Original Article

The prevalence of resistance-associated mutations to protease and reverse transcriptase inhibitors in treatment-naïve (HIV1)-infected individuals in Casablanca, Morocco

Khadija Bakhouch[1], Ahd Oulad-Lahcen[2], Rajae Bensghir[2], Mohamed Blaghen[3], Kamal Marhoum Elfilali[2], Sayeh Ezzikouri[1], Omar Abidi[1], Mohamed Hassar[1], Lahcen Wakrim[1]

[1] *Laboratoire de Virologie, Institut Pasteur du Maroc, 1, Place Louis Pasteur, 20100, Casablanca, Morocco*
[2] *Service des Maladies Infectieuses, CHU Ibn Rochd, Casablanca, Morocco*
[3] *Laboratoire de Microbiologie, Biotechnologie et Environnement, Faculté des Sciences, Université Hassan II-Ain chock. Km 8, Route d'El Jadida, B.P.5366 Mâarif, Casablanca, Morocco*

Abstract

Background: The widespread use of antiretroviral agents and the growing occurrence of HIV-1 strains resistant to these drugs have given rise to serious concerns regarding the transmission of resistant viruses to newly infected persons, which may reduce the efficacy of a first-line antiretroviral therapy.

Methodology: RNA was extracted from plasma samples of 98 treatment-naïve individuals with a plasma HIV RNA viral load of at least 1,000 copies/ml. Both protease (*pr*) and reverse transcriptase (*rt*) were amplified and sequenced using an automated sequencer. National Agency for AIDS Research (ANRS) and Stanford HIV database algorithms were used for interpretation of resistance data.

Results: In the protease segment, various minor mutations were present in the majority of the sequenced samples with high frequencies. Only two major mutations, M46L and V82L, were separately found in three individuals of 71 (4.2%) with one carrying both mutations. In the reverse transcriptase gene, no NNRTIs-associated resistance mutations were detected. Only one patient of 70 (1.4%) carried the F77L mutation that is associated with NRTIs resistance. Genetic subtyping revealed that 74.6% of samples were infected with subtype B, 15.5% with CRF02_AG, 4.2% with CRF01_AE, 1.4% with C, 2.8% with G and 1.4% with subtype F2.

Conclusions: The low prevalence of major mutations associated with resistance to antiretroviral drugs (ARVs) among drug-naïve individuals studied suggests that the routine of drug resistance testing may be unnecessary for all Moroccan individuals newly diagnosed or all patients beginning antiretroviral therapy. Nevertheless, continuous surveillance is required since greater access to antiretroviral drugs is expected in Morocco.

Keywords: *HIV-1, antiretroviral therapy, resistance mutation*

J Infect Dev Ctries 2009; 3(5):380-391.

Received 14 November 2008 - Accepted 28 March 2009

Introduction

Since the introduction of highly active antiretroviral therapy (HAART), the rates of human acquired immunodeficiency syndrome (AIDS)-related morbidity and mortality have significantly decreased [1-3]. However, an undesired consequence of antiretroviral therapy has been the selection and emergence of drug-resistant virus variants which are a major obstacle that limits the long-term efficacy of a successful treatment with currently available antiretroviral drugs (ARVs) [4-7]. Thus, drug resistance is now a widespread and growing problem that not only involves antiretroviral-treated patients, but also drug-naïve individuals infected with HIV-resistant strains [8].

Transmission of HIV-1 drug resistance from treatment-experienced patients to newly HIV-1-infected individuals was observed in developed countries with access to antiretroviral therapy [7,9-13]. The problem of widespread dissemination becomes even more serious when the resistance to two or more classes of drugs can also be transmitted in primary infection and shorten the time to first virological failure [6,7,11,14]. Some studies report very different rates of transmission of drug resistant viruses, ranging from 20% in North America [6,15-

17], to 10% or less in Europe and Latin America [8,18-25].

In developing countries, it is necessary to investigate whether resistant mutants are circulating in untreated patients where much of the burden of HIV-1 infection is concentrated, combination therapy is increasingly available and more intensively introduced, and a sharp increase in drug resistance is expected as patients become more treatment experienced [26,27]. Other factors that contribute to the occurrence of drug resistance in these areas include disruption in supplies of antiretroviral drugs, inappropriate use of drugs, changing medication frequently, administering and taking the wrong dose, and interruptions in treatment due to financial constraints [28].

