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Original

Prevalence of genotypic resistance in untreated HIV-infected patients

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SUMMARY

*The aims of this retrospective study are to assess the prevalence of primary resistances to antiretroviral drugs, both reverse transcriptase and protease inhibitors in untreated patients from Spain, and to determine their possible association with several epidemiological variables. A total of 148 samples belonging to 145 patients were processed using the genotypic technique VERSANT™ HIV 1 (LiPA) in order to study the presence of mutations at codons 41, 69, 70, 74, 75, 103, 106, 151, 181, 184 and 215 of the reverse transcriptase gene (VERSANT™ HIV 1 RT) and at codons 30, 46, 48, 50, 54, 82, 84 and 90 of the protease gene (VERSANT™ HIV 1 Protease). The patients' epidemiological variables which could be relevant to HIV infection were also analyzed. The successful amplification rate was 77.70% for LiPA RT™ and 91.21% for LiPA P™. In the case of LiPA RT™, statistical significance (*p *<0.05) was observed when successful amplification was related to viral load level (*p *<0.001). Global prevalence of resistance was 20.27%. Mutations in the reverse transcriptase gene were found in eight samples (5.40%). Using LiPA P™, mutations were detected in 16.21% of cases, with V82A being the most frequently detected mutation (15/24, 62.50%) in nine samples. The V82A mutation was found alone (66.6%) and it was found together with the I84V mutation in five samples (20.83%). I84V was the second most frequently detected mutation (13/24, 54.16%). No statistical significance was found for any of the epidemiological variables. Due to the problems encountered in a high percentage of samples, the authors concluded that the amplification technique should be improved. The prevalence of resistance detected was around the mean of that found by other authors.*

Key words: HIV - Genotypic resistance - Naïve

Prevalencia de resistencia genotípica en pacientes infectados por el VIH no tratados

RESUMEN

El objetivo de este estudio retrospectivo fue evaluar la prevalencia de las resistencias principales a los fármacos antirretrovirales, tanto los inhibidores de la transcriptasa inversa como de la proteasa, en pacientes infectados por el VIH no tratados, y determinar su posible asociación con diversas variables epidemiológicas. Se procesaron un total de 148 muestras pertenecientes a 145 pacientes utilizando la técnica genotípica VERSANT™ HIV1 (LiPA) con objeto de estudiar la presencia de mutaciones en los codones 41, 69, 70, 74, 75, 103, 106, 151, 181, 184 y 215 del gen de la transcriptasa inversa (VERSANT™ HIV1 RT) y en los codones 30, 46, 48, 50, 54, 82, 84 y 90 del gen de la proteasa (VERSANT™ HIV1 Proteasa). Se analizaron también las variables epidemiológicas que podían ser relevantes para la infección por el VIH. La tasa de amplificación satisfactoria fue del 77,70% para LiPA RT™ y del 91,21% para LiPA P™. En el caso de LiPA RT™ se observó una significación estadística (p <0.05) cuando la amplificación satisfactoria se relacionó con la carga viral (p <0.001). La prevalencia global de la resistencia fue del 20,27%. Se hallaron mutaciones en el gen de la transcriptasa inversa en ocho muestras (5,40%). Utilizando LiPA P™ se observaron mutaciones en el 16,21% de los casos, siendo V82A la mutación más frecuentemente observada (15/24, 62,50%) en nueve muestras. La mutación V82A se observó sola (66,6%) y junto con la mutación 184V en cinco muestras (20,83%). Tras V82A, 184V fue la mutación más frecuentemente observada (13/24, 54,16%). No se halló ninguna significación estadística para ninguna de las variables epidemiológicas. Debido a los problemas hallados en un alto porcentaje de las muestras, los autores concluyen que la técnica de amplificación debe ser mejorada. La prevalencia de la resistencia observada fue aproximadamente la media observada por otros autores.

Palabras clave: VIH - Resistencia genotípica - Pacientes no tratados

INTRODUCTION

Antiretroviral therapies that combine protease inhibitors and reverse transcriptase inhibitors have reduced progression to AIDS and mortality in HIV infection, substantially increasing patient survival (1).

