV-1 has diversified extensively into numerous genetic forms, which include three groups, of which group M, the causative of the pandemic, is subdivided in 9 subtypes (A–K), 6 sub-subtypes, 43 circulating recombinants forms (CRFs) and multiple unique recombinant forms (URFs) (Thomson et al., 2002a; Nájera et al., 2002; Kijak and McCutchan, 2005; Los Alamos HIV Sequence Database, 2009).

HIV-1 genetic variability is an important factor to be considered in the management of antiretroviral (ARV) drug-treated patients, since it may determine the selection of viral populations with decreased susceptibility to currently used drugs (Teixeira et al., 2006; Ríos et al., 2007). The surveillance of ARV resistance mutations is necessary, mainly in resource-limited countries where ARV treatment is being introduced, because the transmission and dissemination of drug-resistant strains may have major public health implications, in order to provide guidelines for the choice of ARV drug regimens and to design lines of rescue treatments directed to resistant variants (Delgado et al., 2001; Pires et al., 2004; Lama et al., 2006; Teixeira et al., 2006; Pérez et al., 2007; Ríos et al., 2007; Vasehus Madsen et al., 2008).
In Panama, 8486 AIDS cases, of which 335 are pediatric (<15 years of age), have been reported from September 1984 through September 2007. The estimated prevalence of HIV-1 infection is 1.0%, corresponding to the second highest prevalence in Central America after Belize (Ministry of Health, National Program on HIV and AIDS and UNAIDS, Panama, 2008; UNAIDS, 2009). The transmission routes are distributed as follows: sexual in 69.3%, perinatal in 3.4%, blood transfusion in 1.6%, and not available in 25.6%. Among sexually acquired infections, 75% correspond to heterosexual transmission, 19% to homosexual men, and 6% to bisexual men (Ministry of Health, National Program on HIV and AIDS, and UNAIDS, 2008). Until September 2007, the annual incidence rate was 12.1/100,000 inhabitants, with the highest rates in the most densely populated areas (Colon Province 41.6, Metropolitan Area of Panama Province 40.7, San Miguelito District 29.3, and the Eastern Districts of Panama Province 25.5) (Ministry of Health, National Program on HIV and AIDS, and UNAIDS, 2008).

In 1999, the Social Security System of Panama began to provide highly active antiretroviral therapy (HAART) for beneficiary patients, and in 2001 the Ministry of Health extended HAART coverage to the rest of HIV-1-infected individuals (Ministry of Health, STD/HIV/AIDS National Program, 2002; Ministry of Health, National Program on HIV and AIDS, and UNAIDS, 2008). The therapy is based mainly in the combination of two nucleoside or non-nucleoside reverse transcriptase inhibitors (NRTI or NNRTI) plus one protease inhibitor (PI) (Ministry of Health, STD/HIV/AIDS National Program, Panama, 2002). The introduction of HAART resulted in a decrease in AIDS mortality and opportunistic infections. Until 2007, a total of 3994 AIDS patients (adults and children) from Panama had received HAART (Ministry of Health, National Program on HIV and AIDS, and UNAIDS, 2008).

Sequence analysis of HIV-1 pol gene provides important information on ARV drug resistance-associated mutations affecting the susceptibility of HIV-1 strains to protease (PR) and RT inhibitors and on subtype diversity (Delgado et al., 2001; Pires et al., 2004; Rios et al., 2007). HIV-1 genetic diversity in Panama was previously examined in gag (p17) and env (C2–C4) genes (Ahumada-Ruiz et al., 2008). However, no studies on HIV-1 pol gene describing the epidemiology of genotypic drug resistance or phylogenetic relationships have been reported. Here we report the first study on the prevalence of ARV drug resistance-associated mutations and on genetic diversity in pol sequences in AIDS patients and drug-naive asymptomatic HIV-1-infected individuals from Panama.