Antiretroviral drug resistance assay has become an important tool in the clinical management of patients infected with HIV-1 [29]. This surveillance of antiretroviral drug resistance helps map regions with high prevalence of primary resistance, where the genotyping test performed in patients without prior treatment would be indicated and would help to determine the initial antiretroviral drugs of choice for the first-line regimen [7,29,30]. Current guidelines recommend the use of antiretroviral resistance testing of drug-naïve subjects who are either acutely or chronically infected, particularly in geographic areas where primary resistance has been consistently documented [3]. Therefore, the primary objective of our study was to estimate the prevalence of mutations associated with antiretroviral drug resistance in a group of newly diagnosed drug-naïve individuals.

Materials and Methods

Study population

A total of 120 samples from antiretroviral-naïve HIV-1 infected individuals were studied. The samples were collected from June 2004 to July 2007.

The study was a collaboration between the Infectious Diseases Unit of the University Hospital Center (CHU Ibn Rochd) and Institut Pasteur du Maroc, Casablanca.

The infected individuals had to be more than 18 years old, newly diagnosed with HIV infection at any stage, and never exposed to antiretroviral therapy with an HIV-RNA load of at least 1,000 copies/ml. The studied group was composed of men (50.8%) and women (49.2%).

After viral load testing, only 98 individuals were eligible for HIV drug resistance genotyping.

Methods

Five-ml blood samples were collected in EDTA blood collection tubes. Plasma was separated and stored in multiple aliquots at -80°C until it was processed.

HIV-1 RNA quantification with real time TaqMan RT-PCR test

The real-time reverse transcriptase test was performed adopting the protocol established by the Working Group for HIV quantification (AC11) of the French National Agency for AIDS Research (ANRS) [31, 32]. The test was conducted with the Generic HIV charge viral kit (Biocentric, Bandol, France).

RNA was extracted from 200 µl of plasma using the QIAmp viral RNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

The TaqMan PCR targeted a conserved consensus region in the long terminal repeat (LTR) region of the HIV-1 major group. The internal HIV-1 TaqMan probe (LTR MLC-1) was (5'-AAGTRGTGTGTGCCC-3'). This probe carried a 5'reporter FAM and a 3'MGB-non fluorescent quencher. The sequences of the forward (NEC005) and reverse (NEC131) primers were (5'-GCCTCAATAAAGCTTGCC-3') and (5'-GGCGCCACTGCTAGAGATTTT-3') respectively. All runs were performed in a 50 µl-volume containing RNA extract (20 µl), primers (200 nM of each), probe (200 nM), 1x PCR buffer, and 1x RT mix (SuperScript^{TM} III Platinum). The external standard was a culture supernatant of an HIV-1 subtype B strain.

For each experiment, one aliquot of this standard was extracted together with the clinical samples and serially diluted (fivefold dilutions) to concentrations from 2.5×10^6 copies/ml to 2.5×10^2 copies/ml. With 0.2 ml of plasma, the threshold of the technique was set at 2.5 $\log_{10}$ copies/ml.

Cycling conditions were as follows: initial incubation at 48°C for 30 minutes of reverse transcription followed by 95°C for 10 minutes before proceeding to 50 cycles of melting at 95°C for 15 seconds and annealing at 60°C for 1 minute. Amplification and data acquisition were conducted using the ABI Prism 7000 Sequence Detection System (Applied Biosystems). The $\log_{10}$ of the number of targets initially present was proportional to the cycle threshold (C_T) and determined using the standard curve.

RNA extraction and sequencing

Plasma (1ml) was centrifuged at 4°C for 1hour and 45 minutes at 21.500 × g. The resulting virus pellet was resuspended in 140 µl of plasma for RNA extraction by use of a QIAmp viral RNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The isolated RNA was resuspended in 80 µl of RNA diluent.

Ten µl of extracted RNA was used as a template to amplify the reverse transcriptase (*rt*) and protease (*pr*) genes with a one-step Access Quick RT-PCR system kit (Promega, Madison, WI) and GoTaq DNA polymerase (Promega) for a first and a second PCR, respectively.