The main objectives of highly active antiretroviral therapy (HAART), introduced in the treatment of HIV infection in 1996, are four: 1) to reduce viral load suppression down to undetectable levels; 2) to restore the immune system, via an increase in CD4 cell levels; 3) to decrease the morbidity and mortality associated with HIV infection; and, last but not least, 4) to improve the patients' quality of life. The efficacy of antiretroviral treatment is limited by the presence of resistance to antiretroviral drugs, by inadequate compliance to therapy and by the side-effects of these drugs (2).

Several studies over recent years have suggested the possibility of transmission of HIV variants harboring mutations associated with resistance and have highlighted the need to establish the transmission rate in each geographical area (1-5).

Progress in the development of techniques to determine phenotypic and especially genotypic HIV resistance to antiretroviral drugs has allowed the introduction of this important parameter in the management of HIV-infected patients in routine clinical care.

The Secretariat of the National Plan on AIDS of the Spanish Ministry of Health, together with European and American HIV resistance expert panels, have determined the situations in which resistance testing should be recommended (6-12). An indication that receives general agreement is in the case of primary HIV infection. In addition, testing naïve patients would achieve two objectives: 1) it would demonstrate regular transmission of HIV resistance variants; and 2) it would avoid the negative impact that the presence of resistance could have in the response to a first-line antiretroviral therapy.

This study has two principal objectives: 1) to determine the prevalence of primary resistance to protease inhibitors and to reverse transcriptase inhibitors, including the analogue and nonanalogue of nucleoside, in HIV-infected naïve patients from the different centers covered by our hospital; and 2) to establish whether the presence of resistance is related to any of the epidemiological variables recorded.

PATIENTS AND METHODS

Patients

Plasma samples from 145 previously untreated HIV-infected patients from seven different centers (hospitals and prisons) in Castille and Leon were analyzed. Samples were collected from November 1996 to March 2003.

The following variables were recorded for each patient: sex, age, date of HIV diagnosis, mode of HIV infection, clinical category (CDC-93), CD4 cell count and viral load level at the time of resistance testing.

Methods

All plasma samples were submitted to the Microbiology Laboratory of the University Clinical Hospital of Valladolid. After viral load quantitation, they were aliquoted and frozen (–80 ºC).

Viral load quantitation was determined by polymerase chain reaction after a previous retrotranscription (RT-PCR) (*Cobas Amplicor HIV-1 Monitor*™, Roche Diagnostics, Branchburg, New Jersey, USA). Both versions of the assay were employed, the "standard" one with a 400 RNA copies/ml threshold until March 2000 and the "ultrasensitive" one able to detect down to 50 RNA copies/ml thereafter.

Viral RNA was isolated from plasma by column filtration using the *SV Total RNA Isolation System* (Promega Corporation, Madison, Wisconsin, USA) according to the manufacturer's instructions. Amplification of the extracted RNA and resistance testing were performed with a commercial Line Probe Assay (LiPA) (*VERSANT*™ HIV 1 RT and *VERSANT*™ HIV 1 Protease, Bayer Corporation. Tarrytown, New York, USA) following the manufacturer's instructions.

Briefly, LiPA is based on a post-PCR hybridation which takes place on nitrocellulose strips where specific oligonucleotide probes are fixed in parallel array. This assay allows the study of wild-type and mutant sequences at codons 41, 69, 70, 74, 75, 103, 106, 151, 181, 184 and 215 of the reverse transcriptase gene (LiPA RT) and at codons 30, 46, 48, 50, 54, 82, 84 and 90 of the protease gene (LiPA P). Mutations in these positions have been reported to be associated with resistance to reverse transcriptase inhibitors and to protease inhibitors, respectively.

The interpretation of the mutations found was conducted following the manufacturer's instructions (13, 14), and according to different national and international guidelines for interpretation (15-19).

Statistical analysis

A descriptive study in percentage terms of all the recorded variables was done with the aid of the statistics program SPSS for Windows, version 9.0. Confidence intervals (CIs)

were calculated using a macro developed by Domènech *et al.* (20). Comparison between variables was performed using the χ^2 test. A p value of less than 0.05 was considered statistically significant.

