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Increase of Recombinant forms CRF20, 23, 24_BG and Several URF of HIV-1 among Newly Diagnosed Cuban Patients: 2013-2014

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Abstract: *The objective of this study was characterize the genetic diversity of HIV-1 in Cuban patients diagnosed during the period 2013-2014. A total of 189 patient samples from all provinces of the country, diagnosed withHIV-1 infection, were studied during the period from April 2013 to April 2014. Viral RNA was isolated for further amplification of the pol gen of HIV-1. The purified products were sequenced and the viral subtype was determined. The recombination was evaluated by boots canning. The relationship between viral variants and epidemiological, immunological and virological variables was determined. 27.5% of the samples were classified as CRF20, 23, 24_BG, followed by subtype B (23.8%) and the presence of other recombinants as CRF19_cpx and CRF18_cpx. 11.1% of the samples were classified as URF and the most frequent combination detected was between CRF18_cpx and CRF19_cpx. The CRF19_cpx variant was detected more frequently in Havana. There was no association between viral variants and sexual orientation. The study confirms the high genetic diversity of HIV-1 in the Cuban population diagnosed between 2013 and 2014 and the predominance, for the first time of CRF20, 23, 24_BG over subtype B in the sample studied, which supports the importance of maintaining close epidemiological surveillance*

Keywords: *molecular epidemiology, HIV-1, subtype, circulating recombinant forms, unique recombinant forms, Cuba*

Abbreviations

Circulating recombinant forms: CRF; Unique recombinant forms: URF ; Men having sex with men: MSM

1. INTRODUCTION

The origin of Human Immunodeficiency Virus type 1 (HIV-1) has been associated with several events of zoonotic transmission of the Simian Immunodeficiency Virus (SIV) of chimpanzees to humans in Central and West Africa, occurring at the beginning of the century XX (1). From the phylogenetic point of view, HIV-1 is classified into four groups: M, N, O and P (2). Group M, responsible for the majority of HIV-1 infections in the world, initially spread throughout Africa (3) and in response to various genetic forces was diversified into different subtypes, sub-subtypes, more than 70 circulating recombinant forms (CRF) and multiple unique recombinant forms (URF) (4). It has been suggested that the presence of circulating recombinant types, subtypes and forms of HIV in a specific geographical area, leads to generations of recombinant forms that emerge in individuals with dual or multiple infections (5).

The HIV epidemic in Cuba began in 1986 and since its inception, the strategies implemented by the Ministry of Public Health have made possible to detect the circulation of HIV-1 and HIV-2 in the HIV-positive population (6). The first molecular epidemiological studies in Cuba described the presence of several HIV-1

subtypes, with a higher frequency in subtype B and the presence of non-B subtypes introduced from the African continent (7). Subsequent studies detected mosaics of genetic variants in the HIV-1 genome in samples from Cuban patients and described the first recombinants in the epidemic context (8-9). The introduction of antiretroviral therapy in Cuba in 2001 led to the monitoring of HIV-1 resistance to antiretrovirals in treated and untreated patients, and in addition to providing the resistance profile to the different antiretrovirals, allowed us to know the Viral subtype that was infecting the individuals (10-12). One of the objectives of epidemiological surveillance is to know the circulating genetic variants of HIV-1 in the newly diagnosed population, due to the possible implications of genetic variability in diagnosis, transmissibility, clinical progression and antiretroviral therapy. These elements motivated the objective of the present study: To characterize the genetic diversity of HIV-1 in Cuban individuals newly diagnosed during the period 2013-2014.

2. Materials And Methods

2.1. Study Population

A cross-sectional analytical study was performed on a representative sample of all patients diagnosed withHIV-1 infection during the period from April 2013 to April 2014. A randomized stratified probabilistic sampling was performed, with a prevalence of 25 % and a 5% of error of the sample. All provinces of the country were represented and 189 patients with HIV-1 viral load values over 1000 HIV-1 RNA copies / mL were studied.

2.2. Ethical Considerations

Ethical procedures were carried out in accordance with the requirements or standards of the Ministry of Public Health of the Republic of Cuba and the Ministry of Science, Technology and Environment (CITMA), which contemplates the principles laid down in the Declaration of Helsinki for medical research in Human beings and the LISIDA and the IPK Ethics Committees. Prior to sample collection, informed consent was obtained from each patient who participated in the study.

2.3. CD4 + Cell Count and Viral Load

The CD4 + cell count was determined using Becton Dickinson technology (Biosciences, USA). Plasma viral load was determined using COBAS Ampliprep / COBAS Taqman HIV-1 Test (Roche Diagnostics GmbH, Mannheim, Germany).

