

Genotypic resistance mutations to antiretroviral drugs in HIV-1 B and non-B subtypes from Cuba

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ABSTRACT

Objectives. To determine the prevalence of drug resistance and to analyze the subtyping in HIV-1 samples from Cuba.

Methods. From an estimated total number of 1 950 HIV-1-infected persons in Cuba, a sample of 103 patients were studied, 76 of whom had received drug treatment for HIV and 27 who had not. The RNA plasma viral load was measured, and automated sequencing was used to assess resistance mutations to reverse transcriptase inhibitors (RTIs) and to protease inhibitors (PIs). Subtyping in the V3 region was performed using heteroduplex mobility assay (HMA). In order to corroborate the HMA results, sequencing of env (C2-V3-C3) was done with one-third of the samples in each of the subtype groups detected by HMA.

Results. Out of the 103 samples, 81 of them (78.6%) were classified as subtype B, 19 (18.5%) as subtype A, and 3 (2.9%) as subtype C. The prevalence of resistance mutations was 26.2% to RTIs, none to PIs alone, and 3.9% to both categories of drugs. The prevalence of resistance to nucleoside RTIs (NRTIs) was 27.6% in treated patients and 7.4% in the untreated patients, and for nonnucleoside RTIs (NNRTIs) it was 5.3% and 0%, respectively. Among treated patients a low frequency (2.6%) of dual resistance to zidovudine (ZDV) plus lamivudine (3TC) and abacavir (ABC) was detected, and multidrug resistance to NRTIs was not found. In relation to PIs together with RTIs, the prevalence of resistance was 5.3% for treated patients and 0% for untreated patients.

Conclusions. Even though Cuba is generally considered an area where subtype B is dominant, we detected a high proportion of non-B subtype viruses. The low prevalence of resistance mutations to RTIs and PIs reflects the delay in introducing these drugs to Cuba. Multidrug resistance to RTIs was not found, so, as of now, the use of these drugs continues to be an option for Cuban patients.

Key words

HIV, AIDS, antiretroviral drugs, drug resistance, Cuba.

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Since 1996, antiretroviral treatment has changed the natural history of HIV type 1 (HIV-1) infection. An increased use of these drugs and the introduction of more potent new molecules has improved the prognosis for HIV-infected patients and has reduced

the mortality rate associated with AIDS.³

³ Palalla F, Moorman A, Chmiel J, Chan C, Holmberg S. Continued low morbidity and mortality among patients with advanced HIV infection and their patterns of HAART usage [conference presentation]. 7th Conference on Retroviruses and Opportunistic Infections, San Francisco, California, United States of America, 30 January–2 February 2000.

In combination with the fact that HIV-1 cannot be eradicated, the problems that are associated with adherence to treatment can result in therapeutic failures that are frequently associated with the selection of viral resistance mutations that are naturally present even in isolates from patients with no prior exposure to the drugs used to treat HIV infection (1-4). Viral recombination can also be an important factor in the propagation of resistance (5-6).

Genotyping methodologies to monitor HIV-1 resistance mutations have been used to study resistant HIV-1 variants in various populations, and this approach's usefulness has even been demonstrated for evaluating mixtures of mutant viruses and wild type viruses (viruses with no primary resistance mutations), according to both published reports (7-9) and unpublished ones.⁴

Most of the antiretroviral resistance studies that have been done so far have been carried out in developed countries, where HIV subtype B is predominant. In Cuba one study (10) has reported that there is a predominance of the B subtype, but more recent research has shown that there is a mosaic of subtypes and recombinant viruses (Cuevas MT, et al. High HIV-1 genetic diversity in Cuba [unpublished manuscript]. 2001). A recent survey of subtypes in Cuba identified the presence of three non-B subtype viruses: A, H, and C (11).

In other countries, studies on genetic resistance have also been carried out on isolates of non-B subtypes, and the implications of this information for vaccine development have been assessed (12, 13).

Because no studies concerning the prevalence of HIV-1 resistance to antiretrovirals had been done in Cuba, we

decided to investigate that issue as well as to look at the subtyping of the Cuban samples. In addition, we explored the importance of both of these types of information for public health on the island.