2. Materials and methods

2.1. Patients and samples

Blood samples were obtained from 82 AIDS patients and 53 asymptomatic drug-naive HIV-1-infected individuals from the largest medical centers of the Panamanian health system, Complejo Hospitalario Dr. Arnulfo Arias Madrid, Caja del Seguro Social de Panamá (CSS), and Hospital Santo Tomás (HST). All samples were collected in 2004 and 2005. Of 135 samples, 109 (80.7%) were from men and 26 (19.3%) were from women. With regard to transmission routes, all patients were infected via sexual contact. Risk exposures were 73 (54.1%) heterosexual, 17 (12.6%) homosexual, 3 (2.2%) bisexual, and 42 unspecified. The distribution of geographic areas of sample collection was 59 Panama District, 28 San Miguelito District, 19 Arraiján District, 7 Colón Province, 5 La Chorrera District, 2 Capira District, and 1 each Chepo District, Coclé Province, Veraguas Province, Chiriquí Province, Bocas del Toro Province, and Comarca Kuna Yala; for 9 samples, data on place of sample collection was not available. To analyze geographical correlations of samples within phylogenetic clusters, two areas were considered, separated by the Panama Canal: East Panama, comprising Panama, San Miguelito, and Chepo Districts, Colón Province, and Comarca Kuna Yala; and West Panama, comprising Arraiján, Capira, and La Chorrera Districts, Coclé, Veraguas, and Chiriquí Provinces.

2.2. RNA isolation, amplification and sequencing

Viral RNA was extracted from 200 µl of plasma with Magna Pure LC Automated Nucleic Acid Extraction System (Roche, Mannheim, Germany), following the manufacturer's instructions. An HIV-1 pol fragment comprising the entire protease and 978 nucleotides (nt) of the RT coding region was amplified by reverse transcription coupled with PCR, followed by nested PCR, from plasma RNA using an in-house method (Villalhemosa et al., 2000). Direct sequencing in PR and RT coding regions was done with ABI Prism BigDye Terminator Kit and ABI PRISM 3700 automated sequencer (Applied Biosystems, Foster City, CA).

2.3. Phylogenetic analyses

The sequence electropherograms were assembled with SeqMan (DNASter, Madison, WI), and manually aligned using BioEdit (Tom Hall, http://www.mbio.ncsu.edu/Bioedit/bioedit.html). Neighbor-joining (NJ) trees, based on Kimura’s 2-parameter distances, were constructed using MEGA v. 3.1 (Kumar et al., 2004). The reliability of tree topologies was assessed by bootstrapping with 1000 replicates. Phylogenetic trees were also constructed using Bayesian inference with MrBayes v. 3.1 (Huelsenbeck and Ronquist, 2001), using the general time reversible nucleotide substitution model, with gamma-distributed among-site rate heterogeneity and a proportion of invariable sites (GTR + I + I). For each dataset, two simultaneous independent runs were performed, with eight chains, sampling every 500 generations. The analyses were run until both runs had reached convergence, as determined by an average standard deviation of split frequencies <0.01. Node support was derived from a majority-rule consensus of trees sampled from the posterior distribution, discarding the first 50% as burn-in.

Recombination analyses were performed with bootscanning using Simplot v. 3.5.1 (Lole et al., 1999). In these analyses, windows of 300 nucleotides were used moving in 20 nt increments; phylogenetic trees were constructed with the NJ method, using Kimura’s 2-parameter distances, with the transition/transversion ratios estimated from the dataset.

The dates of the most recent common ancestors (MRCA) of phylogenetic clusters were estimated using a Bayesian Markov Chain Monte Carlo (MCMC) method as implemented in BEAST v. 1.4 (Drummond and Rambaut, 2007). Since evolutionary rates could not be estimated directly from the sequences of Panama, because samples were collected over a relatively narrow time span, the analysis was performed using mutation rates previously calculated for the same HIV-1 pol segment (Hué et al., 2005) applying a constant molecular clock. The substitution model employed for the analysis was GTR + I + I. Each MCMC chain was run for 80 million states and sampled every 100,000 states, with the first 10% discarded as burn-in. MCMC convergence and effective samples sizes were checked using the program Tracer v. 1.4 (available at http://tree.bio.ed.ac.uk).