2.4. Determination of HIV-1 Subtype and Recombination Analysis

Extraction of viral RNA, amplification of the protease and RT regions of the HIV-1 pol gene by Reverse Transcriptase-nested PCR and subsequent nucleotide sequencing were performed according to the procedures described by Alemán et al, 2015 (13). The HIV-1 subtype was determined by the REGA HIV-1 subtypingtool v 3.0 program (http://dbpartners.stanford.edu:8080/RegaSubtyping/stanford-hiv/typingtool/) and was confirmed by phylogenetic analysis. To corroborate the results obtained, the nucleotide sequences of the 189 samples studied were automatically aligned with reference sequences obtained from the HIV Database of the National Laboratory of Los Alamos (www.hiv-web.lanl.gov) using the tool Clustal W of the MEGA program package version 6.0 (14). For the determination of possible recombinants and breakpoints, boot scaning was performed and the points of similarity between sequences were determined using the Simplot v 3.5 (15) and RDP4 (16) programs. Aditionally, phylogenetic tree was constructed using the neighbor joining method and the genetic distance was estimated according to the two parameters of Kimura. Bootstrap values were calculated based on 1000 replicates. The tree was visualized using the program Fig Tree v 1.1.2 (http://tree.bio.ed.ac.uk/software/figtree/).

2.5. Statistic Analysis

Mean and standard deviation (SD), median and interquartile range (IQR), and frequencies (%) were used to describe patient characteristics. The X2 test and the Fisher exact test were used to compare categorical variables and continuous variables. The non parametric Kruskal Wallis test was used for the comparison of medians of categorical variables. The variables that presented $p<0.05$ were considered in a multivariate logistic regression model after evaluating the multicollinearity of the variance inflation factors. The selected variables were included in a multiple logistic regression model with a significance level of ($p < 0.05$). Odds ratios (ORs) were estimated with a 95% confidence interval (CI). A value of $p < 0.05$ was considered statistically significant. All statistical

analyzes were performed using the SPSS 18 statistical package (SPSS Inc., Chicago, IL).

3. RESULTS

3.1. Characteristics of Patients Included in the Study

The present study included 189 patients diagnosed with HIV-1 in the period between April 2013 and April 2014. The mean age of the patients studied was 33 years with values ranging from 17 to 74 years? 80.9% of the patients were male and 80.3% were in the risk behavior group of men who have sex with other men (MSM). The province of Havana was the region of the country that provided the largest number of patients for the study (38.6%). The median viral load was 58,000 copies of RNA / mL and the CD4 + cell count was 371 cells / mm^3 (Table 1).

Table1: *Clinical and Epidemiological Character - istics of Patients Included in the Study*

Characteristics	Total	%
Age at sampling (years, mean (min-max))	33.5	17-74
Sex (n (%))		
Male	153	80.9
Female	36	19.1
Sexual behaviour (n (%))		
MSM	123	80.3 (123/153)
HT	66	34.9
Region (n (%))		
Havana City	73	38,6
Western region	28	14,8
Central region	32	16,9
Eastern region	56	29,6
CD4 cell count at sampling [c] (cells/mm^3, median (IQR))	371 (270-573)	
HIV-1 viral load at sampling [d] (RNA copies/ml, median (IQR))	58 000 (16 700-127 000)	

MSM: men having sex with men, HT: heterosexual, IQR: interquantil range

3.2. Distribution of Subtypes

In the present study the viral variants CRF20, 23, 24_BG (27.5%) were the most frequently observed, followed by subtype B (23.8%). However, other subtypes were also detected: G (4.2%), C (2.1%), H (1.05%) and circulating recombinant forms such as CRF19_cpx (19.5%), CRF18_cpx (9.5%) and CRF01_AE (0.5 %). In the phylogenetic tree the clusteringof these viruses with their respective reference sequences is observed In contrast, 11.1% of the samples were not grouped with reference sequences according to phylogenetic criteria and they were classified as URF(Fig. 1)..

