New Subtypes and Genetic Recombination in HIV Type 1-Infecting Patients with Highly Active Antiretroviral Therapy in Peru (2008–2010)

Carlos Augusto Yabar, Maribel Acuña, Cecilia Gazzo, Gabriela Salinas, Fanny Cárdenas,
Ada Valverde, and Soledad Romero

Abstract

HIV-1 subtype B is the most frequent strain in Peru. However, there is no available data about the genetic diversity of HIV-infected patients receiving highly active antiretroviral therapy (HAART) here. A group of 267 patients in the Peruvian National Treatment Program with virologic failure were tested for genotypic evidence of HIV drug resistance at the Instituto Nacional de Salud (INS) of Peru between March 2008 and December 2010. Viral RNA was extracted from plasma and the segments of the protease (PR) and reverse transcriptase (RT) genes were amplified by reverse transcriptase polymerase chain reaction (RT-PCR), purified, and fully sequenced. Consensus sequences were submitted to the HIVdb Genotypic Resistance Interpretation Algorithm Database from Stanford University, and then aligned using Clustal X v.2.0 to generate a phylogenetic tree using the maximum likelihood method. Intrasubtype and intersubtype recombination analyses were performed using the SCUEAL program (Subtype Classification by Evolutionary ALgo-rithms). A total of 245 samples (91%) were successfully genotyped. The analysis obtained from the HIVdb program showed 81.5% resistance cases ($n=198$). The phylogenetic analysis revealed that subtype B was predominant in the population (98.8%), except for new cases of A, C, and H subtypes ($n=4$). Of these cases, only subtype C was imported. Likewise, recombination analysis revealed nine intersubtype and 20 intrasubtype recombinant cases. This is the first report of the presence of HIV-1 subtypes C and H in Peru. The introduction of new subtypes and circulating recombinants forms can make it difficult to distinguish resistance profiles in patients and consequently affect future treatment strategies against HIV in this country.

Introduction

HIV/AIDS REMAINS THE MAIN sexually transmitted infection worldwide with around 33 million people living with HIV and 2.7 million new infections in 2007.¹ In Peru, HIV infection continues to be a concentrated epidemic because most cases are detected in men who have sex with men (MSM) and female sex workers. However, this pandemic is also frequently being detected in the heterosexual population.²

The global spread of HIV subtypes and the circulating recombinant forms (CRFs) has dramatically changed in recent years. In South America, the introduction of subtype C and different CRFs has recently been observed in some countries, especially in Argentina and Brazil.^{3–6}

In Peru the most frequent subtype is B. However, subtypes F and CRF17_BF have also been found in lower percentages.^{7–10} Notoriously, infection with some non-B subtypes and CRFs can affect both disease progression¹¹ and clinical treatment,¹²

because they show differential pathogenic and resistance patterns in relation to subtype B. In this context, subtype identification in patients receiving highly active antiretroviral therapy (HAART) might be very important for future studies focused to the discovery and interpretation of new drug-resistant mutations that are subtype specific. For this reason we have initiated the identification of subtypes and recombination events among HIV strains from patients receiving HAART, focusing our analysis on children and adult patients included in the National Antiretroviral Therapy Program from Peru.

Materials and Methods

Clinical samples

A collection of 267 plasma samples collected from HIV-positive patients to monitor HIV antiretroviral resistance

TABLE 1. EPIDEMIOLOGIC CHARACTERISTICS OF THE STUDIED POPULATION

Characteristic	All	Adults	Children
Patients. no. (%)	267 (100)	102 (38.2)	165 (61.8)
Age, mean (range), years	14.3 (100)	37 (18–74)	4.9 (0.2–17)
Sex: no. (%)			
Male	150 (56.2)	60 (40)	90 (60)
Female	117 (43.8)	42 (35.9)	75 (64.1)
Route of transmission: no. (%)			
Sexual	102 (38.2)	102 (100)	0 (0.00)
Vertical	165 (61.8)	0 (0.0)	165 (100)
Region where patients are from: no. (%)			
Lima	215 (80.5)	71 (69.6)	144 (88.3)
Callao	28 (10.5)	13 (12.8)	15 (9.2)
Loreto	14 (5.2)	13 (12.8)	1 (0.6)
Pucallpa	3 (1.1)	0 (0.0)	3 (1.8)
Arequipa	3 (1.1)	1 (1.0)	0 (0.0)
Trujillo	1 (0.37)	1 (1.0)	0 (0.0)
Unknown	3 (1.1)	3 (2.9)	0 (0.0)
Current antiretroviral treatment: no. (%)			
PI only	2 (1.2)	2 (1.2)	0 (0.0)
NRTI only	17 (16.0)	11 (10.8)	6 (3.6)
NNRTI only	0 (0.0)	0 (0.0)	0 (0.0)
PI+NRTI	138 (85.7)	53 (51.9)	85 (51.5)
PI+NNRTI	0 (0.0)	0 (0.0)	0 (0.0)
NRTI+NNRTI	50 (31.1)	20 (19.6)	30 (18.2)
PI+NRTI+NNRTI	3 (1.1)	2 (2.0)	1 (0.6)
No data available	57 (53.8)	14 (13.7)	43 (26.1)

Data were collected from the clinical file of each patient and were sent to the INS from each Health Establishment. In this analysis CD4 and viral load information were not included because these analyses were performed using other samples collected before or after the genotyping assay. Statistical data were calculated by Excel Program 2007.

