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Multiple Resistance and Unusual Mutations from HIV-1 Infecting Peruvian Patients with Highly Active Antiretroviral Therapy

Yabar CA*, Vilcarino G, Yaya M, Espetia S, Acuña M, Mamami E, Santos D, Romero S and Cárdenas F

Abstract

We have analyzed the molecular resistance profile of HIV infecting Peruvian individuals during the first three years that genotyping test was implemented free of charge. According our results, from 297 patients 80% of them were resistant to one or more antiretroviral (ART). M184V was the most frequent resistant mutation (30% for children and 26% for adults). Resistant profile revealed that children showed more resistance to 3TC/FTC (78%), while in adults it was EFV/NVP (50%). Pan-sensitive patients showed similar virological and immunological failure as those of resistant patients. We have also identified six patients showing Pan-resistance, including a 7-year-old subject. Additional sequence analysis revealed two cases of hypermutation with multiple stop codons. Finally, we found twenty-four resistant patients who were genotyped two or more times, however only fifteen (63%) of them were changed to other treatment schedule. As a consequence of this, only five patients (21%) experienced a decrease in their VL. We showed that HIV resistance profile in Peru is complex and follows different molecular evolution depending of age group. Moreover, recent resistant mutations emergency requires a re-planning of therapy strategies that help to diminish the resistance in Peruvian population.

Keywords

HIV; AIDS; FOXP3; Tregs; CD4; Infection

Introduction

Antiretroviral treatment (ART) has improved the global health in HIV-infected patients, reducing the mortality rates especially in resource-poor countries [1]. UNAIDS has reported that ART coverage in Latin America is one of the highest (68%) from low- and middle-income countries [2]. This issue has had an impact on reducing the AIDS cases significantly in the region. In Latin America and the Caribbean there are different factors that have been related to the development of drug resistance such as long-term ART administration, low access to second-line therapy and limited genotyping test [3]. Fortunately, standardized genotyping test is available from most of these countries that have been used to monitor the resistance primary [4]. In Peru, HIV / AIDS is a concentrated epidemic, with 70,000 people living with HIV-1 mainly in Lima and Callao cities. The prevalence of HIV-1 occurs according the risk sexual behavior being 13.8% in trans women, 15.2% for men who have sex with men (MSM) and 0.3% for general population. Of interest, in 2016 about 42 000 people were on Highly Active Antiretroviral Treatment (HAART) [4]. In Peru, HIV genotyping studies were conducted previously, whose prevalence on drug resistance ranged from 1% to 3% for previously untreated patients [5,6] and 27% to 80% patients with experience in ART [5-8]. However, these studies followed different methodological designs, and the human groups recruited were epidemiologically divergent, which makes it difficult to compare them and obviously to have a global idea about drug resistance in Peru. Therefore, not all the available evidence on the resistance profile of Peruvian patients is adequate for political decision-making. In this context, Health Ministry of Peru implemented the National Surveillance of HIV Drug Resistance in 2008. This initiative was initially supported by Global Found and after that by the own government. At this time, our institution is performing the surveillance of molecular resistance in Peruvian population by using an in house HIV genotyping test. This information needs to be analyzed in order to contribute to political decisions of therapy changes and how it could improve the ART effectiveness in Peru.

Thus, the main aim of this study is to study the prevalence of ART resistance during the three first years of surveillance in Peru by using a previously standardized in house genotyping test free of charges. Additionally to this, we want to identify what was the utility of genotyping on making decision for therapy changes.

Materials and Methods

Clinical samples

In this study, we have analyzed the genotyping results of a collection of 297 plasma samples from seropositive patients as part of a National Surveillance for Monitoring HIV Antiretroviral Resistance between 2008 and 2011 by Instituto Nacional de Salud of Peru (Table 1). The samples were taken from patients who had been previously evaluated and approved by the Experts Committee of Ministry of Health (MOH), which decided whether the case would be analyzed for HIV genotyping. The criteria for approval were as follows: 1) Patients whose age ranged from 0 to less than 18 years with a viral load of more than 1,000 copies/ml after six months of receiving first-line antiretrovirals; 2) Patients over 18 years of age, with evidence of more than 1,000 copies/mL after an interval of at least two months of initiation of second-line antiretroviral therapy; And 3) Pregnant women, in case of treatment failure using the two criteria previously indicated. No samples without HAART information were included in this study. All blood samples were immediately processed or maintained at room temperature (20°C-25°C) 24 h before they arrived at the Instituto Nacional de Salud of Peru. They were then homogenized and centrifuged at 4000 rpm for 10 min. Finally, the plasma samples obtained were stored at -80°C until RNA extraction.