2.4. Antiretroviral drug resistance analysis

PR-RT sequences were analyzed for antiretroviral resistance-associated mutations with the HIVdb program at the Stanford
University High Drug Resistance Database (Rhee et al., 2003; http://hivdb.stanford.edu). At ambiguous sequence positions (due to a mixed virus population), a drug resistance-associated mutation was assumed if present in the mixture.

2.5. Statistical analyses

Statistical correlations of phylogenetic clusters with geographical area or transmission route were analyzed with Fisher’s exact test.

3. Results

3.1. Phylogenetic diversity of HIV-1 in Panama

The phylogenetic analysis of HIV-1 pol sequences from Panama revealed that 133 (98.5%) of 135 sequences, including 80 (97%) of 82 AIDS patients and all 53 (100%) asymptomatic individuals, were infected with subtype B viruses, and the two remaining AIDS patients harboured viruses clustering with references of CRF12_BF and sub-subtype A3, respectively (Fig. 1). Bootscan analyses of the two non-subtype B viruses showed that the one clustering with A3 references corresponded to a CRF02_AG/A3 recombinant, with PR mainly of CRF02_AG and RT of sub-subtype A3, and the one clustering with CRF12_BF corresponded to a CRF12_BF/B secondary recombinant, with PR mainly of CRF12_BF and RT recombinant between subtype B and CRF12_BF (Fig. 2).

When analyzing the sequences with a Bayesian phylogeny inference method, 5 clusters of ≥5 sequences were identified within subtype B, which together comprised 87 (65.4%) of 133 subtype B viruses. Clusters were designated B-PA1 (n = 42), B-PA2 (n = 15), B-PA3 (n = 14), B-PA4 (n = 10), and B-PA5 (n = 6) (Fig. 3). Clusters B-PA2, B-PA4 and B-PA5 were supported by a posterior probability (PP) of 1.0, and clusters B-PA1 and B-PA3 by PP of 0.89 and 0.90, respectively. These increased to 1.0 and 0.96, respectively, when the analysis was done after removal of sequences branching as outliers of these clusters. Cluster B-PA1 was significantly associated with East Panama and clusters B-PA2 and B-PA4 with West Panama (p < 0.05, Fisher’s exact test) (Table 1). No significant association between any of the clusters and transmission routes was found. A Bayesian coalescent analysis (Table 1). No significant association between any of the clusters and transmission routes was found. A Bayesian coalescent analysis showed that the data represent calendar years, with the mean values and, in parentheses, the 95% highest posterior density (HPD) confidence intervals. The analysis was performed with BEAST (Drummond and Rambaut, 2007).

3.2. Antiretroviral drug resistance-associated mutations

ARV drug resistance-associated mutations were detected in 8 (6%) individuals, all of them AIDS patients with previous exposure to antiretroviral drugs. The distributions of ARV drug resistance-associated mutations were as follows: one patient with resistance to PI, three to NNRTI, two to PI + NRTI, one to NRTI + NNRTI, and one to all three drug classes. The resistance mutations detected in the eight AIDS patients were consistent with their treatment regimens, except for two patients (PA_23 and PA_59). No ARV resistance-associated mutations were identified in the drug-naïve individuals. A complete list of ARV drug resistance-associated mutations that were found is shown in Table 3.

4. Discussion

In this study, phylogenetic and antiretroviral resistance analyses were combined in partial pol sequences from 135 HIV-1-infected subjects from Panama.
Fig. 1. Neighbour-joining phylogenetic tree of PR-RT sequences from Panama. Names of viruses from Panama start with PA and are in bold type. Names of subtype B references are boxed. The two non-subtype B viruses of Panama are marked with black squares. Names of HIV-1 clades comprising Panamanian viruses are indicated on the right of the corresponding brackets. Only bootstrap values 50% or higher are shown.
Panama has a long time of evolution. This observation is consistent with the report of the first AIDS case in Panama in 1984 (Ministry of Health, National Program on HIV and AIDS, and UNAIDS, 2008).