PI, protease inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, nonnucleoside reverse transcriptase inhibitor.

between 2008 and 2010 was analyzed in this study (see Table 1). Each sample was extracted from a patient who had been previously evaluated and approved by an Instituto Nacional de Salud (INS) Experts Committee, which decided if the patient should be analyzed by HIV genotyping. Criteria for approval were as follow: (1) patients whose age ranged from 0 to less than 18 years old with a viral load of over 10^4 copies/ml after 6 months of receiving first-line antiretrovirals; (2) patients more than 18 years old, with evidence of over 10^3 copies/ml after an interval of at least 2 months of starting second-line antiretroviral treatment; and (3) pregnant women, in case of treatment failure using the two previously indicated criteria.

All blood samples were processed immediately or maintained at room temperature (between 20°C to 25°C) for a maximum of 24 h after arrival at the INS. They were then homogenized and subjected to two centrifugation steps: a first one at 1900 rpm for 10 min and then a second one at 3300 rpm for 20 min. Finally, the plasma samples so obtained were stored at -80°C until RNA extraction.

RNA extraction

For this purpose, a plasma volume of 140 μl was processed with the QIAamp Viral RNA Mini Kit (Qiagen) according to

the manufacturer's recommendations. Each tube containing the purified RNA was stored at -80°C .

One-step reverse transcriptase-polymerase chain reaction and nested PCR

All PCR assays were performed using an Applied Biosystem thermocycler model 9700. The *pol* region (length=1700 bp) was amplified following the Stanford Protocol generously provided by Dr. Robert Grant (Gladstone Institute).¹³ According to this method, 17 μl of RNA was added to a tube containing 1 $\times$ Reaction Mix (0.2 mM of each dNTP and 1.6 mM MgSO_4), 0.5 mM magnesium sulfate, 0.5 pmol of primers RT-21(-) (5'-CTG TAT TTC TGC TAT TAA GTC TTT TGA TGG G-3') (positions 2028–2050 HXB-2) and 1243(-) (5'-ACT AAG GGA GGG GTA TTG ACA AAC TC-3') (positions 3792–3817 HXB-2), 1 pmol of primer MAW-26(+) (5'-TTG GAA ATG TGG AAA GGA AGG AC-3') (positions 2028–2050), and 1 μl of SuperScript III RT/Platinum Taq Mix (Invitrogen) and then incubated at 45°C for 30 min. After that, the enzyme was denatured by 95°C for 2 min followed by 40 cycles to 94°C for 15 s, 55°C for 20 s, and 72°C for 2 min, and finally a hold temperature of 4°C. Each tube was then maintained at 4°C.

Alternatively, a second round was performed in a tube containing 1 $\times$ High Fidelity PCR Buffer Reaction Buffer II (60 mM Tris- SO_4 , pH 8.9; 18 mM ammonium sulfate), 2.5 mM MgCl_2 , 0.15 mM dNTPs, 0.2 pmol of primer PRO-1(+) (5'-CAG AGC CAA CAG CCC CAC CA-3') (positions 2147–2166), 0.1 pmol of primers RT-20(-) (5'-CTG CCA GTT CTA GCT CTG CTT C-3') (positions 3441–3462 HXB-2) and 1205 (5'-CCA GGT GGC TTG CCA ATA CTC TGT CC-3') (positions 3754–3779), 0.25 U of Taq DNA Polymerase (Applied Biosystem), and 5 μl of reaction from the first round (100 μl of final volume). Cycle conditions were as follows: 35 cycles of 94°C for 15 s, 63°C for 20 s, and 72°C for 2 min, followed for one cycle to 72°C for 10 min and a hold temperature of 4°C.

Amplification of *gag* and *env* genes was also performed in order to explore in more detail the genetic characteristics of those viral species classified as subtypes different than B. According to this, the p24-p7 portion of the *gag* gene (460 bp) was amplified by using the primers H1G777(+) (5'-TCACC TAGAAGTTTGAATGCATGGG-3') (positions 777–801) and H1P202(-) (5'-CTAATACTGTATCATCTGCTCCTGT-3') (positions 1874–1898) for the first round and H1Gag1584(+) 5'-AAAGATGGATAATCCTGGG-3' (positions 1123–1141) and g17(-) 5'-TCCACATTTCCAACAGCCCTTTT-3' (positions 1566–1589) for the second round, following the recommendations of Van der Auwera and Heyndrick¹⁴; *env* gene amplification (550 bp) was performed by using the primers ED5(+) 5'-ATGGGATCAAAGCCTAAAGCCATGTG-3' (positions 6556–6581) and ED12(-) (5'-AGTGCTTCCTGCTGCT CCAAGAACCCAAG-3') (positions 7822–7792) for the first round, while primers ED31(+) (5'-CCTCAGCCATTACACA GGCCTGTCCAAAG-3') (positions 6816–6844) and ED33(-) (5'-TTACAGTAGAAAAATCCCCTC-3') (positions 7359–7380) were used for the second round following the recommendations of Delwart *et al.*¹⁵

All PCR products were analyzed by electrophoresis in 1.2% agarose (*pol* region) or 1.5% (*env* and *gag* genes) and registered by using a Gel Doc XDR machine.