RNA extraction

A plasma volume of 140 µl was processed with QIAamp Viral RNA Kit (Qiagen) according to the manufacturer's recommendations. The tubes containing the purified RNA were stored at -80°C.

***Corresponding author:** Carlos Augusto Yabar, HIV / AIDS Laboratory Instituto Nacional de Salud, Peru, Tel: (511) 7480000 Ext: 1544, 1586; E-mail: cyabar@ins.gob.pe

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Table 1: Epidemiological characteristics of Peruvian patients.

Characteristic	All	Adults	Children
Patients. no.(%)	297 (100)	131 (44.1)	166 (55.9)
Age, mean (range), years.	20.5 (0-74)	33.9 (18-74)	7.1 (0-17)
Sex. no.(%)			
Male	174 (58.6)	83 (63.4)	91 (54.8)
Female	123 (41.4)	48 (36.6)	75 (45.2)
Route of transmission: number (%)			
Sexual	131 (44.1)	131 (100)	0 (0)
Vertical	166 (55.89)	0 (0)	166 (100)
Region where patients are from number (%)			
Lima	235 (79.1)	101 (77.1)	134 (80.7)
Callao	29 (9.8)	12 (9.2)	17 (10.2)
Loreto	8 (2.7)	8 (6.1)	0 (0)
Others	25 (8.4)	10 (7.6)	15 (9)
Current antiretroviral treatment number (%)			
PI only	6 (2)	6 (4.6)	0 (0)
NRTI only	0 (0)	0 (0)	0 (0)
NNRTI only	0 (0)	0 (0)	0 (0)
PI+NRTI	10 (3.4)	5 (3.8)	5 (3)
PI+NNRTI	0 (0)	0 (0)	0 (0)
NRTI+NNRTI	85 (28.6)	41 (31.3)	44 (27)
PI+NRTI+NNRTI	159 (53.5)	71 (54.2)	88 (53)
No data available number (%)	37 (12.5)	8 (6.1)	29 (17)
CD4 cell count/μL*	880.5	202.5	678
Viral load (copies/mL)*	199490.57	186129.82	212851.32

*Data available only for 218 patients (121 children and 97 adults)

One-step RT-PCR 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) and described previously [7]. PCR products were visualized by horizontal electrophoresis on a 1.5% agarose gel.

DNA purification and direct sequencing

PCR products were purified using the Qiaquick PCR purification system (Qiagen) following the manufacturer's instructions and then quantified by 1.5% agarose gel electrophoresis, using the low mass ladder molecular weight marker (Invitrogene) and Quantity One software from the Gel Doc Biorad documentation system. Sequencing procedures were performed according to the specifications of the BigDye Terminator v3.1 Cycle Sequencing Kit, using different primers flanking the reverse transcriptase and protease gene regions (Yabar, et al.). The sequencing products were denatured with 15 μl pure formamide and then run using an ABI 3500 XL sequencing analyzer.

Sequences analysis

DNA sequences synthetized by each primer (fragment size between 400 bp-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).

For identification of punctual resistance mutations, consensus sequences were submitted to the HIV DataBase (db) from Stanford University by using the HIVdb program (https://hivdb.stanford.edu/hivdb/by-mutations/).

Ethical considerations

This study was approved by the Experts Committee of Ministry of Health (MOH) of Peru, who analyzed each case and decided what sample should be analyzed for HIV genotyping at Instituto Nacional de Salud of Peru. Additionally to this, we worked with de-identified dataset in order to protect the confidentiality of each patient.

Results

Population characteristics

According Table 1, more than 55% of attended patients were children whose age mean was 7 years old, while adults were 34 years old. Most of them came from urban centers such as Lima (79%) and Callao (10%). Regarding to gender, the male group was slightly larger (59%) than the female. It was seen either adult as children populations. According the ART treatment, the most frequent HAART combination was the Protease inhibitor (PI) plus a Nucleoside Reverse Transcriptase Inhibitor (NRTI) and Non-NRTI (NNRTI) (54%), being NFV (24%) and 3TC (15%) the most frequent drugs used. Although immunological status in children was higher than adults, viral load showed similar values up to 5 logs in both populations.