In the present study, the existence of 5 strongly supported monophyletic clusters within subtype B has been recognized for the first time by using a Bayesian phylogeny inference method. Nearly two thirds of Panamanian subtype B viruses branched within one of these clusters, designated B-PA1 to B-PA5. To analyze geographical correlations of the clusters, Panama was divided in two regions, East and West, using the Panama Canal as the separation line. Three clusters (B-PA1, B-PA2, and B-PA4) were correlated to a geographical area (Table 1). However, no statistically significant correlation between any of the clusters and risk exposure (heterosexual vs. homo- or bisexual) was found. A Bayesian coalescent analysis suggests that these clusters are relatively old, with estimates for the origin of the four largest clusters in the 1980s, and that of the fifth in the early 1990s.

The existence of HIV-1 intraclade phylogenetic clusters, in some cases with epidemiological or geographical correlations, has been reported previously in other countries, including Cuba (subtype B), Brazil (subtype B), Ethiopia (subtype C), and Russia (subtype A) (Thomson and Nájera, 2005; Pérez et al., 2006; Thomson et al., 2009). These clusters may derive from multiple introductions of an HIV-1 subtype or CRF or may reflect the existence of multiple active transmission networks in a country or region. In the case of Panama, a Bayesian analysis suggests an old origin of the clusters, which may support a scenario of multiple subtype B introductions in the country at the onset of epidemic. An alternative possibility is that the clusters reflect the establishment of diverse local transmission networks early in the HIV-1 epidemic in Panama. Those sequences branching outside of the major clusters may represent either separate introductions or intrasubtype (inter-cluster) recombinant viruses. Our preliminary analyses of near full-length genomes (unpublished data) further support the existence of the clusters here identified, and have allowed the identification of intrasubtype B recombinant viruses. Further work will be required to investigate possible biological correlations of the clusters, including susceptibility to immune responses, which may be relevant for the design of vaccines.

Bootscan analyses identified two AIDS patients with recombinant viruses (Fig. 2). The first, PA_15, clustered in PR with CRF02_AG and in RT with sub-subtype A3. The second, PA_39, clustered in PR with CRF12_BF and in RT with subtype B (5' segment) and CRF12_BF (3' segment). In a previous study in Panama which included these same samples using \textit{gag} and \textit{env} segments, PA_15 grouped with CRF02_AG and PA_39 with CRF12_BF in both regions (Ahumada-Ruiz et al., 2008). The bootscan analyses suggest that both samples correspond to unique recombinant forms, but characterization of full length genome sequences will be required to fully determine the recombination profile.

The CRF02_AG and sub-subtype A3 predominates in West and Central African countries (Meloni et al., 2004; Delgado et al., 2008). However, the sporadic presence of CRF02_AG has been reported recently in Ecuador, Brazil and Cuba (Carrion et al., 2003; Eyer-Silva and Morgado, 2007; Pérez et al., 2007). CRF12_BF has been reported in countries of South America such as Argentina, Uruguay and Chile (Thomson et al., 2000, 2002b; Carr et al., 2001; Ríos et al., 2007). Travel-associated infections from different categories contribute to an increase in HIV diversity, as recently observed in countries such as Cuba (Thomson and Nájera, 2001; Pérez et al., 2006).