DNA purification and direct sequencing

The PCR product was purified with a Qiaquick PCR purification System (Qiagen) according to the manufacturer's instructions and then quantified by Agarose Gel Electrophoresis using the Low Mass Ladder Molecular Weight Marker (Invitrogen) and the Quantity One software of the Gel Doc documentation system (Bio-Rad).

To determine the reverse transcriptase and protease sequence, 10 ng of DNA was used for direct sequencing (BigDye Terminator v3.1 Cycle Sequencing Kit), which was mixed with 4 μ l of Ready Reaction Premix containing Big Dye, 1 $\times$ of BigDye Sequencing Buffer, 0.25 pmol of the following primers: MAW-46 (5'-TCC CTC AGA TCA CTC TTT GGC AAC GAC-3'), DSPR (5'-GGG CCA TCC ATT CCT GGC-3'), PSR-2 (5'-ATG CCT TTA TTT TTT CTT CTG TC-3'), RT-a (5'-GTT GAC TCA GAT TGG TTG CAC-3'), RT-b (5'-CCT AGT ATA AAC AAT GAG ACA C-3'), RT-y (5'-GTG TCT CAT TGT TTA TAC TAG G-3'), and HXB2-89 (5'-AAT CTG ACT TGC CCA ATT CAA TTT-3'). Alternatively, a list of primers was used as well as RT-z (5'-TAG GCT GTA CTG TCC ATT TAT C-3'), B (5'-GGA TGG AAA GGA TCA CC-3'), Brev (5'-GGT GAT CCT TTC CAT CC-3'), HXB2-88 (5'-TAA AAT TAA AGC CAG GAA TGG ATG-3'), and MAW-70 (5'-TAA TCC CTG CGT AAA TCT GAC TTG CCC A-3'). For *env* sequencing, the primers used were ED31 (+) and ED33 (-).

Sequencing mix was subjected to one hold to 96°C for 1 min, 25 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. Sequencing products were mixed in a chilled clean-up buffer containing 3 M sodium acetate, pH 5.4, and 78% of ethanol. The mix was incubated to 20°C–25°C for 30 min and then centrifuged to 13,000 rpm for 30 min. After a washing process with 70% alcohol and a centrifugation step to 13,000 rpm for 15 min, sequencing products were dried to 20°C–25°C for 20 min. The sequencing products were denatured with 15 μ l of pure formamide and then run using a 3100 Avant sequencing analyzer.

Sequences analysis

DNA sequences synthesized by each primer (fragment size between 400 bp and 600 bp) were either analyzed as assembled in FASTA format by using the softwares sequencing analysis version 5.1.1 and SeqScape version 2.5, respectively (Applied Biosystem). Then, FASTA sequences were aligned with Clustal W, version 2.0.11¹⁶ to perform phylogenetic analysis by the maximum likelihood (ML) method¹⁷ using the HKY85 model,¹⁸ considering a 1000 bootstrap and gap deletion.

We have used the program SCUEAL¹⁹ (Subtype Classification by Evolutionary Algorithms) for HIV to identify subtypes and detect intersubtype and intrasubtype recombination breakpoints. According to this, intrasubtype/intersubtype recombination is detected phylogenetically using different parts of the query strain and then clustered with different subtype reference sequences. Significance is established by phylogenetic model averaging, and the breakpoints are mapped using a genetic algorithm.

For identification of punctual resistance mutations, consensus sequences were submitted to the HIV Database (db) from Stanford University by using the HIVdb program (<http://sierra2.stanford.edu/sierra/servlet/JSierra?action=sequenceInput>).

Results

Population characteristics

According to Table 1, 61.8% of the people on whom a resistance test was performed were children whose mean age was approximately 5 years old, while the mean age for the adult population was 37 years old. Most of them (81% and 11%) came from urban centers such as Lima and Callao. Regarding gender, the male group was slightly larger (56%) than the female group in both the adult and children populations. According to treatment protocols, the most frequent HAART combination was the protease inhibitor (PI) with a nucleoside reverse transcriptase inhibitor (NNRTI) (85.71%) in contrast with other drug combinations. The available database did not show information about the actual course of therapy for an important number of children ($n=43$, 26%).

Genetic diversity analysis revealed one case of HIV subtype H and various recombination events

Out of 267 samples, 245 (92%) were successfully genotyped while the other 22 samples failed to be amplified by PCR assay. The phylogenetic analysis performed using the ML method (see Materials and Methods) revealed that 98% ($n=241$) of the samples belonged to subtype B. Of interest, we have found two cases of subtype A, one case of subtype C, and for the first time in Peru, one sample subtyped as H (see Fig. 1).

