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GENETIC ANALYSIS OF PROTEASE AND REVERSE TRANSCRIPTASE OF HIV-1 FROM SOUTHERN BRAZIL NAÏVE CLINICAL ISOLATES

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Title: Genetic analysis of protease and reverse transcriptase of HIV-1 from Southern Brazil naïve clinical isolates.

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ABSTRACT

Background: Rio Grande do Sul, the southernmost state of Brazil, presents an atypical epidemiological situation where HIV-1 subtype C co-circulates with subtypes B and CRF31_BC, contrasting with the other Brazilian regions were subtypes B and F dominates. These infections have been effectively managed with highly active antiretroviral therapy (HAART) since 1996.

Objective: To evaluate the profile of mutations and polymorphisms in the protease and reverse transcriptase regions in diferent subtypes of HIV-1 from untreated patients living in Southern Brazil.

Methods: Blood samples from 99 HIV-1 positive antiretroviral/drugs-naïve, which were not in drug therapy, were collected from 2005 to 2007, in Porto Alegre Brazil. Protease and reverse transcriptase genes were amplified, sequenced and subtyped through phylogenetic analyses. HIV strain genotyping was performed by partial *pol* sequence analysis with the HIV Drug Resistance Database of Stanford University.

Results: Phylogenetic analyses of the 99 *pol* samples, were classified according their subtypes: B (26.2%), C (39.4%), F (1.1%), CRF31_CB (19.2%) and URF (14.1%). Eight (8.1%) samples showed primary resistance mutations to Calibrated Population Resistance tool based in the 2009 Surveillance Drug Resistance Mutation list. Two samples presented PI resistance mutations: I54T and I54L; two NRTI resistance mutation: D67G, D67N and K70R, and three NNRTI resistance mutation: V106M, Y181C and K103N. There is no significant association between presence of resistant genotypes and subtypes, but resistance mutations seem to be less frequent at subtype C.

Conclusions: Primary resistance mutations represented 8.1% of sequences demonstrating an increase of 5% in the last years in that region. Here, we describe for the first time the mutational profile of CRF31_BC. In this light, the results of genetic analysis of HIV-1 from naïve patients appear to be a promising and important tool for the surveillance of HIV infection.

INTRODUCTION

The genetic diversity of human immunodeficiency virus type 1 (HIV-1) allows its classification in several groups, subtypes, subsubtypes, circulating recombinant forms (CRF) and unique recombinant forms (URF) [1]. Subtype C is highly prevalent and worldwide spread, it is responsible for more than 50% of HIV infections worldwide [2, 3]. However antiretroviral drugs used to treat HIV infected-patients were developed using biophysical, biochemical, and *in vitro* studies of subtype B isolates [4]. Most data on the genetic mechanisms of HIV-1 drug resistance are also derived from subtype B viruses. At Southern Brazil the subtypes C, B and CRF31_BC are co-circulating since mid-1990 [5, 6, 7, 8], and a significant portion of the HIV-infected population has been exposed to antiretroviral drugs since 1996.

Transmitted or primary resistance represents a challenge for the control of HIV-1 infection since it can reduce the efficacy of first line therapy antiretroviral and may impact clinical outcomes [9]. Surveillance of resistance to antiretroviral drugs has been performed worldwide and genotyping strategies are based on these data [10]. For instance, surveys conducted in developed countries during the past decade and targeting recent seroconverters have shown prevalence rates of 10 to 17% in France, 13% in German, 14% in the United Kingdom, 15 to 26% in North America, and 23 to 26% in Spain [11, 12]. In Brazil, a recent study revealed a low prevalence of 8.1% of primary mutations resistance [13].

Previous studies have reported differences in the mutational profile among HIV subtypes [14, 15]. Such high viral genetic diversity among subtypes is involved in differences in the rate of disease progression and response to antiretroviral therapy, including the development of resistance [2, 16]. Therefore, it is crucial to acquire further knowledge concerning the real significance of these differences; it may be important to determine strategies of initial treatment for infected individuals. The objective of this study was to evaluate the profile of mutations and polymorphisms in the protease (PR) and reverse transcriptase (RT) genes in different subtypes of HIV-1 untreated patients living in Southern Brazil.