The genotypic drug resistance analyses revealed that 8 (9.7%) AIDS patients and no drug-na"\i"ve individuals harbored mutations conferring high or intermediate resistance levels to ARV drugs (Table 3). These figures are lower than those reported in similar studies in Latin America. Thus, in Chile the prevalence of high or
Fig. 3. Bayesian phylogenetic tree of PR-RT subtype B sequences from Panama. Names of viruses from Panama start with PA and are in bold type. Names of subtype B references are boxed. Only node posterior probabilities ≥0.90 are shown, except for cluster B-PA1 (PP = 0.89). Panamanian subtype B cluster names are indicated on the right of the corresponding brackets. For clusters B-PA1 (PP = 0.89) and B-PA3 (PP = 0.90), the node posterior probabilities increased to 1.00 and 0.96, respectively, when sequences PA_46 and PA_208, branching as outliers of cluster B-PA1, and PA_58, branching as outlier of cluster B-PA3, were removed in a subsequent analysis.
intermediate resistance level was 77% among 66 ARV drug-treated subjects and 2.5% among 79 drug-naive individuals (Ríos et al., 2007). In Venezuela the prevalence of resistance to RT inhibitors was 26% in ARV treated patients and 3% in drug-naive individuals (Delgado et al., 2001). In Peru, the prevalences of ARV drug resistance mutations were 31.3% among treated patients and 3.3% among drug-naive individuals (Lama et al., 2006). The only data on HIV-1 drug resistance in Central America is from a study in Honduras in 2008, where the drug resistance prevalence was 9.2% among drug-naive individuals (Lloyd et al., 2008).

The low prevalence of ARV drug mutations in AIDS patients of Panama may derive from the relatively short time elapsed from generalization of ARV drug therapy in Panama until the time of sample collection for this study. Differences with other countries may also derive from differences in the degree of adherence to the therapeutic regimens. Failure to detect drug resistance mutations among 53 drug-naive individuals might suggest that it may not be necessary to present at routine test for drug resistance prior to the start of ARV drug treatment in Panama. However, since this may change in the future, it is important to continue the epidemiologic surveillance of ARV drug resistance in Panama.

In PA_14 sample, the T215S mutation was detected. The T215S/C/D/E/I/V/A/N mutations represent reversions from the NRTI resistance mutation T215Y. Most of these mutations do not reduce NRTI susceptibility, but their presence suggests that a resistant virus may have been transmitted (Goudsmith et al., 1996). In this patient, the sample collection and the start of the ARV therapy was in the same month, which suggest that the T215S mutation might derive from a transmitted virus.

In two samples (PA_23 and PA_59) NNRTI resistance mutations were detected. However, both patients reportedly were never treated with NNRTI drugs. This finding suggests the possibility of reinfection with viruses with NNRTI resistance mutations (Table 3).

In conclusion, this is the first study on HIV-1 pol gene in Panama, analyzing genetic diversity and ARV drug resistance among treated and drug-naive individuals. Only subtype B was found to be circulating in Panama, although sporadic recombinant viruses (CRF02_AG/A3 and CRF12_BF/B) were identified. Drug resistance mutations were detected in 9.7% AIDS patients and revealed the presence of acquired mutations to ARV drugs. This information data may be relevant for the management of patients and for implementing prophylactic measures to prevent increases in acquired resistance mutations. Finally, we identified for the first time 5 phylogenetic intrasubtype clusters comprising the majority of subtype B viruses, with the origin of 4 of the largest clusters estimated in the 1980s, which suggests either multiple HIV-1 subtype B introductions, or the establishment, early in the epidemic, of local transmission networks, which gave rise to most current HIV-1 infections in Panama.

Acknowledgments

The authors thank all patients who participated in this study. We also gratefully acknowledge all the physicians and nurses at the CSS and HST who contributed to the collection of samples. We also thank Dr. Moisés Espino, Miss Esther Puga (CSS), Dr. Rigoberto Samaniego and Dr. Antonio Torres (HST) for their invaluable collaboration. We are also grateful to Pablo Martínez, Aurora de Miguel, and Ana Parejo of the Genomic Unit, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Majadahonda, Madrid, Spain, for technical assistance in sequencing. Sara Ahumada Ruiz was supported through a fellowship from the Government of Panama.

References


Los Alamos HIV Sequence Database Available at: http://hiv-web.lanl.gov.