To explore the genetic characteristics of subtype H in more detail, we have performed the analysis of the *gag* and *env* genes by phylogeny. According to this, the PCR assay failed to amplify the *gag* fragment (data not shown); however, we have got to amplify 550 bp of *env*. The phylogeny analysis was performed using different strains of HIV-1 subtyped H as reported in various countries (see Fig. 2). Our phylogenetic analysis using the *env* gene revealed that the Peruvian sample was clustered with strains from Central and South Africa (e.g., Angola, Democratic Republic of Congo, Cameroon), which was named subtype "H1" in this work, while other sequences from the Central African Republic, Belgium, and Spain were clustered to another phylogenetic group, which was named subtype "H2."

On the other hand, we have performed recombination analysis in order to find recombinant cases along the *pol* region. For this purpose we used the SCUEAL algorithm. According to the results obtained, we have identified 20 cases of intrasubtype recombination (among B subtype viruses) and nine different cases of intersubtype recombination (A1/B, B/C, B/D, B/F1, and a CRF17-like case) (see Fig. 3). This algorithm also confirmed the presence of subtypes A, C, and H in the Peruvian population.

HIV recombinant species showed drug resistance mutations different from nonrecombinants

We have analyzed the most prevalent resistance mutations among HIV species showing either recombination (intrasubtypes and intersubtypes) and not recombination (see Fig. 4). This analysis was performed to determine if recombination is involved in transmission of some specific resistance mutations different than the nonrecombinant species. Our results showed that mutations M46I (11.8–14.3%), L90M (12.8–17.6%), M184V (24–28%), and K103N (15.8–21.4%) were

FIG. 1. Phylogenetic analysis of HIV *pol* sequences obtained from Peruvian patients receiving highly active antiretroviral therapy (HAART). This analysis was performed with the maximum likelihood method using a bootstrapping factor of 1000. Capital letters show each referential subtype included in this analysis and the letters in large font size indicate genetic subtypes found in this population. The number at the bottom shows the scale by which the phylogenetic tree was designed.

FIG. 2. Phylogenetic analysis of HIV *env* sequences (550 bp) for a Peruvian case subtyped as H (●) compared to other HIV subtype H referential strains and other subtypes. This analysis was performed using the maximum likelihood method using a bootstrapping factor of 500. Capital letters "H1" and "H2" indicate two phylogenetic groups of subtype H found in this analysis. The number at the bottom shows the scale by which the phylogenetic tree was designed. GenBank accession numbers: 01AOHJM63 Angola (AY456298), 01AOHJM06 Angola (AY456280), 01AOSNS22 Angola (AY456288), 01AOSNS23 Angola (AY456289), 01AOSNS01 Angola (AY456278), 02DC.KS033 Democratic Republic of Congo (AJ877649), 02DC.KBS187 Democratic Republic of Congo (AJ877579), 02DC.LBTB031 Democratic Republic of Congo (AJ877804), 040135609 Peru (in process of submission), 01CM.1296NG Cameroon (AY371162), 02DC.KTB026 Democratic Republic of Congo (AJ877681), 05CVHAN19 Cape Verde (JF267404), 01AOSNS51 Angola (AY676573), CU4 Cuba (AF425490), 01AOHDC230 Angola (AY456308), 02DC.KSFE110 Democratic Republic of Congo (AJ877727), 97DC.KP93 Democratic Republic of Congo (AJ877875), 01AOHDP70 Angola (AY676581), 02DC.KSTB022 Democratic Republic of Congo (AJ877746), United Kingdom (FJ711703), UNC6316.11 United States (HM215438), HIV Central African Republic (AF005496), 93AOHDC251 Angola (HQ738344), TV 228 gp41 South Africa (EF547498), 93JTS10-9499 South Korea (JQ248224), 93JTS10-9498 South Korea (JQ248223), VI991 Belgium (AF190127), VI997 Belgium (AF190128), 90CR056 Central African Republic (AF005496), and LMM12131193 Spain (HQ426900). Sequences of other referential strains were provided by the HIV Reagent Program.

FIG. 3. Intersubtype HIV-1 recombination from the Peruvian population. Recombination analysis was performed by the SCUEAL algorithm (see Materials and Methods) and the illustration was designed using the Recombinant HIV-1 Drawing Tool (www.hiv.lanl.gov/content/sequence/DRAW_CRF/recom_mapper.html). Numbers on the top to the left indicate the sample code, numbers oriented vertically indicate the position of breakpoint recombination according to SCUEAL analysis, and regions on the HIV genomic map and boxes shadowed with black and gray colors indicate the subtype recombination identified.

FIG. 4. Comparison of resistance mutations to protease inhibitors (A), nucleoside analog reverse transcriptase inhibitors (B), and nonnucleoside reverse transcriptase inhibitors (C) among HIV-1 samples showing intrasubtype (■)/intersubtype (■) recombination and nonrecombination (□). The x-axis shows mutation prevalence (%), which was calculated for each recombinant and nonrecombinant group. The y-axis shows resistance mutation. Arrows indicate the most frequent mutations for intrasubtype/intersubtype recombinants.

highly frequent for either intrasubtype/intersubtype recombinants as nonrecombinant species. However, we found recombinants showing some specific mutations with more frequency than others. In the case of intersubtype recombinants, they showed a high frequency of mutations N88S (14%), K101Q (20%), and E138K (10%), while for intrasubtype

recombinants the most prevalent mutations were I54L (6%), D67N (14%), K101E/H (11%), G190A (16%), and K219Q (12%). Additionally, we found that recombinant cases showed more resistance (85–100%) than the nonrecombinants (14%), with children (85%) mainly showing HIV with intrasubtype recombination (data not shown).