MATERIALS AND METHODS

Study population

Blood samples from 99 HIV-1 positive and antiretroviral therapy naive patients were collected from 2005 to 2007 at the metropolitan region of Porto Alegre, Southern Brazil. Demographic and clinical data from patients (age, gender, first positive serology for HIV-1, CD4 T-cell counts, HIV viral load and presumed transmission) were compiled. All participants read and signed an informed consent. This study was approved by the FEPPS Ethical Research Committee (process 18/2005).

DNA Extraction, Polymerase Chain Reaction, and Sequencing

DNA samples were extracted from 200µl of whole blood by a method of salting-out followed by PCR amplification using nested primers as described elsewere [17]. Amplification of the PR/RT region was performed using K1 and K2 as outer primers, and DP10 and F2 as inner primers [18, 19]. Entire PR (corresponding to positions 2253–2549 relative to HXB2 genome) and a 720bp fragment of RT (corresponding to positions 2550–3270 relative to HXB2 genome) were amplified. The PCR products were purified using PureLink PCR Purification kit (Invitrogen Inc.) according to the manufacturer's protocol. Purified DNA was sequenced by using the ABI BigDye Terminator v.3.1 cycle sequencing ready reaction kit (Applied Biosystems, Foster City, California, USA) and processed with an automated ABI 3130xl Genetic Analyzer (Applied Biosystems).

Phylogenetic Analysis

Sequences were edited with SeqMan software (DNASTAR), and then aligned with reference sequences obtained from Los Alamos Sequence Database - LASDB (www.hiv.lanl.gov) in the ClustalX [20]. Subtype determination was inferred by phylogenetic analysis based on Neighbor Joining, Kimura two-parameter model using the software MEGA 4.0 [21]. Boostrap values (1000 replicates) above 70% were considered significant. Recombination analysis was performed by bootscanning using SimPlot 3.5.1 in 200 bp sliding window advanced in 20 bp steps size increments (1000 replicates) [22].

Resistance Mutation and Polymorphism Analysis

Edited sequences were submitted to Stanford University HIV Drug Resistance Database. Primary drug resistance was obtained using the Calibrated Population Resistance (CPR) tool based in the Surveillance Drug Resistance Mutation list (SDRM - 2009). Mutation and polymorphisms profiles were inferred based on HIVdb: Genotypic Resistance Interpretation Algorithm (hivdb.stanford.edu) [23]. Polymorphisms were defined as mutations that occurred in more than 5% of sequences and subtype-specific polymorphisms were defined as those significantly more prevalent in subtype.

Statistical methods

Statisticals comparisons of different group proportions were made using the Chi square test and Fisher's exact test when appropriate and the significance level was set at $p < 0.05$. Multiple logistic regression analysis was carried out to estimate the odds ratios (OR) with 95% confidence intervals in order to assess fold increase for subtype and mutations. Statistical analysis was performed using the SPSS 16.0 statistical package.

RESULTS

Study samples and *HIV*-1 *pol* (*PR/RT*) subtyping

The patients' ages ranged from 19 to 76 years (mean 35 ± 10.01) and 54% of the studie individuals were men. Overall samples were obtained from drug-naïve individuals with CD4+ T-cell counts ranging from 64 to 1115 cells/mm^3 (average, 383.9 ± 205.6), and viral loads varying from 1.76 to 5.70 × 10^3 mRNA copies/ml (average, 4.2 ± 0.8). The estimated time of infection (first positive serology) ranged from 7 to 60 months (average, 33 ± 10.17). Presumed HIV-1 infection transmission occurred through homosexual (n = 18; 20.5%), heterosexual (n = 66; 75%) and blood contact (n = 4; 4.5%). HIV-1 PR/RT samples were classified as subtypes B (26.2%), C (39.4%), F (1.1%), CRF31_CB (19.2%) and URF (14.1%). The URF sequences showed different points of recombination between subtypes B/C (12) and B/F (02). There is no significant difference between the subtypes regarding age, viral load, CD4 T-cell counts, time of presumed infection and gender. However there is a positive relationship (p=0.0194) between subtypes and routes of transmission, subtype

C was more frequent in heterosexuals. Table 1 summarizes the epidemiologic, clinical, and laboratorial data of the 99 patients.