ART and resistant mutations profile reveal differences among children and adults

From total ART-experienced participants (n=297) we have found 237 (80%) cases showing drug resistance. Analyzing the resistance profile separately from children and adults we found either common as divergent characteristics. At first, both populations showed that the major ART administrated was 3TC, and it was evidenced through the high frequency mutation M184V, (30% for children and 26% for adults). However, analysis of IP-resistant mutations showed that N88D and D30N were the most frequent mutations in children (21%-23% respectively) while adults were I54V (13%), V82A and M46I (14%). Likely wise, K103N was the most prevalent NNRTI-resistant

mutation for both populations, being higher in adults (23%) than children (15%). Taking all these data together, resistance frequency in children was higher (90%) than adults (67%) showing that resistance profile for both population was different (Table 2).

Patients infected with HIV drug-sensible showed immunological decay and high viral load similar to those infected with resistant viruses

We have analyzed the immunological and virological status between pan-sensible and resistant patients. According our database (Table 3), 242 patients (either pan-sensible as resistant) with complete information about CD4/CD8 cell count and viral load, showed both immunological and virological fail. Of interest, viral load from pansensible patients were higher than resistance ones.

HIV genotyping showed pan-resistance viral strains

We have identified six patients (2%, 6/297) showing resistant genotype to every known antiretroviral drug, which were named pan-resistant patients (Table 4). Of interest, in this group a seven years old patient showing a high diversity of resistance mutations along protease and reverse transcriptase gene was identified.

As was expected, pan-resistance individuals showed low CD4/CD8 cell count (average of 96 cell/μL) and high viral load (up to 5 log, average of 161026 copies/mL). Analyzing the ART scheme, all of them received any PI and NTRI, however none of them received NNTRI. The most frequent mutations were M41L and M184V (12%) related to NTRI resistance.

Two samples with unusual mutation and stop codons showed persistent high viral load and resistance profile

We have detected two cases of patients showing HIV genotypes with evidence of unusual mutations and numerous stop codons along the Reverse Transcriptase gene. These viral species were reported as APOBEC 3G/F hypermutants by the HIV db program (Stanford database). According this, the first case (Hypermut 1) revealed five stop codons, three APOBEC residues (G152KR, W153S, G155E) and several unusual punctual mutations along 15 positions (146-161). The Hypermut 2 case showed four stop codons, one APOBEC mutation

Table 2: General Data of drug resistance mutations and drug resistance profile found from Peruvian patients receiving HAART.

	ART administration more frequent (>10%)	Resistant mutation most frequent (>10%)			Resistant population (%)	Drug resistance profile (>20%)
		IP-related	NRTI-related	NNRTI-related		
Children (n=166)	3TC (70), AZT (54), NFV (41), D4T (19), NVP (13), EFV (10)	D30N (23), N88D (21), L90M (19), M46I (18)	M184V (30)	K103N (15), Y181C (11)	90	3TC (77.7), FTC (77.1), NFV (52.4), ATV/r (50.6), ABC (46.4), D4T (45.2), NVP (43.4), AZT (42.8), EFV (42.8), DLV (41.8), DDI (40.4), IDV/r (30.7), SQV/r (30.7), ETR (27.7), FPV/r (25.9), TDF (25.3)
Adults (n=131)	3TC (69), LPV/r (39), AZT (33), DDI (30), EFV (19), ABC (19), ATV (16), D4T (15), NVP (12)	V82A (14), M46I (13), I54V (13)	M184V (26)	K103N (23)	67	EFV (49.6), NVP (49.6), ABC (48.0), FTC (47.2), 3TC (43.6), DLV (32.5), ETR (28.5), RPV (27.3), D4T (26.8), DDI (26.8), AZT (26.0), ATV/r (23.6), NFV (23.6), IDV/r (21.1), SQV/r (21.1), LPV/r (20.3), FPV/r (20.3)

Table 3: Virological and immunological status in pansensible and pan-resistant patients.

	Immunological and virological values (media)		
	CD4 (cell/μL)	**CD8 (cell/μL)**	**Viral load (copies/mL)**
Pan-sensible (n=45)	306,6	1025,4	390733,2
Resistant (n=197)	485,3	1241,1	148946,7

Table 4: Mutations and frequency among six cases of pan-resistance patients.