TABLE 2. RESISTANCE MUTATIONS AT NON-B SUBTYPES AND RECOMBINANT CASES

Code	Subtype reported in this study	Resistance mutations according to the Stanford HIV Database			Resistance mutation in non-B subtypes ¹²	% Frequency at subtype B samples reported in this study
		IP major mutations	NRTI mutations	NNRTI mutations		
040135609	H	None	M184V	V108I, Y181C	M36I	34.44
051830710	A	None	D67N, K70R, M184V, K219Q	K101Q, G190A	M36I	34.44
110216810	A	None	None	None	M36I	34.44
070406409	C	None	None	None	M36I, I93L	34.44, 73.86
40528410	B/C	None	None	None	None	—
40520809	B/F (CRF-12-like)	V32I, M46I, I47V, I54L, V82F	M41L, D67N, K70R, V75M, M184V, T215F, K219Q	K103N	G196E	15.1
41644709	B/F1	D30N, N88D	M184V	K101Q, K103N	I93L	73.86

Low frequency of mutations related to non-B subtypes

We have analyzed a list of new resistance mutations recently reported for non-B subtypes¹² and have matched them with the sequence from four species reported here as subtypes A, C, and H, including some intersubtype recombinant mutation cases. Table 2 shows mutations M36I, I93L, and G196E, which were present at least once in four non-B subtype cases, except for one B/C recombinant. All these mutations were not considered as resistance mutations by the Stanford HIV Database. Of interest, the frequency of I93L was high in HIV subtype B circulating among the Peruvian population (73.86%), while the frequency for M36I and G196E was low (34% and 15%, respectively).

Discussion

In this study we have performed an analysis of the genetic variability of HIV subtypes circulating among Peruvian patients receiving HAART, focusing our analysis within the context of drug resistance. Thus, we have studied HIV-infected patients in the Peruvian National Antiretroviral Therapy Program who are currently receiving antiretroviral drugs. This population is very important because most of the patients come from different regions of Peru in which a high HIV/AIDS prevalence has been reported (i.e., Lima and Callao).²

According to our results, the molecular information gathered indicates that the HIV genetic profile in Peru has not changed significantly since the first molecular study was performed between 1994 and 1998.⁷

The high frequency of subtype B was persistent in Peruvian populations regardless of their sexual behavior and epidemiologic characteristics.^{5,8,9,20} This information suggests that subtype B has gained important epidemiologic success in this country in contrast to other subtypes previously identified such as A and F.^{7,9,21} In this work we have identified cases of subtypes A, C, and H ($n=4$). In regard to subtype A, there is only one case reported in South America from a Peruvian heterosexual male patient,⁷ who denied having had sexual contact with any individuals from other countries. In this respect, the fact that subtype A was first found in Africa and

Asia (reviewed by Requejo²²) and then identified in South America is intriguing. In particular, this subtype was not identified in Peruvian populations engaging in high-risk sexual behavior,²⁰ which suggests that infection was concentrated in individuals engaging in low-risk sexual behavior; consequently only sporadic cases have originated within the 10 years of recorded cases.

Another explanation about the low frequency of this subtype might result from differences in pathogenic properties and routes of transmission between subtype A and other subtypes,²² factors that would make it difficult for expansion in this country. Obviously, though, further genetic, epidemiologic, and clinical studies are needed to elucidate the origin, pathogenic features, and transmission of this subtype in South America.

In the case of subtype C, which is highly prevalent in South Africa and East Asia,²² there exists robust evidence that it arrived in South America from Middle-Eastern Africa.²³ Having been first introduced into the United Kingdom by homosexual contact,²⁴ it then finally disseminated into different countries in South America: it was first reported in Porto Alegre and São Paulo, Brazil,²⁵ then in Argentina, Paraguay, Uruguay,⁵ and finally Venezuela.²⁶ In this study, subtype C was detected in an Argentinean patient (Dr. La Rosa, personal communication, 2011) who represents the first recorded imported case in Peru. Of interest, evidence of B/C recombination reported previously⁸ and in this article suggests that this finding does not necessarily represent an isolated case. A program of continued subtype surveillance is necessary, especially in border areas with Brazil, in order to determine additional cases of subtype C in this country.

Regarding subtype H, it was identified in an 8-year-old patient with a viral load of 135357 particles/ml and a cell count of 564 cells/ml. This sample was newly evaluated by resequencing the *pol* and *env* genes twice, in both cases producing the same data and confirming its classification as subtype H. Further phylogenetic analysis using the *env* gene revealed that this viral strain might have originated from Central and South of Africa in concordance with the prevalence of subtype H in this continent (5–9%), while few isolated

cases have been reported in Europe and Asia.^{27,28} Additionally, we showed that other strains detected in Spain, Belgium, the United States, and South Korea were clustered to another phylogenetic group named by this work as "H2." This finding suggests that transmission of subtype H to other countries might result in new genetic variants possibly by recombination with other subtypes. In fact, most of the sequences included in subtype "H2" were recombined with segments of other subtypes such as subtype A and some of unclassified origin,²⁸ suggesting that currently subtype H is not completely pure. Full-length genomic analysis is required to determine if the subtype H reported in Peru has retained the same genetic characteristics as the ones of other species previously reported.