Protease inhibitor (PI) and reverse transcriptase inhibitor (RTI) resistance-associated mutations

Eight (8.1%) samples showed primary mutations related to CPR – SDRM (Table 2). Two samples presented PI resistance mutation: I54T and I54L; three NRTI resistance mutation: D67G+D67N and K70R, and three NNRTI resistance mutation: V106M, Y181C and K103N. No individuals were found carrying primary mutations of more than one class of antiretroviral drugs. There was no significant association between presence of resistant genotypes and subtypes, but resistance mutations seem to be less frequent at subtype C. Accessory mutations were found in PR region of twelve samples, at following positions: A71V/T [4/99], T74S [3/99], L89V [3/99], L10I/F/V [2/99]. In addition, fifteen sequences showed unusual mutations in PR codons (30, 32, 47, and 82), which had been reported as resistance mutations or accessories mutations.

Polymorphisms associated to protease and reverse transcriptase

Natural sequence polymorphisms were detected at 18 and 29 positions in PR and RT respectively (Figure 1). The PT polymorphisms that showed significantly ($p>0,05$) association with subtype B were E35D, R41K, I62V, L63P and with subtype C were I15V, M36I, N37K, R41N, H69K, L89M, I93L. Polymorphisms K20R, I135T, F214L were associated with subtype B at RT gene and V35T, E36A, T39D, K43R, S48T, V60I, K122E, K173A, D177E, T200A, Q207E, R211K with subtype C. Polymorphisms verified in CRF_31 corresponded predominantly to the same of subtype C, but in different frequencies (Table 3).

DISCUSSION

At Rio Grande do Sul, the southernmost state of Brazil, molecular epidemiological studies have revealed that HIV-1 subtype B co-circulates with subtype C and CRF31_BC in populations under similar socio-demographic conditions. Since 1996 the Brazilian government implemented a free-of-cost access of AIDS patients to licensed therapy

antiretroviral. Such conditions made the Southern Brazil an interesting model to explore the potential differences between subtypes B and C, especially in naïve populations.

Not surprisingly most of HIV isolates circulating at Southern Brazil were subtype C, accounting for 39%, followed by subtype B (26%), F (1.1%), CRF31_BC (19%) and unique recombinant forms (14%) accordingly to previous studies [5, 7, 8]. Due to this equitative circulation in a drug naïve population it is important to constantly monitorate the frequency of primary mutations on both subtypes. Brazil has reported to 2009 one of the lowest rates of primary drug resistance of the countries that offer access to HAART, with only 8.1% of total resistance [11, 12, 13]. At this point, we found a total of 8.1% of samples presenting primary resistance. Evaluation of such patterns conduced in the same population at 2005 revealed a prevalence of 3.0% demonstrating an increase of 5% in the last years in that region [24].

Viral replicative fitness is negatively affect by resistance mutations and may no longer be detectable in the absence of selective drug pressure. Additionally, sequencing methods used in this study does not identify variants that constitute less than 20–30% of the viral population. Therefore a current limitation on HIV drug resistance studies is the possible underestimation of the prevalence of resistance mutations among grug-naïve patients due to its high variability [9, 35].

Two mutations involved in resistance to protease inhibitors were found in codon 54 of PR: I54T in a sample of subtype B and I54L in a sample of subtype C. The same was true for the codon 67 of RT: D67N were found in subtype B and D67G in subtype C, mutations conferring resistance to NRTIs. Some codons seem to be more plastic than others, but the exchange of aminoacids may be subtype-specific [2, 25, 26]. M184V is frequently related to NRTI resistance mutation [27]. Furthermore, this mutation affects the 3TC action, one of the most commonly used NRTI [28]. Published papers reported reduced fitness, in vitro HIV replication in patients carrying the M184V mutation [29, 30]. A recent hypothesis for the frequent occurrence of mutations which lead to reduction in viral fitness in the absence of selective drug pressure suggest a combined result of HIV-1 high error rate and cytotoxic T lymphocyte (CTL) mediated immune selection pressure [26]. Resistance to the NNRTs was due K103N, V106M and Y181C mutations often found in drug-naïve patients [9, 31]. K103N mutation is associated to a lower deleterious impact on viral

fitness [28, 32] that may facilitate its persistence among chronically infected individuals. Generally NNRTs not lead to valuable changes in viral fitness, i.e., does not cause a high genetic barrier [26, 29].