Both genes were amplified using the ANRS consensus sets of primers [33]: for the *rt* amplification, MJ3 and MJ4 were used for a first PCR (RT-PCR), and A(35) with NE1(35) as inner primers for nested PCR. For the protease gene amplification, we used the following outer primers: 5'Prot1 and 3'Prot1 and the inner primers: 5'Prot2 and 3'Prot2 (Table 1).

RT-PCR involved a reverse transcription step at 45°C for 45 minutes followed by 40 cycles of 95°C for 5 minutes, 55°-60°C (depending on primers) for 1 minute and 72°C for 1 minute, with a final extension step of 7minutes at 72°C. The RT-PCR products were then subjected to nested PCR with the following cycling conditions: a denaturation step of 5 minutes at 94°C, and then 40 cycles with 1 cycle consisting of 30 seconds at 94°C, 45 seconds at 61-63°C (depending on primers) and 1 minute at 72°C with a final extension at 72°C for 7 minutes.

The PCR products were analyzed in 1.5% ethidium bromide stained agarose electrophoresis gel, to evaluate the purity and the amount of DNA, and photographed under ultraviolet illumination. The resulting PCR-amplified DNA fragments were purified using both a QIAquick Gel Extraction Kit (Qiagen, Germany) and the Exonuclease I (ExoI) with Shrimp Alkaline Phosphatase (SAP) method (Amersham, GE Healthcare). The purified products of both strands were sequenced with the BigDye Terminator Cycle Sequencing Kit v.3.1 (Applied Biosystems, Foster city, CA) using the forward and reverse primers. Labelled fragments were precipitated with EDTA/Ethanol method, and then resuspended in 20 µl of formamid to be analysed on an ABI Prism 3130 Genetic Analyser (Applied Biosystems). Obtained sequences were aligned and compared to the HXB2 reference sequence (Genbank accession number K03455) using SeqScape software version 2.5.

Resistance-associated mutations were identified referring to the ANRS (AC11) and the Stanford's HIV drug-resistance database (Stanford HIVdb) genotypic resistance interpretation algorithms and the International AIDS Society (IAS-USA) mutations list (2007 update). The genetic subtyping and recombination analyses were performed using both the Stanford sequence analysis program (http://hivdb.Stanford.edu/) and the NCBI HIV subtyping tool (http://www.ncbi.nlm.nih.gov/) by

Table 1. Sequences of consensus primers used to amplify reverse transcriptase and protease regions.

Region			Sequence of the following primers (bases)	Tm (°C)
pr	Outer	MJ3	5'-AGTAGGACCTACACCTGTCA-3'	55
		MJ4	5'-CTGTTAGTGCTTGGTTCCTC-3'	
	Inner	A(35)	5'-TTGGTTGCACTTTAAATTTTCCCATTAGTTATT-3'	63
		NE1(35)	5'-CCTACTAACTTCTGTATGTCATTGACAGCAGCT-3'	
rt	Outer	5'Prot1	5'-TAATTTTTTAGGGAAGATCGGCTTCC-3'	60
		3'Prot 1	5'-GCAAATATGGAGTATTGTATGGATTTTCAGG-3'	
	Inner	5'Prot2	5'-TCAGAGCAGACAGAGACCAACAGCCCCA-3'	61
		3'Prot2	5'-AATGCTTTTATTTTTTCTTCTGTCAATGGC-3'	

comparing the study sequences to a set of reference sequences using BLAST. The phylogenetic tree was constructed by the neighbour-joining method using the protease sequences.

Results

Among 120 newly diagnosed HIV-infected subjects enrolled in this study, the quantitative real-time RT-PCR results showed that only 98 had a plasma HIV-1 RNA viral load ≥ 1000 copies/ml. The baseline characteristics of these 98 subjects are shown in Table 2.

Table 2. Baseline characteristics of 98 HIV-1 infected individuals naïve for antiretroviral treatment.

Baseline Characteristics	Patients (n=98)
Gender	
Males no. %	51.1 (50)
Females no. %	48.9 (48)
Age (years)	
Range	20-50
Median	35
Plasma HIV RNA (Log_{10} copies/ml)	
Range	3.50-6.12
Median	5.62
CD4 T-cell counts (cells/mm^3)	
Range	17-420
Median	180

Protease (*pr*) and reverse transcriptase (*rt*) genes were successfully amplified and sequenced for 71 of 98 (72.45%; CI95%, 69.25 to 75.65) and 70 of 98 (71.43%; CI95%, 68.23 to 74.83) samples, respectively. Among these amplified samples, 67 were amplified and sequenced for both genes. Genotypic resistance results were interpreted using the ANRS algorithm updated in July 2007, the 2007 (IAS-USA) list, and Stanford HIVdb algorithm.