MATERIAL AND METHODS

This research in Cuba was part of a pilot study of the WHO-UNAIDS (World Health Organization-Joint United Nations Programme on HIV/AIDS) to monitor HIV resistance to antiretroviral drugs in Latin America. The UNAIDS study was carried out in Argentina, Brazil, Cuba, and Venezuela, with the Department of Viral Pathogenesis of the National Center of Fundamental Biology of the Carlos III Institute of Health, of Madrid, Spain, serving as the coordinating center for the study.

In this survey we analyzed Cuban samples, which were collected in 1999. Written informed consent was obtained from the subjects, and a standardized questionnaire was used to obtain demographic, clinical, and risk-factor data on each of those persons.

Cuba has the lowest HIV prevalence in the Americas (0.03%), with an estimated number of infections of 1 950 at the end of 1999 in a total population of 11.2 million persons (14). For our study we selected a sample population of 103 patients. We placed each of the 103 into one of four categories that were related to the antiretroviral regimen that each patient had been receiving at the beginning of his or her treatment.

The first category consisted of patients who had never received any antiretroviral drug. There were 27 persons in this "untreated patients" category, or 26.2% of the 103 subjects in the study.

The second category consisted of 29 patients (28.2% of the 103) who had started their treatment with zidovudine (ZDV). In 3 of these 29 patients, the monotherapy treatment was later changed to bitherapy (two-drug therapy), using zidovudine plus another reverse transcriptase inhibitor (RTI). Nine of these 29 patients were not re-

ceiving any antiretroviral drug when the sample was obtained.

The third category consisted of 28 patients (27.2% of the 103) who had begun treatment with bitherapy. In 24 of these 28 persons the treatment was ZDV plus another RTI. Two of these 24, after one month of bitherapy, had a protease inhibitor (PI) added to their treatment. Three of these 24 patients were not receiving any antiretroviral drug when the sample was obtained. The remaining 4 patients undergoing bitherapy had been taking one RTI plus one PI for 1 to 3 years. In 2 of these 4 cases, after 1 year of treatment, a second RTI was added. All of these 4 patients were undergoing treatment when the samples were collected.

Finally, the fourth category consisted of 19 individuals (18.4% of the 103) who had started their treatment with triple therapy. Of these 19, 17 of them were receiving two RTIs plus one PI, and 1 of them was receiving one RTI plus two PIs. The last of these 19 patients had received triple therapy for only 6 months during 1998 and was not receiving any antiretroviral drug when the samples were collected.

Out of the 103 patients in our study, 42 of them (40.8%) were classified in the "C" AIDS clinical stage, according to the classification system of the Centers for Disease Control and Prevention of the United States of America (15). Sexual contact had been the predominant route of infection for the 103 study patients, with it being heterosexual contact for 42 of the patients (40.8%) and homosexual contact for 60 of them (58.3%). There was 1 case of perinatal transmission.

For the 103 patients, the mean viral load was 80 000 copies/mL (range, < 200 to 1 800 000), and the mean CD4+ count was 356 cells/mL (range, 10 to 1 284). We performed DNA extraction from 10⁶ peripheral blood mononuclear cells using the Casas method (16). Amplification and direct sequencing of *pol* gene coding for reverse transcriptase and protease were performed using the ABI PRISM[®] Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, California, United States) with AmpliTaq[®] DNA poly-

⁴ Little S, Daar E, Keiser P, D'Aquila R, Connick E, Hellmann C, et al. The spectrum and frequency of reduced antiretroviral drug susceptibility with primary HIV infection in the United State [conference presentation]. 7th Conference on Retroviruses and Opportunistic Infections, San Francisco, California, United States of America, 30 January-2 February 2000.

merase FS (Roche Molecular Systems, Alameda, California, United States). The HIV-1 RNA plasma viral load was measured using Nucleic Acid Sequence Based Amplification (NASBA) technology (Organon Teknika, Boxtel, Netherlands). Genotyping of the envelope was performed by heteroduplex mobility assay (HMA), using an HMA subtyping kit (National Institutes of Health, AIDS Research and Reference Reagent Program, Bethesda, Maryland, United States). In order to corroborate the HMA results, sequencing of *env* (C2-V3-C3) was done in one-third of each subtype that had been detected by HMA.