Since this sample comes from a child, it is possible that one or both parents might have been infected either abroad or by sexual contact with a foreign partner. Epidemiologic data about this case revealed that the mother was Peruvian while the father was Chinese. Although this information suggests that this case of subtype H might have a possible Asian origin, serological and molecular assays revealed that the father was HIV negative (data not shown). Since the mother mentioned that she has had other sexual partners from Peru and the United States, it is possible that subtype H might have been transmitted from a foreign sexual partner before pregnancy. This information suggests that subtype H might be transmitted in this country, making it necessary to perform more surveillance studies of genetic subtypes.

Despite the fact that subtype F represents the second genetic variant most frequently found in Peru, phylogenetic analysis was not able to detect this; however, we have evidence of two cases of B/F recombination (B/F1 and CRF12-like). These findings indicate that these recombinants might have come from Argentina, Uruguay, Chile, or Brazil, countries where they have been reported in high frequency.²⁹ In addition, Carrion *et al.*⁹ found two cases of B/F recombination in HIV-infected children using a 1915-bp region of the *gag-pol* genes (positions from 1575 to 3565 based on the HXB2 strain). According to our data, we have not identified recombination B/F in the pediatric population. This finding probably is due to the fact that the *gag-pol* region considered in this study did not provide enough information to detect recombination (it is about 42 nucleotides of the *gag* region) in comparison with Carrion *et al.*⁹ who considered about 727 nucleotides. Therefore, we need to perform further analyses in order to find more evidence of recombinant events in HIV infecting different human groups.

It is important to mention that recombination may have an important impact on public health because it offers evidence that a previous dual infection has occurred³⁰ with the concomitant risk of disease progression.³¹ In addition to this, recombination has been involved in improving viral fitness³² and increasing the risk of developing drug resistance species (reviewed by Rambaut *et al.*³³). In this context, we have analyzed cases of recombination focusing our attention on molecular resistance to antiretroviral therapy. According to our data, intrasubtype/intersubtype recombinant cases not only showed a higher frequency of resistance in comparison to nonrecombinants, but they also contained some more frequent resistance mutations. In particular, we have found the mutation N88S, which showed more prevalence in the intersubtype recombinant group. Of interest, this mutation was

previously described from recombinants A/E, A/G, and F subtypes,^{12,34} suggesting that it was recently incorporated into some specimens of B virus, giving an additional resistance characteristic to this genetic variant. Regarding E138K, it has been reported as a novel resistance mutation associated with susceptibility to ETR *in vitro*, and which has been similarly attributed to either B or non-B subtypes.³⁵

In addition, the mutations D67N, G190A, and K219Q found from intrasubtype recombinants have been described as having no significant differences between B and non-B subtypes,³⁶ suggesting that all these mutations are selected by antiretroviral treatment independently of subtype.

Taking all these data together, the recombination might be an important factor that contributes to changing the resistant profile of some HIV strains. However, the drug resistance suggests a multifactorial problem that should be analyzed from different angles using a more comprehensive perspective. On the other hand, we confirm that M36I, a novel resistance mutation reported for non-B subtypes,¹² was found in A, C, and H (4/4, 100%) subtypes, while it was present in only 34% of the total B subtypes analyzed in this study. This finding is concordant with previous reports in which M36I from non-B subtypes was significantly prevalent (85.5%, $p < 0.0001$) in comparison with subtype B species (26.5%).³⁶ Of interest, some samples with B/non-B recombination have not shown this mutation, revealing that recombination might contribute to modifying the resistance profile of non-B viruses. On the other hand, M36I was found in both treated³⁷ and treatment-naïve patients,³⁶ suggesting that genetic diversity factors but not antiretroviral therapy are a conditional factor to select for this mutation. Therefore, the contribution of M36I to drug resistance is not totally clear, although some studies have suggested that this mutation may act as a minor polymorphism against protease inhibitors.³⁶

Regarding G196E, considered as a new resistance mutation in non-B viruses, it has also been frequently seen in treated patients infected with subtype B showing NRTI resistance.³⁸ Recently Martínez-Cajas *et al.*¹² observed that this mutation seems to have an impact on non-B viruses; however, they did not elaborate on this observation. Saeng-aroon *et al.*³⁹ found that G196E was significantly prevalent in HIV subtype CRF01_AE detected in treated patients (5/45, $p < 0.05$) but not in naïve subjects. Altogether, we can infer that G196E can be selected by treatment independently of genetic subtype.

We found mutation I93L, which is considered a single polymorphism and was also analyzed in our study, in non-B viruses; however, according to the analysis of the HIVdb algorithm, it was more frequent in subtypes B (74%). This mutation has been strongly implicated on hypersusceptibility to lopinavir for HIV subtype C.¹² In agreement with our study, I93L was found in a sample identified as subtype C; however, we do not have data available about current therapy to make it possible to make inferences.