Analysis of the protease sequences revealed various minor mutations/polymorphisms in the majority of studied individuals at the following positions: L10I/V (15.4%; 11 of 71), I13V (46.4%; 33 of 71), I15V (22.5%; 16 of 71), G16E (14%; 10 of 71), K20I/M/R (32.4%; 23 of 71), M36I/L/V (53.5 %; 38 of 71), D60E (5.6%; 4 of 71), I62V (21.1%; 15 of 71), L63P (42.2%; 30 of 71), I64L/V/M (46.4%; 33 of 71), H69K (23.9%; 17 of 71), A71T (2.8%; 2 of 71), V77I (19.7%; 14 of 71), L89V (8.4%; 6 of 71) and I93L (12.9%; 9 of 71).

Two major mutations were detected in three different individuals of 71 (4.2%; CI 95%, 1 to 7.4), with one individual carrying both M46L and V82L mutations.

Single amino substitutions were identified in 4 of 71 (5.6 %) protease sequences, whereas 94.4% of sequences harboured dual (16.9%, 12 of 71), triple (18.4%, 13 of 71), quadruple (26.8%, 19 of 71), quintuple (23.9%, 17 of 71), sextuple (7%, 5 of 71) and septuple (1.4%, 1 of 71) substitutions (Table 3).

Regarding the *rt* gene, the accessory mutation F77L occurred in one patient of 70 (1.4%; CI 95%, -1.8 to 4.6). According to Stanford HIVdb algorithm, this mutation is associated with reduced sensitivity to commonly used NRTIs. No mutations associated with NNRTIs were detected.

Subtyping and phylogenetic analyses of the *pr*-*rt* sequences revealed that 53 (74.6%) of the sequences were subtype B, 11 (15.5%) were subtype CRF02_AG, three (4.2%) were subtype CRF01_AE, one was (1.4%) C, two (2.8%) were G and one (1.4%) was subtype F2 (Table 3).

Table 3. Subtypes and distribution of resistance-associated mutations in drug-naïve HIV-1 infected individuals as mentioned in Stanford HIVdb and ANRS algorithms.

Samples	Subtypes	NRTIs	NNRTIs	PIs	
				Major mutations	Minor mutations
No.1	B	-	-	-	K20R, M36I, M36L, L63P
No.2	B	-	-	-	V77I
No.3	B	-	-	-	I15V, G16E, K20M, M36I, I64V
No.4	B	-	-	-	G16E, M36I, I64V
No.5	B	ND	ND	-	I13V, L63P
No.6	B	-	-	-	I62V, L63P, I64L, I93L
No.7	B	-	-	-	K20R, M36I
No.8	B	-	-	-	I15V, G16E, K20M, I64V
No.9	B	-	-	M46L	M36I, I62V, L63P, L89V
No.10	B	-	-	M46L, V82L	L10I, I62V, L63P, L89V, I93L
No.11	B	-	-	-	I15V, G16E, K20M, I64V
No.12	B	-	-	-	L63P, I64L, V77I
No.13	B	-	-	-	L63P, I64L
No.14	B	-	-	-	L63P, I64V
No.15	B	-	-	V82L	I15V, M36I
No.16	B	-	-	-	I15V, M36I, I64V
No.17	B	-	-	-	I13V, I64V, V77I
No.18	B	-	-	-	I13V, M36V, I62V, L63P
No.19	B	-	-	-	K20R, L63P, V77I
No.20	B	-	-	-	L63P, V77I
No.21	B	-	-	-	I13V, L63P
No.22	B	ND	ND	-	L63P
No.23	B	-	-	-	L63P, I64L
No.24	B	-	-	-	L63P, V77I
No.25	B	-	-	-	M36I, I64V
No.26	B	-	-	-	I13V, L63P, I64V, V77I
No.27	B	-	-	-	I13V, L63P, I64V, V77I
No.28	B	-	-	-	I13V, L63P, I64V, V77I, L89V
No.29	B	-	-	-	I13V, I62V, L63P, V77I
No.30	B	ND	ND	-	I15V
No.31	B	-	-	-	I15V
No.32	B	-	-	-	M36I, I62V, I64V
No.33	B	-	-	-	I13V, G16E, M36I, I62V, I64V
No.34	B	-	-	-	L10V, G16E, M36I, I62V, I64V
No.35	B	-	-	-	L63P, I93L
No.36	B	-	-	-	I15V, M36I, I64L, H69K
No.37	B	-	-	-	I15V, M36I, I62V, L63P, I93L
No.38	B	-	-	-	M36L, I62V, I64M
No.39	B	-	-	-	I13V, I15V, K20M, M36V, I64V
No.40	B	-	-	-	D60E, I62V, L63P, A71T, I93L