The resistance mutation positions were defined following criteria previously established (4).

An analysis comparing the proportions of B and non-B viruses harboring the various mutations was carried out using the RSIGMA BABEL statistical software package (Horus Hardware, Madrid, Spain).

RESULTS

Resistance mutations to RTIs were detected in 25 of the 76 treated patients and in 2 of the 27 untreated patients. (The absence of primary resistance mutations was defined as "wild type.") The data on the resistance mutations we found are shown in Table 1.

Resistance to nucleoside reverse transcriptase inhibitors (NRTIs) was found in 21 of the 76 treated patients and in 2 of the 27 untreated patients (data not shown). The most common of these mutations, among treated patients, was T215Y/F, which was found in 12 isolates. This was followed by K70R in 9 isolates, M184V in 6, and T69D in 4. All these 4 persons had been receiving ZDV when they were enrolled in the study. T215Y/F mutation was also observed in 1 of the untreated patients, and T69D in another one.

We did not find insertion of two amino acids following RT codon 69, nor did we find the Q151M multidrug resistance mutation to RTIs (data not shown). Resistance mutations associ-

TABLE 1. Number of resistance mutations to antiretroviral drugs found among 103 HIV-1-infected untreated and treated patients in Cuba, according to HIV subtype, 1999

Gene resistance ^a	Untreated patients (27 patients) Subtype			Treated patients (76 patients) Subtype			Total	
	A	C	B	A	C	B	No.	(%)
RT	0	0	2	3	1	21	27	(26.2)
RT plus PR	0	0	0	2	0	2	4	(3.9)
Wild type	5	1	19	9	1	37	72	(69.9)
Total	5	1	21	14	2	60	103	(100.0)

^a RT = reverse transcriptase; PR = protease; "wild type" is used when no primary resistance mutations were found.

ated with nonnucleoside RTIs (NNRTIs) were detected in 4 of the 76 treated patients (5.3%) but not in any of the 27 untreated patients (data not shown). The K103N resistance mutation was found in 1 patient who was receiving delavirdine; Y181C in 2 patients, treated with nevirapine (NVP) plus ZDV in 1 case and with only ZDV in the other case; and Y188C in 1 case without NNRTI treatment but with ZDV. The predominant secondary mutations and polymorphisms among the 76 treated patients were: L214F in 35 cases (46% of the 76 treated patients), R211K in 10 of them (13.2%), M41L in 9 (11.8%), D67N in 8 (10.5%), and S68G in 7 of them (9.2%). The respective frequencies of these mutations among the 27 untreated patients were: 33.3%, 7.4%, 3.7%, 3.7%, and 14.8%.

A low prevalence of resistance mutations to PIs was also found: 4 of the 76 treated patients, with L90M in 3

cases, and G48V in 1 other. These 4 patients were all receiving saquinavir (SQV), and all of them had resistance mutations to RTIs.

Secondary resistance mutations to PIs found were: L63P in 55 cases, M36I in 48, V77I in 20, L10V/I in 9, K20R in 8, and A71V/T in 6 samples.

There were 29 treated patients with resistance mutations to RTIs (Table 2). In these 29, the plasma HIV-1 RNA viral load was higher than 1 000 copies/mL in 21 of them, between 200 and 1 000 copies in 1 of them, and undetectable (< 200 copies/mL) in 7 of them. There were also 47 treated patients in whom a wild-type genotype in the reverse transcriptase (RT) sequence was detected; in 10 of those 47 persons, the viral load was higher than 10⁵ copies/mL.