RESULTS

Overall, 148 samples from 145 patients were analyzed. Most were men (70.27%) and 89.19% were patients from the two main hospitals of Valladolid, *i.e.,* 50% (CI 95%, 41.70 to 58.30) from the Río Hortega University Hospital and 39.19% (CI 95%, 31.08 to 47.30) from the University Clinical Hospital of Valladolid.

With respect to the mode of infection, 54.05% (CI 95%, 45.77 to 62.33) were intravenous drug users, 10.13% (CI 95%, 5.12 to 15.14) reported homosexual contact and 5.4% (CI 95%, 1.65 to 9.15) reported only heterosexual contact. Blood transfusion was the cause of infection in 1.35% (CI 95%, 0 to 3.27), and data regarding mode of infection were not available for 32.43% (CI 95%, 24.65 to 40.20).

HIV was diagnosed before 1998 in 52.02% (CI 95%, 43.72 to 60.32) and after 1998 in 34.45% (CI 95%, 26.55 to 42.34). Data on the date of HIV infection were not available for 13.51% (CI 95%, 7.83 to 19.18).

In terms of viral load levels, 54.73% (CI 95%, 46.46 to 62.99) had values equal to or greater than 100,000 RNA copies/ml, and 45.27% (CI 95%, 37 to 53.54) had values less than the aforementioned value. CD4 cell count ranges were less than 200 CD4/mm^3 in 47.29% (CI 95%, 38.99 to 55.58) and greater than or equal to 200 CD4/mm^3 in 40.54% (CI 95%, 32.38 to 48.69). Data on viral load levels were not available for 12.16% (CI 95%, 6.73 to 17.58).

Out of the 148 samples analyzed, successful amplification prior to mutation detection was achieved in 77.7% (CI 95%, 70.78 to 84.61) for LiPA RT and in 91.21% (CI 95%, 86.50 to 95.91) for LiPA P (Table 1).

We studied whether achieving successful amplification was related to any of the patient variables recorded, and statistical significance was only found for LiPA RT and viral load level (viral load levels over 100,000 RNA copies/ml rendered better amplification rates). For LiPA P, none of the variables compared showed statistical significance (Tables 2 and 3).

Table 1. Sample distribution according to amplification*.

	Amplification N (%)	No amplification N (%)
LiPA RT	115 (77.7)	33 (22.29)
LiPA P	135 (91.21)	13 (8.78)

N = 148.

Table 2. Epidemiological variables studied for reverse transcriptase inhibitors according to overall sample amplification*.

Epidemiological variables	Amplified samples N = 115	Non-amplified samples N = 33	*p*
Sex			
Men	82 (80.40)**	20 (19.60)	0.24
Women	33 (71.74)	13 (28.26)	
Mode of infection			
Parenteral	64 (78.05)	18 (21.95)	0.98
Sexual	18 (78.27)	5 (21.73)	
No data	33 (76.75)	10 (23.25)	
Date of diagnosis			
Before 1998	58 (75.33)	19 (24.67)	0.73
After 1998	37 (72.55)	14 (27.45)	
No data	–	–	
Hospital of origin			
University	45 (77.59)	13 (22.41)	0.23
Río Hortega	55 (74.33)	19 (25.67)	
Penitentiary	3 (100.00)	–	
Others	12 (92.31)	1 (7.69)	
RNA viral load (copies/ml)			
<100,000	42 (62.69)	25 (37.31)	**<0.001**
≥100,000	73 (90.12)	8 (9.88)	
CD4 count (cells/mm^3)			
<200	61 (87.15)	9 (12.85)	0.009
≥200	44 (73.34)	16 (26.66)	
No data	10 (55.56)	8 (44.44)	
HIV infection stage			
AIDS	50 (84.75)	9 (15.25)	0.20
Not AIDS	36 (70.59)	15 (29.41)	
No data	29 (76.32)	9 (23.68)	

*N = 148. **Percentages by lines are shown in parentheses.

In our study, the prevalence of primary resistance to antiretroviral drugs was 20.27%. Mutations in the reverse transcriptase gene were found in eight cases (5.40%; CI 95%, 1.65 to 9.15), but none were of relevance. In the case of LiPA P, the proportion of mutations detected was 16.21% (CI 95%, 10.09 to 22.33), with V82A being the most frequent mutation (15/24, 62.5%). The I84V mutation was also frequent (13/24, 54.16%), and in five samples (20.83%) it was found in association with the V82A mutation. Coincidence of mutations conferring resistance to both drug families was observed in two cases (1.74%) (Table 4).