			Cell count (μl/mL Blood)						
Code	**Gender**	**Age**	**CD4**	**CD8**	**Viral load (copies/mL blood)**	**Current ART therapy**	**IP-resistance mutations**	**NRTI-resistance mutations**	**NNTRI-resistance mutations**
PanRes1	M	74	280	887	56933	ATV/r, D4T, DDI	I50L, V82F	M41L, E44D, L210W, T215F	Y188L
PanRes2	M	53	37	286	97004	LPV/r, ABC, DDI	M46I, I54V, V82A, L90M	A62V, D67N, K70E, V751, F77L, Y115F, F116Y, Q151M, M184V	K101E, Y181C, G190A
PanRes3	M	47	19	548	205289	ATV/r, LPV/r, SQV/r, 3TC, ABC, AZT, D4T	L24I, I54V, I84V, V82A, V82T, M184V	M41L, E44D, L74V, V75M, V118I, M184V, L210W, T215Y	K101E, G190A
PanRes4	M	19	8	187	259967	LPV/r, 3TC, DDI	V32I, M46I, I47V, I54L, V82F	M41L, D67N, K70R, V75M, M184V, T215F, K219Q	K103N
PanRes5	M	7	76	2001	253941	LPV/r, 3TC, D4T	L24I, L33F, M46I, 154V, V82A	M41L, D67N, T69N, K70R, L74I, M184E, M184V, T215F, K219E	A98G, K101Q, Y181C, G190A
PanRes6	M	43	156	1017	93022	LPV/r, D4T, DDI	M46L, G46Q, I54V, V82S, I84V	M41L, L74I, M184V, L210W, T215Y	K103N, Y181C, P225H

(W229KR) and nine unusual residues located along 13 positions. Both cases were sequenced twice finding the same results. We have also re-analyzed these sequences by using the Hypermutant v.2 program, showing not significant p value (Data no shown). Additional to this, both samples showed several resistance mutations related to both protease and reverse transcriptase inhibitors. Of interest, viral load from one sample showed a value up to 5 log and CD4 very low cell count (113 cell / mL) (Table 5).

No therapy changes experienced Peruvian patients after their first genotyping result

We have analyzed if genotyping information was used for Physician for therapy changes and its effect on reducing the virus load and improving the immunological status. For this, we have selected 24 cases that were genotyped twice or more during a time interval of six month as minimum containing full data of ART therapy, viral load and CD4 cell count. Of importance, they showed high viral load count and low CD4 cell count (Table 6). Although twenty cases (83%) showed resistant profile before the first genotyping test, only 15 (63%) patients were changed to other therapy scheme. This therapy change was partially effective since most of patients showed a resistant profile similar to the first time, while only five cases reduced their viral load to less than 1000 copies/mL.

Discussion

In this paper we described the resistance profile in HIV-infected Peruvian patients receiving HAART during the first three years since a validated genotyping test was available. According our epidemiological data, we have included patients with different age, sex and geographic origin, which offer an approximation about of general population of this country. Most of patients are predominately from Lima, Callao and Loreto, regions where high prevalence of AIDS have been reported [9]. We have found that ART therapy scheme was predominately HAART, especially using the combination NRTI plus NNRTI and PI plus NRTI plus NNRTI. This data is similar than others Latin American countries, where HAART started immediately [3] which suggest that ART resistance in Peru would be very similar to other countries in the Region. In this study, every patients showed virological failure, however twenty percent of them did not showed any drug resistant mutations. To this regard, not only resistant virus have been related to virological failure, but also suboptimal adherence [10,11] low drug absorption [12], run out of medication supplies [13] and one recently described factor which involve the presence of minor viral subspecies holding drug resistant mutation [14]. Therefore, assessment to every of these factors is needed to determine the exactly origing that might causes the virologic failure from patients infected with HIV susceptible according the Sanger method.

It is important to mention that resistance prevalence in this group was 80% (237/297). Considering that between 2008 and 2011 there were 22,148 patients receiving HAART (population estimated by Health Ministry of Peru [15], the resistance prevalence was about 1%. This agrees with the report by Pineda et al. [3]. In 2012, which shows that Peru has a prevalence of resistance to transmitted drugs of less than 5%.

This index is very low comparing to other countries from Latin America [16]. Although the short size of the sample included in this study might be a limiting factor, the poor access to clinical attention and disinformation about monitor assays could also be decreasing significantly this prevalence.

On other hand, we have found that M184V was the most prevalent mutation in this population, similarly to other studies performed in Peru [5,7], Latin America [3] and worldwide [17]. Although this mutation confers high resistance to both 3TC and FTC, it also increases the susceptibility to AZT, delays the emergence of resistant mutations associated with AZT and d4T, and affects the viral fitness and transmission [18]. All these points explain why this mutation is still maintained in patients receiving HAART in Peru and other countries.