Table 3. Continued.

Samples	Subtype	NRTIs	NNRTIs	PIs	
				Major mutations	Minor mutations
No.41	B	F77L	-	-	G16E, A71T
No.42	B	-	-	-	L10V, I13V, M36I, I64V
No.43	B	-	-	-	L10V, I13V, M36I, I64V, L89V
No.44	B	-	-	-	I62V, L63P, I93L
No.45	B	-	-	-	I13V, D60E, I64V, V77I
No.46	B	ND	ND	-	I62V, L63P
No.47	B	-	-	-	L10V, L63P, I64L, V77I
No.48	B	-	-	-	L10V, I13V, I15V, M36I
No.49	B	-	-	-	L10V, I13V, I15V, L63P
No.50	B	-	-	-	I62V, I64L, V77I
No.51	B	-	-	-	I62V, L63P, I64L, L89V, I93L
No.52	B	-	-	-	I15V, D60E, L63P
No.53	B	-	-	-	L63P, V77I, I93L
No.54	02_AG	-	-	-	L10I, I13V, K20I, M36I, I64L, L89V
No.55	02_AG	-	-	-	I13V, K20I, M36I, I64M, H69K
No.56	02_AG	-	-	-	I13V, K20I, M36I, H69K
No.57	02_AG	-	-	-	I13V, K20I, M36I, I64L, H69K
No.58	02_AG	-	-	-	I13V, K20I, M36I, H69K
No.59	02_AG	-	-	-	I13V, K20I, M36I, L63P, I64L H69K
No.60	02_AG	-	-	-	I13V, K20I, M36I, H69K
No.61	02_AG	-	-	-	I13V, K20I, M36I, I64L, H69K
No.62	02_AG	-	-	-	L10V, I13V, G16E, K20I, M36I, H69K
No.63	02_AG	-	-	-	I13V, K20I, M36I, H69K
No.64	02_AG	-	-	-	I13V, I15V, K20I, M36I, H69K
No.65	02_AE	-	-	-	I13V, M36I, H69K
No.66	02_AE	-	-	-	I13V, I15V, M36I, H69K
No.67	02_AE	-	-	-	I13V, G16E, K20R, M36I, H69K
No.68	G	-	-	-	I13V, K20I, M36I, I64M, H69K
No.69	G	-	-	-	L10I, I13V, I15V, K20I, M36I, H69K
No.70	C	-	-	-	I13V, K20R, M36I, D60E, H69K, I93L
No.71	F2	-	-	-	L10I, I13V, G16E, K20R, M36I
No.72	U	-	-	ND	ND
No.73	U	-	-	ND	ND
No.74	U	-	-	ND	ND

NRTIs: Nucleotide reverse transcriptase inhibitors, NNRTIs: Non-nucleotide reverse transcriptase inhibitors, PIs : Protease inhibitors, ND: not determined PCR negative, U: Unclassifiable

The distribution of the detected mutations in the protease gene between HIV-1 B and non-B subtypes is presented in Table 4. The minor mutation L63P was observed in 54.7 % of subtype B sequences followed by I64V (33.9%), 62V (28.3%) and V77I (26.4%). Frequent minor mutations observed in non-B subtype sequences were M36I (100%), I13V (100%), H69K (88.8%) and K20I (72.3%).