When we performed the genotyping of the envelope by HMA, out of the 103 samples, 81 of them (78.6%) were clas-

TABLE 2. Resistance mutations to reverse transcriptase inhibitors and to protease inhibitors in HIV-1-infected patients in Cuba, in relation to viral load, 1999

Viral load (copies/mL)	Untreated patients (27 patients)		Treated patients (76 patients)	
	Wild type	Resistance mutations	Wild type	Resistance mutations
< 200	3	0	15	7
200-10 ³	3	0	5	1
> 10 ³ -10 ⁴	6	0	7	7
> 10 ⁴ -10 ⁵	10	1	10	6
> 10 ⁵	3	1	10	8
Total	25	2	47	29

TABLE 3. Resistance mutations in reverse transcriptase in HIV-1 B and non-B subtype viruses among treated and untreated patients, Cuba, 1999

Position	Subtype B			Subtype non-B			Total B and non-B (103 patients)
	Treated (60 patients)	Untreated (21 patients)	Total (81 patients)	Treated (16 patients)	Untreated (6 patients)	Total (22 patients)	
M41L ^a	9	1	10	0	0	0	10
D67N	6	1	7	2	0	2	9
S68G ^b	4	1	5	4	2	6	11
T69D ^c	4	1	5	0	0	0	5
K70R	7	0	7	2	0	2	9
K103N	1	0	1	0	0	0	1
Y181C	2	0	2	0	0	0	2
M184V	4	0	4	2	0	2	6
Y188C	1	0	1	0	0	0	1
L211K	9	1	10	1	1	2	12
L214F	27	8	35	8	1	9	44
T215Y/F	9	1	10	3	0	3	13

^{a,b,c} In these three positions the difference in the proportion of B and non-B viruses harboring the indicated mutation was statistically significant: a: $P < 0.001$; b: $P = 0.03$; c: $P = 0.02$.

sified as subtype B, 19 (18.5%) as subtype A, and 3 (2.9%) as subtype C. The sequencing of *env* that was done in one-third of the samples of each subtype—27 B, 6 A, and 1 C—found results that were the same as those with HMA.

In the group of 19 isolates classified by HMA as subtype A, resistance mutations to both RTIs and PIs were detected in 2 of the treated patients, and to RTIs alone in 3 of the treated patients. In this group of patients infected with subtype A viruses, T215Y/F mutation to ZDV was observed in 3 samples and M184V in another 3 cases, 1 of them in association with T215Y/F mutation.

Resistance mutations were not detected in any of the untreated patients infected with subtype A viruses.

The resistance mutations in protease and in reverse transcriptase that we found in the B and non-B subtype viruses are listed in Tables 3 and 4.

DISCUSSION

This is the first survey providing the prevalence of HIV-1 resistance mutations together with genotyping in Cuba. The low frequency of primary resistance mutations to RTIs and/or PIs that we found among HIV-1-in-

ected patients in Cuba reflects the delay in introducing this antiretroviral therapy to the island.

Overall, the prevalence of RTI and PI resistance mutations are close to the values found in other Latin American countries studied in our laboratory under this UNAIDS Program.⁵ This is the case of Venezuela (17) and of Argentina,⁶ indicating that these drugs have been recently introduced in these two countries. The remaining country included in the UNAIDS study, Brazil, shows higher resistance prevalence values, probably due to an earlier and more extended use of the drugs.

All the 13 patients in whom a T215Y/F mutation was found had been treated with ZDV in monotherapy or dual therapy, the predominant therapeutic schemes in Cuba. This T215Y/F mutation has been reported as indicating a poor patient prognosis (18).

The presence of an M184V mutation, which is related to resistance to 3TC and ABC, was found in 6 of the 76 treated patients, and was not found among the untreated patients. This is indicative of the limited use of these drugs in Cuba.

Various studies carried out in developed countries (19–21) have indicated that there is still a low prevalence of HIV-1 strains with multidrug resistance (MDR) to NRTIs. In this study in Cuba, while MDR to RTIs was not detected, it will be important to maintain the surveillance over MDR emergence.

The NNRTIs have had limited use in the HIV-1-infected population in Cuba, which is reflected in the low frequency (5.3%) of resistance mutations to these drugs, as compared to the 17.2% that we found in a study that we conducted in Spain (21). In this Cuba study there were two cases with Y181C and Y188C mutations associated with the use of NNRTIs, corresponding to two patients who had only received ZDV monotherapy, and thus could be considered resistant strains that had been transmitted. The absence of resistance mutations to ZDV in these two patients with prolonged ZDV monotherapy treatment could be due to the suppression of the T215Y mutation by the presence of Y181C, as previously described (22).