In conclusion, we have shown that HIV subtype B is the most prevalent (98%) variant in patients receiving HAART in Peru. However, evidence of new subtypes containing subtype-specific drug resistance mutations suggests that surveillance studies of new subtypes are required, especially in human groups with different sexual behaviors. These investigations have the potential of providing relevant information to improve the surveillance of resistance in Peru and to find a new transmission focus.

Sequence Data

Sequence data from this article have been deposited with GenBank under accession numbers JQ430749 to JQ430992.

Acknowledgments

We thank the experts from Pediatric Net and Adult Net and physicians from different health establishments who treated and evaluated HIV-infected patients included in this study, particularly Dr. Jorge Arevalo and Dr. Lenka Kolevic. We thank Dr. José Castillo and Dr. Heinner Guio whose scientific opinions and criticism have improved the scientific content of this article.

We thank the NIH AIDS Research and Referent Reagent Program for providing the primers and protocol to amplify the *gag/env* region and sequences of HIV referential subtypes. We also thank Mr. Ronal Briceño and Mrs. Benedicta Yana, technicians from the Laboratorio de VETS/VIH-SIDA, Instituto Nacional de Salud, who contributed their dedication, time, and technical expertise. This research was supported by the Instituto Nacional de Salud of Peru.

Author Disclosure Statement

No competing financial interests exist.

References

1. WHO, UNAIDS, UNICEF: Towards universal access. Scaling up priority HIV/AIDS interventions in the health sector. Progress Report 2009. Available at <http://www.who.int/hiv/pub/2009progressreport/en/>. Last access: December 23, 2011.
2. Pun M, Escudero A, Suárez LA, and Alvarado R: Análisis de la situación de la epidemia del VIH-SIDA. Available at http://www.dge.gob.pe/notas_prensa/2011/ResumenSit_VIH2011_01Dic.pdf. Last access: December 26, 2011.
3. Passaes CP, Guimarães ML, Bello G, and Morgado MG: Near full-length genome characterization of HIV type 1 unique BC recombinant forms from Southern Brazil. *AIDS Res Hum Retroviruses* 2009;25(12):1339–1344.
4. Sanchez GI, Bautista CT, Eyzaguirre L, *et al.*: Molecular epidemiology of human immunodeficiency virus-infected individuals in Medellín, Colombia. *Am J Trop Med Hyg* 2006;74(4):674–677.
5. Carrion G, Eyzaguirre L, Montano SM, *et al.*: Documentation of subtype C HIV Type 1 strains in Argentina, Paraguay, and Uruguay. *AIDS Res Hum Retroviruses* 2004;20(9):1022–1025.
6. Carrion G, Hierholzer J, Montano S, *et al.*: Circulating recombinant form CRF02_AG in South America. *AIDS Res Hum Retroviruses* 2003;19(4):329–332.
7. Russell KL, Carcamo C, Watts DM, *et al.*: Emerging genetic diversity of HIV-1 in South America. *AIDS* 2000;14(12):1785–1791.
8. Yabar CA, Salvatierra J, and Quijano E: Polymorphism, recombination, and mutations in HIV type 1 gag-infecting Peruvian male sex workers. *AIDS Res Hum Retroviruses* 2008;24(11):1405–1413.
9. Carrión AG, Laguna-Torres VA, Soto-Castellares G, *et al.*: Molecular characterization of the human immunodeficiency virus type 1 among children in Lima, Peru. *AIDS Res Hum Retroviruses* 2009;25(8):833–835.
10. Laguna-Torres VA, Olson J, Sánchez JL, *et al.*: Distribución de los subtipos del VIH-1 en nueve países de América del Sur, 1995–2002. *Rev Peru Med Exp Salud Publica* 2005;22(1):12–18.
11. Kiwanuka N, Laeyendecker O, Robb M, *et al.*: Effect of human immunodeficiency virus type 1 (HIV-1) subtype on disease progression in persons from Rakai, Uganda, with incident HIV-1 infection. *J Infect Dis* 2008;197(5):707–713.
12. Martinez-Cajas JL, Pai NP, Klein MB, and Wainberg MA: Differences in resistance mutations among HIV-1 non-subtype B infections: A systematic review of evidence (1996–2008). *J Int AIDS Soc* 2009;12(1):11.
13. HIV Genotyping–Antiviral Resistance Test ABI 3100 genetic analyzer: Adapted from Stanford Hospital and Clinics Laboratories. Revised at 01-10-2008. pp36.
14. Van der Auwera G and Heyndrickx L: HIV-1 group M gag Heteroduplex Mobility Analysis (HMA) Subtyping Kit. Protocol version 3. 2000.
15. Delwart EL, Herring B, Rodrigo AG, and Mullins JI: Genetic subtyping of human immunodeficiency virus using a heteroduplex mobility assay. *PCR Methods Appl* 1995;4:S202–S216.
16. Thompson JD, Higgins DG, and Gibson TJ: CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 1994;22(22):4673–4680.
17. Felsenstein J: Evolutionary trees from DNA sequences: A maximum likelihood approach. *J Mol Evol* 1981;17(6):368–376.
18. Hasegawa M, Iida Y, Yano T, *et al.*: Phylogenetic relationships among eukaryotic kingdoms inferred from ribosomal RNA sequences. *J Mol Evol* 1985;22(1):32–38.
19. Kosakovsky Pond SL, Posada D, Stawiski E, *et al.*: An evolutionary model-based algorithm for accurate phylogenetic breakpoint mapping and subtype prediction in HIV-1. *PLoS Comput Biol* 2009;5(11):e1000581.
20. Yabar C, Salvatierra J, and Quijano E: Variabilidad del gen de la envoltura del VIH-1 en tres grupos humanos con diferentes conductas sexuales de riesgo para adquirir ITS-VIH. *Rev Peru Med Exp Salud Publica* 2007;24(3):202–210.
21. Hierholzer J, Montano S, Hoelscher M, *et al.*: Molecular epidemiology of HIV type 1 in Ecuador, Peru, Bolivia, Uruguay, and Argentina. *AIDS Res Hum Retroviruses* 2002;18(18):1339–1350.
22. Requejo HI: Worldwide molecular epidemiology of HIV. *Rev Saude Publica* 2006;40(2):331–345.
23. Jones LR, Diletnia DA, Manrique JM, *et al.*: In-depth analysis of the origins of HIV type 1 subtype C in South America. *AIDS Res Hum Retroviruses* 2009;25(10):951–959.
24. de Oliveira T, Pillay D, Gifford RJ, and the UK Collaborative Group on HIV Drug Resistance: The HIV-1 subtype C epidemic in South America is linked to the United Kingdom. *PLoS One* 2010;5(2):e9311.
25. Csillag C: HIV-1 subtype C in Brazil. *Lancet* 1994;344(8933):1354.
26. Castro E, Moreno M, Deibis L, *et al.*: Trends of HIV-1 molecular epidemiology in Venezuela: Introduction of subtype C and identification of a novel B/C mosaic genome. *J Clin Virol* 2005;32(3):257–258.
27. Kandathil AJ, Ramalingam S, Kannangai R, *et al.*: Molecular epidemiology of HIV. *Indian J Med Res* 2005;121(4):333–344.
28. Janssens W, Laukkanen T, Salminen MO, *et al.*: HIV-1 subtype H near-full length genome reference strains and analysis of subtype-H-containing inter-subtype recombinants. *AIDS* 2000;14(11):1533–1543.