An independent test was carried out to determine the possibility of a relationship between the patient variables

Table 3. Epidemiological variables studied for protease inhibitors according to overall sample amplification*.

Epidemiological variables	Amplified samples N = 135	Non-amplified samples N = 13	*p*
Sex			
Men	93 (91.18)**	9 (8.82)	0.98
Women	42 (91.30)	4 (8.70)	
Mode of infection			
Parenteral	74 (90.24)	8 (9.76)	0.46
Sexual	20 (86.95)	3 (13.05)	
No data	41 (95.35)	2 (4.65)	
Date of diagnosis			
Before 1998	69 (89.62)	8 (10.38)	0.15
After 1998	47 (90.39)	5 (9.61)	
No data	19 (100.00)	–	
Hospital of origin			
University	54 (93.10)	4 (6.90)	0.69
Río Hortega	66 (89.18)	8 (10.82)	
Penitentiary	3 (100.00)	–	
Others	11 (91.66)	1 (8.34)	
RNA viral load (copies/ml)			
<100,000	59 (88.05)	8 (11.95)	0.22
≥100,000	76 (93.82)	5 (6.18)	
CD4 count (cells/mm^3)			
<200	64 (91.42)	6 (8.58)	0.14
≥200	53 (88.33)	7 (11.64)	
No data	18 (100.00)	–	
HIV infection stage			
AIDS	55 (93.22)	4 (6.78)	0.28
Not AIDS	70 (89.74)	8 (10.26)	
No data	37 (97.37)	1 (2.63)	

*N = 148. **Percentages by lines are shown in parentheses.

recorded and the presence of mutations in both genes studied, but no relationship was found in any of the cases studied (Tables 5 and 6).

DISCUSSION

The epidemiological features of our sample of HIV-infected individuals, *i.e.,* sex, mode of infection and clinical stage, are in general accordance with those described for the rest of Spain, and they are in agreement with data reflected in reports evaluated during the same period of time (21).

Data from the recent inclusion of genotypic resistance testing in routine clinical care show no support for the benefits of amplification previous to the LiPA assay. Inhibitions are said to be an inherent problem in PCR techniques (22), and have therefore represented important difficulties

Table 4. Mutations found conferring resistance to reverse transcriptase inhibitors (RTIs) and protease inhibitors (PIs).

Case	RTI		PI	Antirretrovirals
	NRTI	NNRTI		
1	M41L, K70R, M184V			3TC, ABC, AZT, d4T, ddC, ddI
2	K70R			ABC, AZT, d4T
3	K70R, D69N			ABC, AZT, d4T
4	M184V			3TC, ABC, ddC, ddI
5	M41L, L74V, M184V, T215F/Y		M46I, 154V, V82A	3TC, ABC, AZT, d4T, ddC, ddI, APV, IDV, RTV, SQV
6	M41L, L74V, T215Y		I84V, V82A	ABC, AZT, d4T, ddC, ddI, APV, IDV, RT
7		Y181C		DLV, NVP
8		Y181C		DLV, NVP
9			M461	IDV
10			V82A	IDV, RTV
11			V82A	IDV, RTV
12			V82A	IDV, RTV
13			V82A	IDV, RTV
14			V82A	IDV, RTV
15			V82A	IDV, RTV
16			V82A	IDV, RTV
17			V82A	IDV, RTV
18			V82A	IDV, RTV
19			V82A, 184V	APV, IDV, RTV
20			V82A, 184V	APV, IDV, RTV
21			V82A, 184V	APV, IDV, RTV
22			V82A, 184V	APV, IDV, RTV
23			I84V	APV, IDV, RTV
24			I84V	APV, IDV, RTV
25			I84V	APV, IDV, RTV
26			I84V	APV, IDV, RTV
27			I84V	APV, IDV, RTV
28			I84V	APV, IDV, RTV
29			I84V	AVP, IDV, RTV
30			I84V	AVP, IDV, RTV

3TC: lamivudine; ABC: abacavir, APV: amprenavir; AZT: zidovudine; d4T: estavudine; ddC: zalcitabine; ddI: didanosine; DLV: delavirdine; IDV: indinavir; NVP: nevirapine; RTV: ritonavir; SQV: saquinavir.

in our study, mainly for the reverse trancriptase gene. This phenomenon is influenced by several factors, some of which can be controlled but others cannot. Those factors that can be brought under control as techniques are continuously and systematically used tend to disappear, and the level of

Table 5. Epidemiological variables studied for reverse transcriptase inhibitors in amplified samples*.