⁵ Contreras G, Cuevas MT, Manjón N, Vázquez de Parga E, Delgado E, Villahermosa ML, et al. WHO-UNAIDS HIV-1 antiretroviral resistance studies [conference presentation]. 4th International Workshop on Drug Resistance and Treatment Strategies, Sitges, Spain, 12–16 June 2000.

⁶ Vázquez de Parga E, Ávila M, Cuevas MT, Salomon H, Thomson MM, Osmanov S, et al. Sequencing analysis of HIV-1 reverse transcriptase and protease genes in a cohort of patients from Argentina. The Fifth European Conference on Experimental AIDS Research, Madrid, Spain, 16–19 June 2000.

TABLE 4. Resistance mutations in protease in HIV-1 B and non-B subtype viruses among treated and untreated patients, Cuba, 1999

Position	Subtype B			Subtype non-B			Total B and non-B (103 patients)
	Treated (60 patients)	Untreated (21 patients)	Total (81 patients)	Treated (16 patients)	Untreated (6 patients)	Total (22 patients)	
L10I/V	4	2	6	3	0	3	9
K20R	4	1	5	3	0	3	8
M36I	29	6	35	12	1	13	48
G48V	0	0	0	1	0	1	1
L63P	35	8	43	8	4	12	55
A71V/T	4	0	4	1	1	2	6
V77I ^a	15	4	19	1	0	1	20
L90M	2	0	2	1	0	1	3

^a In this position the difference in the proportion of B and non-B viruses harboring the indicated mutation was statistically significant ($P < 0.01$).

Resistance mutations to RTIs were found in 2 of the 27 untreated patients, or 7.4% of them. This percentage is noticeably lower than the 22% to 29% that has been found in studies with untreated patients in some other countries (23–25).

Resistance mutations to PIs were only found in 4 of the 76 treated patients (5.3%); in all those cases it was in association with the use of the corresponding inhibitor. This frequency is still low for these drugs in comparison to that in other studies from some other countries. For example, in our study in Spain (21) we found an overall level of 25.1%, similar to results from other developed countries, according to at least one published study (26) and one unpublished one.⁴ Nevertheless, the 5.3% level in Cuba is in line with other countries where this type of treatment has been recently introduced, such as Côte d'Ivoire, where a prevalence of 6% was recently reported (27). That same Côte d'Ivoire study reported that resistance among patients infected with non-B subtypes is conferred by mutations similar to those documented for subtype B infections. We also saw the same profile for primary resistance mutations in subtypes A, B, and C in Cuba (Table 1).

With regard to the viral load that we saw in Cuba, it is interesting to note the high percentage of cases presenting undetectable levels of RNA in plasma associated with the presence of resistance mutations. This may indicate that the samples were taken be-

fore the viral load had increased. This also suggests the usefulness of performing resistance studies in the follow-up with treated patients, regardless of their low viral load. There is a simple new procedure for nucleic acid extraction and amplification that permits sequencing studies of resistance mutations in patients with an undetectable viral load, thus facilitating treatment decisions (28).

Also in this Cuban study, we found a group of 10 patients in whom the viral load was higher than 10^5 copies/mL but who did not have mutations associated with resistance (Table 2). This may be indicative of a lack of adherence to treatment during the early treatment phase.

An R211K/L214F natural polymorphism in the RT gene was found in 21 of the 76 treated patients and in 9 of the 27 untreated patients. In addition, a polymorphism in the protease gene at positions 63 was found in 55 out of the total of 103 patients and at position 36 in 48 of the 103.

In this paper we have reported on the first study of the prevalence of antiretroviral drug-associated resistance mutations in Cuba. It is important to note the absence of MDR to NRTIs, and the low prevalence of resistance to RTIs and to PIs, thus allowing the continued use of these drugs for the HIV-1-infected population in Cuba. Our findings may also provide valuable epidemiological information to consider for vaccine design.

The detection of several non-B subtype viruses circulating in Cuba supports the relevance of phylogenetic surveillance studies, given the possibility of spread of these genetic forms and the potential emergence of recombinant viruses.⁷

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⁷ Cuevas MT, Thomson MM, Villahermosa ML, Ruibal-Brunet JJ, Diaz-Torres H, Vázquez de Parga E, et al. High proportion of non-B subtype and intersubtype recombinant viruses among HIV-1 individuals in Cuba [conference presentation]. 101st General Meeting of the American Society for Microbiology, Orlando, Florida, 20–24 May 2001.