29. Kijak GH and McCutchan FE: HIV diversity, molecular epidemiology, and the role of recombination. *Curr Infect Dis Rep* 2005;7(6):480–488.
30. Fang G, Weiser B, Kuiken C, *et al.*: Recombination following superinfection by HIV-1. *AIDS* 2004;18(2):153–159.
31. Gottlieb GS, Nickle DC, Jensen MA, *et al.*: Dual HIV-1 infection associated with rapid disease progression. *Lancet* 2004;363(9409):619–622.
32. Turk G, Carobene M, Monczor A, *et al.*: Higher transactivation activity associated with LTR and Tat elements from HIV-1 BF intersubtype recombinant variants. *Retrovirology* 2006;3:14–25.
33. Rambaut A, Posada D, Crandall KA, and Holmes EC: The causes and consequences of HIV evolution. *Nat Rev Genet* 2004;5(1):52–61.
34. Dumans AT, Barreto CC, Santos AF, *et al.*: Distinct resistance mutation and polymorphism acquisition in HIV-1 protease of subtypes B and F1 from children and adult patients under virological failure. *Infect Genet Evol* 2009;9(1):62–70.
35. Asahchop EL, Oliveira M, Wainberg MA, *et al.*: Characterization of the E138K resistance mutation in HIV-1 reverse transcriptase conferring susceptibility to etravirine in B and non-B HIV-1 subtypes. *Antimicrob Agents Chemother* 2011;55(2):600–607.
36. Montes B, Vergne L, Peeters M, *et al.*: Comparison of drug resistance mutations and their interpretation in patients infected with non-B HIV-1 variants and matched patients infected with HIV-1 subtype B. *J Acquir Immune Defic Syndr* 2004;35(4):329–336.
37. Kantor R, Katzenstein DA, Efron B, *et al.*: Impact of HIV-1 subtype and antiretroviral therapy on protease and reverse transcriptase genotype: Results of a global collaboration. *PLoS Med* 2005;2(4):e112.
38. Svicher V, Sing T, Santoro MM, *et al.*: Involvement of novel human immunodeficiency virus type 1 reverse transcriptase mutations in the regulation of resistance to nucleoside inhibitors. *J Virol* 2006;80(14):7186–7198.
39. Saeng-aroon S, Tsuchiya N, Auwanit W, *et al.*: Drug-resistant mutation patterns in CRF01_AE cases that failed d4T+3TC+nevirapine fixed-dosed, combination treatment: Follow-up study from the Lampang cohort. *Antiviral Res* 2010;87(1):22–29.

Address correspondence to:

Carlos Augusto Yabar
Laboratorio de VETS/VIH-SIDA
Instituto Nacional de Salud
Defensores del Morro Ave 2268, Chorrillos
Lima LI09
Peru

E-mail: cyabar@ins.gob.pe