Discussion

Resistance genotyping has become an important tool in therapeutic management protocol of HIV-1 infection [34,35]. Such assessment of anti-HIV drug resistance is currently recommended in European, as well as American, guidelines. Recent European guidelines recommend resistance testing for all drug-naïve patients with acute or recent HIV infection, while for chronically infected patients, in whom treatment is to commence, resistance testing is recommended if the suspicion of resistance is high or the prevalence of resistance in this population exceeds 10% [36]. IAS-USA guidelines recommend resistance testing in drug-naïve patients when the regional prevalence of resistance is at least 5% [3,30].

Our results showed that most of the 71 individuals who were successfully genotyped carried many minor mutations with high frequencies in the protease region. These mutations are often found at polymorphic positions and may have limited effect on susceptibility to antiretroviral drugs. However, these mutations should be considered when assessing the possible impact on the therapy response as they have been reported to be associated with high viral fitness in strains with major mutations [21,29,37-42]. Among the minor mutations encountered, I15V is not listed by the IAS-USA, while mutations I13V, I64L/M/V, I93L are not included in the ANRS algorithm.

M36I/L/V were the most common minor mutation (53.5 %), followed by both I13V (46.4%) and I64L/V/M (46.4%), L63P (42.2%) and K20I/M/R (32.4%). It was observed that the presence of minor protease inhibitor mutations such as M36I may increase viral replication capacity [43-45].

The two major mutations M46L and V82L were observed in three different individuals. One of these individuals carried both mutations. According to the Stanford HIVdb system, the M46L mutation decreases susceptibility to different protease inhibitors (PIs): Indinavir (IDV), nelfinavir (NFV), fosamprenavir (FPV), lopinavir (LPV), atazanavir (ATV), tipranavir (TPV) and possibly saquinavir (SQV) and darunavir (DRV) when present with other mutations, while the V82L mutation is considered a rare tipranavir (TPV)-selected mutation, and its effect on other PIs has not been characterized.

Considering subtype, genotyping results revealed the presence of multiple subtypes carried by our study population which may explain the large number of minor mutations observed in protease sequences. Subtype B was the most frequent (74.6%), followed by CRF02_AG (15.5%) and CFR02_AE (4.2%). The analysis of the distribution of observed mutations between subtype B and non-B subtypes showed that the minor mutations L63P, I64V, I62V and V77I were more prevalent in subtype B, whereas I13V, K20I, M36I and H69K mutations were found at a higher frequency in non-B subtypes as described by previous reports [20, 41, 46, 47]. Major resistance mutations (M46L and V82L) were only observed in subtype B.

Although little information is available on the source of HIV-1 infection of studied individuals, our results are consistent with previous studies reporting that in the nineties, subtype B was largely predominant in Morocco [48,49]. This high subtype B prevalence can be explained by the fact that tourism constitutes the main source of HIV-1 infections in Morocco [48]. The increasing prevalence of non-B subtypes, especially CRF02_AG recombinant, showed in our results is also in agreement with a previous report by Akrim *et al*. (2006) that showed a significant augmentation and rapid evolution of the non-B subtypes in Morocco [50]. The introduction of non-B subtypes in Morocco, especially CRF02_AG which is the most prevalent non-B subtype, can be mainly associated with the increasing phenomenon of Sub-Saharan Africa immigration to European countries via Morocco. During the last years, many of the migrants who fail to enter Europe become permanent settlers in Morocco. This hypothesis is enhanced by other studies that showed a predominance of CRF02_AG recombinant in West Africa [51-54] where most of these migrants originate.

Table 4. Prevalence of mutations and polymorphisms in HIV-1 protease sequences of 71 drug-naïve subjects and their distribution between subtype B and non-B subtypes.

Mutation	Number of individuals			
	Subtype B	Non-B subtype	Total	Percentage of total individuals (%)
L10I	1	3	4	5.6
L10V	6	1	7	9.8
I13V	15	18	33	46.4
I15V	13	3	16	22.5
G16E	7	3	10	14
K20I	0	13	13	18.3
K20M	4	0	4	5.6
K20R	3	3	6	8.4
M36I	16	18	34	47.8
M36L	2	0	2	2.8
M36V	2	0	2	2.8
M46L	2	0	2	2.8
D60E	3	1	4	5.6
I62V	15	0	15	21.2
L63P	29	1	30	42.2
I64L	8	4	12	16.9
I64M	1	2	3	4.2
I64V	18	0	18	25.3
H69K	1	16	17	23.9
A71T	2	0	2	2.8
V77I	14	0	14	19.7
V82L	2	0	2	2.8
L89V	5	1	6	8.4
I93L	8	1	9	12.6

In the reverse transcriptase gene, the F77L mutation, that is not included in the ANRS algorithm, was found in one individual (with subtype B) of 70 without the presence of other mutations. Considered of minor importance, this mutation was not included in our estimate of prevalence. However, it is encompassed in the evolution of a set of five mutations, beginning with Q151M, followed by F77L and F116Y and later by A62V and V75I that confers multiple drug resistance [55].