We have also found differences to resistant profile of adults and children, specifically against NFV whose resistance was higher in children than adults. This issue is probably due to NFV was usually administered to children population, mostly because it was well-tolerated and available for paediatric formulation [19]. Nowadays, this drug have been discontinued definitely (Dr Rober W Shafer, personal communication). In the case of adults, the resistance frequency was high for NNTRI whose regimen is very frequent in this population in comparison to children. An explanation of this fact is that PI-based regimen is associated with drug-drug interaction (XDDIs) in adult population, in comparison with NNTRI where percent of XDDIs is very low (about 5%) [20].

Table 5: Hypermutation cases and resistant profile.

			Cell count/mL								
Code	**Age**	**Gender**	**CD4**	**CD8**	**Viral load copies/mL**	**Subtype**	**Stop codons positions**	**APOBEC 3G/F**	**IP resistance mutations**	**NTRI resistance mutations**	**NNRTI resistance mutations**
Hypermut 1	9	Female	113	1794	206707	B	146, 153, 154, 155, 156	G152K/R, W1535, G155E	D30N, N88D	M41L, D67N, K70R, L74I, V118I, M184V, T21SF, K219Q	A98G
Hypermut 2	63	Male	ND	ND	ND	B	188, 224, 227, 229	W229KR	150V	A62V, V75M/I, F77L, F116Y, Q151M, M184V	K103s, V106M, F227L/Y

Table 6: Summary of genotyping information, viral load and immunological status from patients genotyped twice or more.

	Samples analysed twice or more	**Viral and immunological status (Median)/ Viral load (copies/mL) CD4 (cell/uL)**		**Resistant cases according first therapy (n (%))**	**Patients whom changed their treatment scheme according first genotype (n (%))**	**Second Viral and immunological status (Median)/ Viral load (copies/mL) CD4 (cell/uL)**		**Resistant cases according second therapy (n (%))**	**Cases(n (%)) showing less than 1000 copies after of therapy change**
Children	16	17080	565	13 (81.3)	9 (37.5)	14120	485	14 (87.5)	4 (25)
Adults	8	105994	170	7 (87.5)	8 (25)	60118	153	7 (87.5)	1 (12.5)
Total	24	72439	434	20 (83)	15 (62.5)	89632	374	21 (87.5)	5 (20.8)

Also, our study revealed six patients showing resistance to every antiretroviral drugs (pan-resistant patients). According the HIV Data Base of Stanford University, these patients showed different levels of resistance for all drugs (Data no shown). Because of historical data of ART treatment of these patients is not fully available, it is difficult to analyse what would be the exactly factor involved on this multiple resistance. However, some reports have evidenced that recombination events might be implicated on producing multiple resistance as a consequence of a superinfection [21] or enhancing the molecular evolution by the ART itself into the same subject [22]. Although, only one sample showed the recombination F1/CRF12 according SCUEAL analysis [23] other factors such as heavy ART therapy, adherence problems, genetic diversity or mother-to-child transmission (in the case of a child included in this group) might be involved on pan-resistance. However, each case should be studied separately in order to find the possible cause related to this problem.

Two cases of HIV hypermutated were found circulating from patients with evidence of high viral load, suggesting that these viruses were persistently replicating into the host despite the fact they contained stop codons and high frequency of G-to-A changes. This data are intriguing because hypermutated virus are not able to be infectious and usually are found integrated to the host DNA as a provirus [24]. However, electropherogram analysis and cloning procedures (no shown in this article) revealed numerous nucleotides mixtures in the same positions and gave evidence of two different strains (hypermutant and non-hypermutant viruses) circulating simultaneously in blood from the same host. These evidences suggest that defective strains would be restoring their pathogenicity probably by recombination with infective strains, recovering not only their replicative capacity but also conferring themselves drug resistance as it was previously shown *in vitro* assays [25]. Further cloning procedures and *in vitro* infection assay should be performed in order to show this issue.

At last, we have shown that genotyping results are not totally used by physician to treat Peruvian patients. Only five of twenty four cases got reduce their viral load to less than 1000 copies. This data suggests that despite the fact genotyping results might be useful to improve the patient's health, availability of antiretroviral drug is critical in order to complete the attention and improve the quality life of them. In summary, the ART resistance profile during the first three years after the HAART beginning in Peru revealed moderate resistance frequency, that follow a different evolution course between adults and children. This information might be useful for the National AIDS Strategy that monitor and treat this infection; and also to understand what would be the next research proposal that help to reduce the resistance to ART and increase the efficiency of care of Peruvian patient.

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Conflict of Interest

The Authors declare no conflict of interest related to this work. This research was supported by the Instituto Nacional de Salud of Peru.

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Author Affiliations

Top

Department of Biotechnology and Molecular Biology, Instituto Nacional de Salud, Peru