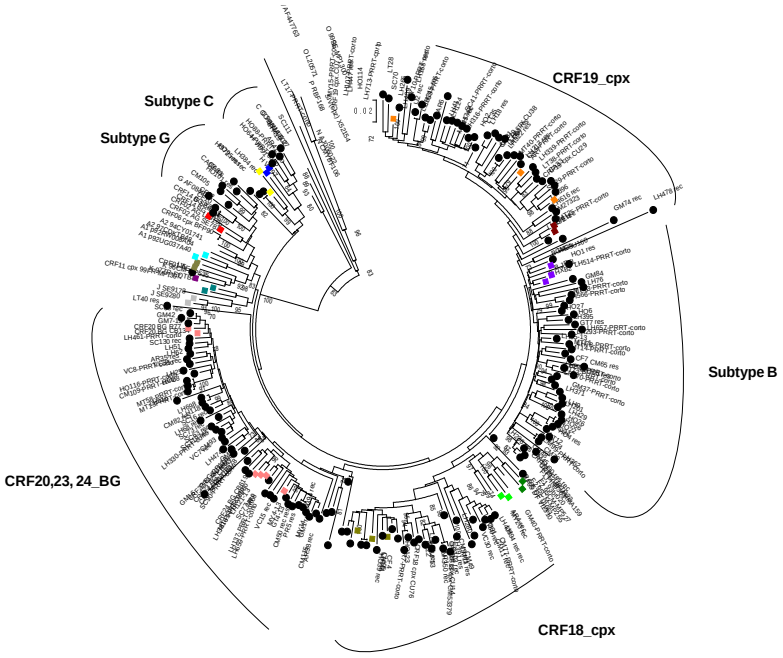
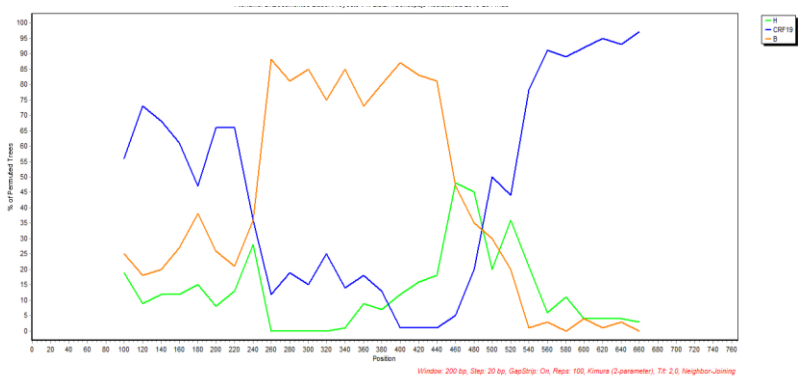
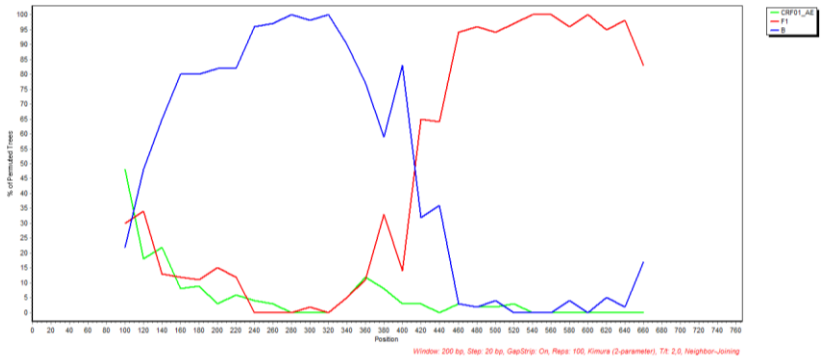


Figure1. *Neighbor-Joining Tree Using Pol Sequences from the 189HIV-1-Infected Cuban Patients Diagnosed During 2013 and 2014. The Tree was Constructed Using Kimura's Two Parameter Distances. Bootstrap Values ≥ 70 % are Shown. Subtype and CRF Reference Viruses are Denoted by the Corresponding Subtype or CRF Names Followed by the Name of the Isolates and Colors.*

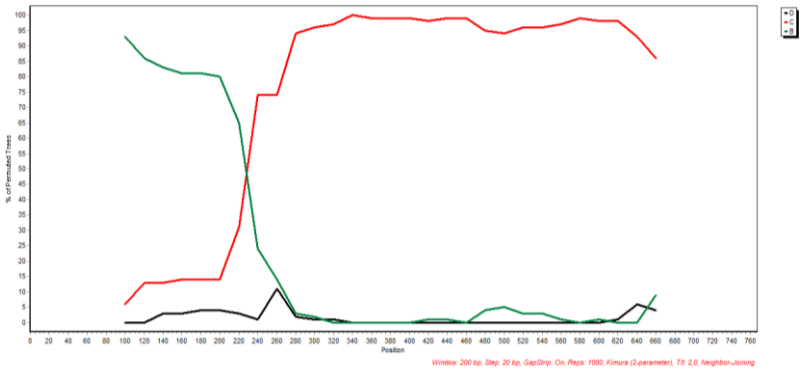
The recombination analysis detected as more frequent URF combinations: CRF19_cpx/B, B/F1, B/C and CRF18_cpx/CRF19_cpx, as illustrated in Figure 2.