Epidemiological variables	Mutated N = 8	Not mutated N = 107	*p*
Sex			
Men	7 (8.53)**	75 (91.47)	0.43
Women	1 (3.03)	32 (96.97)	
Mode of infection			
Parenteral	3 (4.69)	61 (95.31)	0.21
Sexual	–	18 (100.00)	
No data	5 (15.15)	28 (84.85)	
Date of diagnosis			
Before 1998	1 (1.72)	57 (98.18)	0.32
After 1998	2 (5.40)	35 (94.60)	
No data	5 (25.00)	15 (75.00)	
Hospital of origin			
University	3 (6.67)	42 (93.33)	**0.003**
Río Hortega	1 (1.82)	54 (98.18)	
Penitentiary	–	3 (100.00)	
Others	4 (33.33)	8 (66.67)	
RNA viral load (copies/ml)			
<100,000	–	42 (100.00)	0.03
≥100,000	8 (10.95)	65 (89.04)	
CD4 count (cells/mm^3)			
<200	5 (8.20)	56 (91.80)	0.46
≥200	2 (4.54)	42 (95.46)	
No data	1 (10.00)	9 (90.00)	
HIV infection stage			
AIDS	3 (6.00)	47 (94.00)	0.21
Not AIDS	1 (2.78)	35 (97.22)	
No data	4 (13.80)	25 (86.20)	

*N = 115. **Percentages by lines are shown in parentheses.

Table 6. Epidemiological variables studied for protease inhibitors in amplified samples*.

Epidemiological variables	Mutated N = 24	Not mutated N = 111	*p*
Sex			
Men	17 (18.27)**	76 (81.73)	0.82
Women	7 (16.66)	35 (83.34)	
Mode of infection			
Parenteral	17 (22.97)	57 (77.03)	0.19
Sexual	3 (15.00)	17 (85.00)	
No data	4 (9.76)	37 (90.24)	
Date of diagnosis			
Before 1998	16 (23.18)	53 (76.82)	0.16
After 1998	6 (12.76)	41 (87.24)	
No data	2 (10.52)	17 (89.48)	
Hospital of origin			
University	7 (12.96)	47 (87.04)	0.5
Río Hortega	14 (21.21)	52 (78.79)	
Penitentiary	2 (66.67)	1 (33.33)	
Others	1 (8.33)	11 (91.67)	
RNA viral load (copies/ml)			
<100,000	9 (15.25)	50 (84.75)	0.5
≥100,000	15 (19.73)	61 (80.27)	
CD4 count (cells/mm^3)			
<200	10 (15.62)	54 (84.38)	0.74
≥200	11 (20.75)	42 (79.25)	
No data	4 (21.05)	15 (78.95)	
HIV infection stage			
AIDS	10 (18.18)	45 (81.82)	0.68
Not AIDS	9 (20.93)	34 (79.07)	
No data	5 (13.51)	32 (86.49)	

*N = 135. **Percentages by lines are shown in parentheses.

amplification failure reaches an assumable value probably due to not yet established uncontrollable factors. In our series, in an attempt to justify the low successful amplification rate, all recorded variables were contrasted to the fact of whether amplification was achieved or not. Statistical significance was only found for LiPA RT as related to viral load level, and this association was directly proportional, which is logical given that starting from a bigger inoculum would render a greater amplification product.

The presence or absence of mutations in both genes when contrasted to the epidemiological variables recorded showed no statistical significance. A few studies have shown a relationship between the presence of mutations and the therapeutic history or the source of HIV infection (2, 23-25). In our retrospective study, we did not have access to the patients' treatment history, a fact which partially limits our contribution.