Accessories mutations that confer potential low-level resistance at PI nelfinavir were found in 6% of samples; T74S (3%) occurs in untreated persons with subtype C viruses and mutations L89V (3%) generally emerges during treatment [26]. Other accessories mutations reported were L10I and A71T/V, both of them could occurs in untreated persons. Codon 63 is the most polymorphic found among samples (H/T/Q/S/A/V/C/I/F), being the L63P a molecular signature subtype B-specific. Other polymorphisms encountered were K20R (8%) specific-subtype B which is associated with resistance to multiple PIs when is present with other mutations, and M36I, specific-subtype C which doesn't resistance associated.

In addition to resistance mutations, polymorphisms also contribute with lower or higher drug's effectiveness degree, since they reflect the protein three-dimensional structure such is directly related to antiretroviral actions. Papers that describe and relate the polymorphisms to subtypes are important to monitoring the virus evolution face to different environments. Furthermore, the use of molecular modeling has been providing important insights about viral proteins form-function.

When analyzing primary drug resistance mutations among subtypes B, C, and CRF31_BC we found no correlation between subtype and resistance genotype due to reduced number of samples. However, subtype C seems to present fewer resistance mutations than subtype B and CRF31_BC. A recent study conducted in the same region evaluating mutational profile in patients under antiretroviral therapy revealed this same pattern [14]. These results in combination suggest that subtype B naturally harbors more drug resistance mutation than subtype C probably due to a wide range of genetic barriers [33]. Moreover, subtype C presents poor relative replication efficiency related to slower disease progression, longer survival of the human host, and thus more time for transmission answering the efficient spread through southern Brazil [16]. Here, we described for the first time the mutational profile of CRF31_BC. Recombinant forms are constantly arisen from co-circulation of subtypes; therefore it is important to monitorate the mutational evolution over time and its consequences to clinical disease.

In regard to transmission route our data supports an association of subtype C to heterosexual contact. Previous studies suggested a higher potential of subtype C viral particles to infect dendritic cells of the female genital tract [34]. Moreover, the transmission group of viral introduction should be taken into consideration in regard of the dynamic of the epidemic.

Our data are particularly important in view of the recent increase in prevalence of drug-resistance mutations in a region under a regimented antiretroviral therapy. This data should be an advertisement on focus the subtyping concomitant to the genotyping of viral genome before the beginning of drug treatment. Therapy could be optimized if treatment was subtype-directed accounting for genetic barriers of each HIV-1 subtype. In this light, the results of genetic analysis of HIV-1 from drug-naïve patients appear to be a promising and important tool for the surveillance of HIV infection.

FIGURES

Table 1 - Summarizes the epidemiologic, clinical, and laboratory data of the 99 patients HIV+ from Southern Brazil.

	B (n=26)	**C (n=39)**	**CRF_31 (n=19)**	**URF (n=14)**	**P**	**overall (n=99)***
Age (years)	36.6 (±11.7)	33.2 (±10.1)	36.8 (±8.7)	32.8 (±7.9)	NS	35 (±10,01)
Gener (mens)	61.50%	46.20%	47.4%	71.4%		54.4%
CD4+ (cellsmm^3)	396 (±187.2)	384.6 (±155.9)	346.9 (±278.9)	411.3 (±260.3)	NS	383,9 (±205,60)
Viral load (ln)	4.3 (±0.68)	4.1 (±0.92)	4.2 (±0.74)	4.2 (±1.07)	NS	4,2 (±0,80)
HIV time (months)	31.6 (±9.3)	34.4 (±8.9)	29.5 (±8.9)	36.4 (±15.4)	NS	33 (±10,17)
Presumed transmission					0.019	
homosexual	39.1%	2.9%	25.0%	28.6%		20.5%
heterosexual	56.5%	91.4%	68.8%	71.4%		75.0%
blood contact	4.4%	5.7%	6.2%	0.0%		4.5%
Primary resistance mutations					NS	
PI	1	0	1	0		
NRTI	2	1	0	0		
NNRTI	0	1	1	1		
	12.5%	5.1%	10.5%	7.1%		8.1%
Primary accessories mutations					NS	
PI	15.4%	7.7%	10.5%	14.3%		11.1%

PI: protease inhibitors; NRTI: nucleoside reverse transcriptase inhibitors; NNRTI: non-nucleoside reverse transcriptase inhibitors. NS = $p < 0.05$.