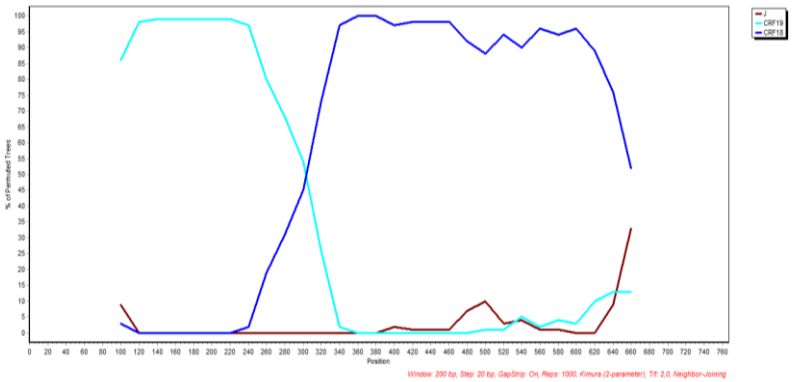
2(a)



2(b)



2(c)



2(d)

Figure.2: *Recombinats analysis by boots canning. a) CRF19_cpx/B, b) B/F1, c) B/C, d) CRF19_cpx/ CRF18_ cpx.*

3.3. Relationship between Detected Viral Variants and Epidemiological, Immunological and Virological Variables

The MSM population of the present study was more frequently infected with subtype B (31/123, 25.2%), followed by CRF20, 23, 24_BG (28/123, 22.8%), CRF19_cpx (23 / 123, 18.6%), URF (15/123, 12.1%), and CRF18_cpx (13/123, 10.5%). However, the same proportion of patients infected with CRF20, 23, 24_BG, in both MSM and HT (53.8% (28/52) and 41.6% (24/52), respectively) is observed. Subtype B was associated with the age range of 46-61 years (p = 0.019, OR = 2844 (1.262-6.406)), and the CRF20, 23, 24_BG variants with the ranges of 15 to 30 years (p = 0.035, OR = 2,053 (1,069-3,944)) (Table 2). Linear regression analysis showed that CRF19_cpx was two times more frequently detected amongpatients living in Havana (p = 0.039, OR = 2.198 (1.062-4.549)) while it was three times more likely to detect CRF18_cpx in patients residing in the central region than in the rest of the country (p = 0.017, OR = 3,716 (1,316-10,496)). URF was associated with CD4 + cell counts below 200 cells (p = 0.050, OR = 3.152 (1.150-8.640)) (Table 2). No significant differences were found when comparing medians of viral load values between the different subtypes using Kruskal Wallis non-parametric test (results not shown).

Table2: *Relationship among Viral Variants and Epidemiological, Clinical, Immunological and Virological Variables*

	Subtype HIV-1 (n)								
	B (45)	**CRF20, 23, 24_BG (52)**	**CRF19_cpx (37)**	**CRF18_cpx (18)**	**URF (21)**	**G (8)**	**C (4)**	**H (2)**	**CRF01_AE (1)**
Age, years (n)									
15-30 (92)	18	**32*** ***(p=0.035)***	16	7	12	4	2	0	1
31-45 (64)	14	16	13	6	7	4	2	2	0
46-61 (31)	**13*** ***(p=0.019)***	3	7	5	2	0	0	0	0
62-77 (2)	0	1	1	0	0	0	0	0	0
Sexual behaviour (n)									
MSM (123)	31	28	23	13	15	7	3	2	0
HT (66)	14	24	14	5	6	1	1	0	1
CD4 count, cél/mmm^3 (n) [a]									
<200 (30)	7	6	7	2	**7*** ***(p=0.05)***				
200-350 (50)	10	14	11	7	6				
351-500 (40)	8	14	8	1	3				
>500 (53)	14	15	9	6	2				
Region (n)									
Havana City (73)	21	16	**20*** ***(p=0.039)***	7	8	0	1	0	0
Western region (28)	4	9	6	1	6	0	1	0	1
Central región(32)	6	9	3	**7*** ***(p=0.017)***	1	**5*** ***(p=0.004)***	0	0	0
Eastern region (56)	14	18	6	3	6	3	2	2	0

MSM: men having sex with men, HT: heterosexual, CRF: Circulating recombinants forms, URF: unique recombinants forms,

a-Data were available for 173 patients.