The overall prevalence of major resistance was thus (4.2 %; CI 95%, 1 to 7.4%) (3 of 71), mainly due to major resistance to PIs, since minor mutations/ polymorphisms were not included. This prevalence is lower when compared to some studies conducted in North America and Europe with an overall resistance percentage of (18 %; $P = 0.007$) for USA [56], (25%; $P = 0.001$) for Greenland [57], (20.27 %; $P = 0.001$) and for Spain [58], while the reported prevalence in Portugal (7.78%; $P = 0.410$) [25], Belgium (9.5 %; $P = 0.231$) [59] and in one study conducted in 40 US cities (10%; $P = 0.166$) [60] was not so different. In contrast to most of the international studies we have reviewed, no major resistance mutations in reverse transcriptase gene were detected.

These differences between studies could be explained by different factors: the longstanding availability of antiretroviral therapy in the transmitter pool in industrialized countries, the cross-sectional rather than longitudinal nature of the studies, the sample size, a lack of knowledge of the relative rates of antiretroviral drug use within the community, the timing of the sampling, and also the differential medication adherence that was commonly reported and was associated with an increased risk of initial virological failure with antiretroviral resistance emergence [61].

In our study, most of the individuals were diagnosed at an advanced stage of infection (1-3 years after infection) and the exact lapse of time between the moments of infection and sample collection is unknown. Therefore, the overall low prevalence of resistance may have been influenced by this long period. Theoretically, newly infected

patients are more likely to harbour resistant HIV strain variants than chronically treatment-naïve patients. This might be explained by the fact that wild type strains may become predominant in the absence of selective drug pressure, when individuals are infected with both wild type and mutant viruses. In our studied individuals, wild type variants may have emerged as prevailing within the long period of infection, rendering false negative resistance testing results [58,62,63], and the resistance-associated mutations would no longer be detected in the absence of the drug selection pressure [64]. However, several studies have demonstrated the persistence of mutations of major resistance for periods as long as five or seven years [65-67].

In addition, the low prevalence of transmitted resistance mutations can be explained by the limited number of HIV infected and treated individuals, compared to other countries, since Morocco is a low-infection prevalence country according to the World Health Organization (WHO) classification. In Morocco, the population of treated patients is well monitored and the virological failure cases are limited and well documented. This may imply that the transmission of resistance from treated patients to treatment-naïve individuals is unlikely to occur frequently. In conclusion, our study indicates for the first time that a number of major mutations associated with resistance to PIs exist among HIV-1 drug-naïve individuals in Morocco, although at a low frequency (4.2%). These results suggest that routine drug resistance testing may be unnecessary for all Moroccan individuals newly diagnosed with HIV-1 or all patients before the initiation of antiretroviral therapy. However, due to the increasing number of HIV-1 infected individuals and access to treatment and availability of ARVs for Morocco's infected population, larger studies and continuous surveillance among drug-naïve populations are required, in order to recognize early significant changes which may occur in the future.

Acknowledgements

We are deeply indebted to the patients who agreed to participate in this study. The study was supported by Association de Lutte contre le SIDA (ALCS) and the Coopération Technique Belge (CTB) and Le Ministère Français des Affaires Etrangères (Fond de Solidarité Prioritaire 2001/168 "Appui au Réseau de Surveillance des Résistances aux Antimicrobiens").

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Corresponding Author:
Khadija Bakhouch
Mailing address: Laboratoire de Virologie, Institut Pasteur du Maroc, 1, Place Louis Pasteur, 20100, Casablanca, Morocco.
Fax: +(212)522 26 09 57
Tel: +(212)522 43 44 50
Email: bakhouch.khadija@yahoo.com

Conflict of interest: No conflict of interest is declared.