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Mutaciones de resistencia genotípica a los fármacos antirretrovíricos en los subtipos B y no B del VIH-1 en Cuba

RESUMEN

Objetivos. Determinar la prevalencia de la resistencia a los fármacos y analizar la presencia de mutaciones de resistencia genotípica en los subtipos B y no B del VIH-1 en Cuba.

Métodos. Entre un total de 1 950 personas que se estima que están infectadas por el VIH-1 en Cuba, se estudió una muestra de 103 pacientes, de los cuales 76 habían recibido antirretrovíricos y 27 no. Se determinó la carga de ARN vírico en el plasma y se utilizó la secuenciación automatizada para detectar mutaciones de resistencia a los inhibidores de la transcriptasa inversa (ITI) y de la proteasa (IP). También se procedió a la subtipificación de la región V3 con prueba de movilidad de heteroduplex (HMA). Para confirmar los resultados de esta prueba se secuenció el gen *env* (C2-V3-C3) en un tercio de las muestras de cada uno de los subtipos detectados por HMA.

Resultados. De las 103 muestras, 81 (78,6%) fueron clasificadas como pertenecientes al subtipo B, 19 (18,5%) al A y 3 (2,9%) al C. La prevalencia de mutaciones de resistencia fue del 26,2% para los ITI y 3,9% para los ITI más IP. Para los ITI nucleosídicos fue del 27,6% en los pacientes tratados y del 7,4% en los no tratados; para los ITI no nucleosídicos, las cifras correspondientes fueron del 5,3% y 0%, respectivamente. En los pacientes tratados se detectó una baja frecuencia (2,6%) de resistencia a la zidovudina más lamivudina y abacavir, y no se encontró multirresistencia a los ITI nucleosídicos. Para las combinaciones de IP e ITI, la prevalencia de la resistencia fue del 5,3% en los pacientes tratados y del 0% en los no tratados.

Conclusiones. Aunque generalmente se considera que en Cuba predomina el subtipo B, en este estudio se detectó una alta proporción de virus del subtipo no B. La baja prevalencia de mutaciones de resistencia a los IP e ITI refleja la introducción más tardía de estos fármacos en el país. Como no se observó multirresistencia a los ITI, el empleo de estos fármacos sigue siendo una opción válida para los pacientes cubanos.

Epidemiology may be considered the study of the circumstances under which a disease arises and flourishes in its natural surroundings. It constitutes an approach to the unravelling of nature's secrets pertaining to the development of a disease through the identification of factors which are directly or indirectly associated with the disease. It involves the study of the distribution and determinants of disease prevalence and incidence. This requires analysis and interpretation of distribution patterns of disease in terms of possible causal factors. However, in the final analysis, the epidemiologic study suggests guilt by association. Proof of causal association between a factor and a disease is seldom provided by an epidemiologic study. More often, important clues to pathogenesis of disease are uncovered which require more fundamental laboratory or clinical research to demonstrate a cause and effect relationship.

[La epidemiología puede considerarse como el estudio de las circunstancias en las cuales surge y se desarrolla una enfermedad en su ambiente natural. Constituye un enfoque para descubrir los secretos de la naturaleza con referencia a la evolución de una enfermedad, mediante la identificación de factores asociados con ella directa o indirectamente. Incluye el estudio de la distribución y determinantes de las prevalencias e incidencia de las enfermedades; esto implica el análisis e interpretación de los patrones de distribución de las enfermedades en función de los posibles factores causales. Sin embargo, en última instancia, el estudio epidemiológico apunta a la causalidad por asociación. Rara vez un estudio epidemiológico aportará pruebas concluyentes de una asociación causal entre un factor y una enfermedad; con más frecuencia descubre indicios de la patogenia de la enfermedad, los cuales necesitan otras investigaciones más exhaustivas, clínicas o de laboratorio, para demostrar una relación entre causa y efecto.]

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"An approach to longitudinal studies in a community: the Framingham study"
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