* Only a sample of subtype F was found, therefore their data were not presented in the table (no showed resistance mutation).

Table 2 - Amino acid substitutions in the PR and RT resistance-related codons of naive patients HIV+ from Southern Brazil.

Sample	Subtype sample	Subtype mutations*	PI Major Mutations	PI Accessories Mutations	NRTI Mutations	NNRTI Mutations	Resistance profile			
							Potential Low	Low	Intermediate	High
RS012	B	B	-	-	**D67N + K70R**	-		D4T, TDF	AZT	
RS021	URF	C	-	L89V	-	-	NFV	-		
RS028	B	B	**I54T**	A71V	-	-		DRV, NFV	FPV	
RS038	B	B	-	L89V	-	-	NFV	-		
RS045	CRF_31	C	-	T74S	-	-	NFV	-		
RS052	B	B	-	A71V	-	-		-		
RS053	URF	B	-	A71T	-	-		-		
RS055	C	C	-	T74S	-	-	NFV	-		
RS059	C	C	-	L89V	-	-	NFV	-		
RS069	CRF_31	C	-	T74S	-	-	NFV	-		
RS072	C	C	-	L10I	**D67G**	-		AZT		
RS095	C	C	-	-	-	**V106M**		ETR		NVP, DLV, EFV
RS098	CRF_31	B	-	-	-	**Y181C**			EFV, ETR	DLV, NVP
RS104	B	B	-	-	**M184V**	-				3TC, FTC
RS117	CRF_31	C	**I54L**	-	-	-		NFV, SQV		
RS126	URF	C	-	-	-	**K103N**		ETR		NVP, DLV, EFV
RS152	B	B	-	A71V	-	-		-		
RS156	C	C	-	L10I	-	-		-		

PI: protease inhibitors; NRTI: nucleoside reverse transcriptase inhibitors; NNRTI: non-nucleoside reverse transcriptase inhibitors. Resitence mutations are in indicate in bold.
AZT: zidovudine; DLV: delavirdine; d4T: stavudine; EFV: efavirenz; ETR: etravirine; FPV: fosamprenavir; NFV: nelfinavir; NVP: nevirapine, SQV: saquinavir and DRV: duranavir; TDF: tenofovir; 3TC: lamivudine.

* Sequence subtype at mutation position.

Table 3 - Linear regression demonstrating the correlation subtype-specific polymorphism from protease and reverse transcriptase.

	Polymorphism	Subtype	Fold increase	95% C.I.	*p* Value
Protease	I15V	C	2.7	(1.1 - 6.4)	P=0.022
	M36I	C	2.9	(1.2 - 6.9)	P=0.015
		CRF_31	18	(2.3 -141.3)	P=0.006
	N37K	C	10.9	(3.7 - 32.1)	P=0.001
	R41K	B	3.1	(1.1 - 8.4)	P=0.026
	R41N	CRF_31	5.9	(1.6 - 21.8)	P=0.008
		C	9.2	(3.4 - 24.5)	P>0.001
	I62V	B	4.7	(1.1 - 19.1)	P=0.32
	L63P	B	4.6	(1.7 - 12.1)	P=0.002
	H69K	C	20.7	(5.7 - 75.2)	P>0.001
		CRF_31	7.3	(1.6 - 33.7)	P=0.011
	L89M	C	3.1	(1.3 - 7.4)	P=0.009
		CRF_31	5.1	(1.3 - 18.7)	P=0.015
	I93L	C	2.6	(1.1 - 6.1)	P=0.022
Reverse Transcriptase	K20R	B	12.2	(2.3 - 65.3)	P=0.004
	V35T	C	7.7	(2.6 - 22.4)	P>0.001
		CRF_31	13.3	(1.7 - 104.5)	P>0.014
	E36A	C	8.1	(3.2 - 20.3)	P>0.001
	T39D	C	3.7	(1.6 - 8.6)	P=0.002
	S48T	CRF_31	14	(1.8 - 110.1)	P=0.012
		C	11.4	(3.6 - 36.1)	P>0.001
	I135T	B	7.7	(2.7 - 22.1)	P>0.001
	K173A	C	59.9	(7.5 - 474.1)	P>0.001
	D177E	C	7.4	(3.0 - 18.3)	P>0.001
	T200A	C	18.9	(6.3 - 56.6)	P>0.001
	Q207E	C	7.5	(3.1 - 18.8)	P>0.001
	R211K	C	3.8	(1.3 - 11.2)	P=0.004
	F214L	B	4.4	(1.5 - 12.9)	P=0.007

95% confidence interval.