Although our overall prevalence of resistance is 20.7%, it is mainly due to primary resistance to protease inhibitors (16.21% *vs.* 5.40% due to reverse transcriptase inhibitors). The prevalence of mutations in the reverse transcriptase gene obtained in our study is lower than that found in most Spanish series (23, 25, 31, 32), with the exception of preliminary data reported for a multicenter study by the Study Group of Primary HIV Resistance conducted by Guerrero *et al.* (26). The preliminary data from this study show a frequency of 2.96%, which is inferior to ours. Results obtained in the international studies that we have reviewed are variable, with overall resistance percentages ranging from the 5% reported by Weinstock *et al.* (27) to the 28.9% obtained by Horban *et al.* (28). The results for the reverse transcriptase gene obtained in the aforementioned series are also variable. For example, while Weinstock *et al.* (27) found a prevalence of 6%, Salomon *et al.* (29) recorded a

prevalence 24% in intravenous drug users. In the recent CATCH study (30), conducted in collaboration with 16 European centers, the resistance rates were 7.1% for nucleoside reverse transcriptase inhibitors and 2.7% for non-nucleoside reverse transcriptase inhibitors.

Our prevalence for protease gene mutations is greater than that reported by Puig *et al.* (31) in the ERASE-2 study. Surprisingly, Guerrero *et al.* (26) in the aforementioned study did not find any primary mutations in this gene. Results from the international studies reviewed are also variable in terms of resistance to protease inhibitors, with a prevalence of 1% in the Weinstock *et al.* (27) study, 2.3% in the CATCH (30) study, 4% in the Yerly *et al.* (2) study and 24% for intravenous drug users in the study by Salomon *et al.* (29).

In our study, we found resistance to reverse transcriptase inhibitors in eight patients. Of these, six cases presented mutations associated with nucleoside reverse transcriptase inhibitors, two of whom also presented mutations associated with protease inhibitors, and two cases presented resistances to non-nucleoside reverse transcriptase inhibitors. The mutations found for reverse transcriptase were: M41L, K70R, D69N, L74V, M184V, T215F/Y and Y181C in those with resistances to non-nucleoside reverse transcriptase inhibitors. These mutations conferred resistance to all the nucleoside reverse transcriptase inhibitors and to the non-nucleoside reverse transcriptase inhibitors delavirdine and nevirapine. Our results conflict with those reported by other Spanish authors (26, 31, 32) because they present a large number of resistances due to the increase in resistances associated with each mutation over the years of our study. In different international studies, the T215Y mutation plays a more important role (2, 27, 29, 33) than in ours, where its prevalence is 1.74%.

Among the mutations detected by LiPA P, the most remarkable observation is the presence of the V82A mutation in 15 out of 24 samples (62.5%) and of the I84V mutation in 13 out of 24 (54.16%). In five cases, patients presented both mutations, which means that they were resistant to amprenavir, indinavir and ritonavir. The mutations M46I and I54V were also found. These results are similar to those obtained by Puig *et al.* (31) in the ERASE-2 study, one of the few Spanish series that includes the analysis of mutations associated with resistance to protease inhibitors. In the study by Guerrero *et al.* (26), all mutations detected in the protease gene were considered to be secondary and were therefore not listed. The V82A mutation is also the most frequently detected mutation in international studies (2, 27, 29), followed by the L90M (2, 23).

The low prevalence of resistance obtained may have been influenced by the long time period between the moment of infection and sample collection, because within this period of time wild-type variants may have emerged as prevailing, rendering false-negative resistance testing results (34, 35). In our study, we used the first sample sent for viral load quantitation after HIV diagnosis, but we do not know the exact lapse of time that occurred between the two events.

Due to the prevalence of mutations found in treatment-naïve patients in our study and in other international ones (28, 30, 36, 37), in our opinion resistance testing should be done prior to initiating an antiretroviral therapy in these patients.

It seems appropriate to promote studies to correlate these findings with those documented by phenotypic assays, as the latter have the advantage of being a direct measure of HIV resistance.

In any case, the report of results on this topic, even if limited by a low number of subjects and by incomplete information in some of them, is nevertheless very important and any initiative on the subject should be welcomed.

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