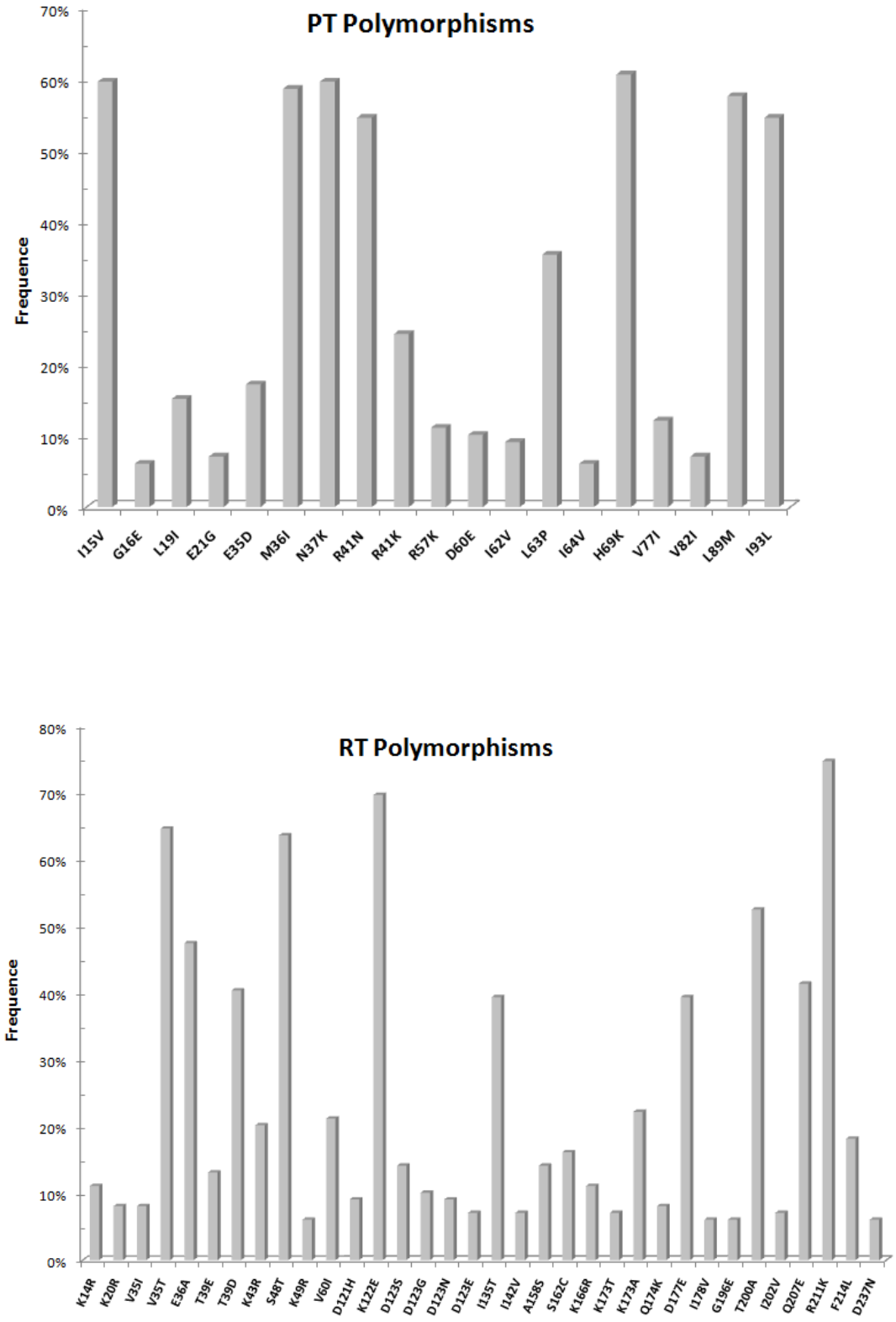

Fig 01 – Frequence of polymorphisms in protease and reverse transcriptase genes of naïve patients.

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