4. DISCUSSION

Since the detection of the first case of HIV in the 1980s, the genetic diversity of this retrovirus has played a fundamental role in its dissemination, consequently, epidemiological surveillance of circulating viral variants is a fundamental premise for better management and control of the epidemic. At present, the scientific community has givenspecial attention to the non-B subtypes, due to the increase of these variants in areas such as North America and Europe, where previously subtype B prevailed. This could have implications in the response to antiretroviral therapy, progression of the disease and the design of vaccines (4). Although Cuba has a low prevalence of HIV infection (0.19%), several studies have described a high genetic diversity of HIV-1, rendering into a wide circulation of subtypes, sub-subtypes, CRFs and multiple URFs (7 -12), comparable to the variability reported in some African countries (4). Contrary to what has been described so far in the Cuban epidemic, where subtype B has been the most frequent genetic form, in the present study CRF20, 23, 24_BG were the predominant genetic variants (28%) in the studied population. Previous studies described a decrease in subtype B with an increase in non-B subtypes and several recombinants in samples collected in 2003 (9). The increase in the frequency of detection of CRF20, 23, 24_BG in several studies carried out in the newly diagnosed population from 2009 to the present, reinforces the fact of the displacement of subtype B by these recombinant forms (11, 12). Similar events are reported in some localities of Brazil with the recombinant BF and BC (17), as well as in Russia with the recombinant AB (18) and in China with the recombinant BC (19, 20), mainly in intravenous drug users IDU). It is not known exactly whether the rapid expansion of BG recombinants is due to viral biological characteristics or increased risk sexual behaviors, or both, but such diversity reflects the dynamic nature of the genetics of the HIV-1 epidemic, through which new genetic forms, introduced or generated by recombination, can rapidly expand into an established epidemic (9).

Interestingly, 11.1% of the viral infections of the present study were caused by URFs. These recombinants are very common in regions where multiple subtypes circulate, such as sub-Saharan Africa; But recentlyit has been detected relatively frequently in developed countries (21). Despite the spread of recombinants in the HIV-1 pandemic, the time of origin is not well known. It has been suggested that early recombination was a common phenomenon in the historical evolution of HIV-1. However, the detection of viral URFs could increase in the global epidemic context if more regions of the viral genome were analyzed. Recombination seems to be very important in the evolution of HIV-1, since it can provide a biological advantage over parental viruses, facilitate biological adaptation and increase evolutionary fitness or viral capability. In addition, it has complex consequences on the estimation of the time of divergence, due to an apparent increase in the ranges of variation between the nucleotide sites and because it reduces the genetic distance between the sequences (21). However, the prevalence of URF at the global level (estimated at around 20%) is still underestimated. Genetic complexity is not always detected, and this is mainly due to the subtyping of only one genetic region and not the complete viral genome (4). The MSM population of the present study was more frequently infected insubtype B (25.2%). Subtype B has been associated with MSM in the Cuban epidemic and worldwide (9, 23, 24). Statistical analysis of the present study showed no association between sexual orientation with this subtype, nor with the rest of the genetic forms detected (Table 2), a result that differs from that described by previous studies conducted in Cuban patients infected with HIV-1 (7, 9).

The most frequent detection of CRF19_cpx in Havana and CRF18_cpx in the central region reflect the dynamics of this retrovirus in the Cuban epidemic context. In a study carried out by Perez et al., 2007, patients from the central region of the country were associated with CRF19_cpx, while URF CRF18_cpx, B / CRF18_cpx and subtype H were associated with patients residing in the eastern region (25). The discrepancy between these results could be explained by the time elapsed between the two studies, the diversity of genetic forms that circulate in the Cuban epidemic, which, together with the migration of infected individuals from one region to another, has made possible an increase in some viral variants in regions where their proportion was lower.

The detection of URF was associated with CD4 + cell counts below 200 cells; interestingly10 of

these viruses contained the combinations ofCRF19_cpx / CRF18_cpx. It has been argued that the genetic diversity of HIV-1 might affect the rate of progression to AIDS. However, several authors associate subtypes C, and D with a rapid progression of the disease and categorize themas more aggressive than the G, CRF01_AE variants,CRF02_AG and A, being subtype A considered as the less aggressive HIV-1 subtype. One previous study showed the association of CRF19_cpx with the rapid progression to AIDS in Cuban patients (27). Therefore, continuous virological, immunol - ogical and clinical monitoring of individuals infected with this recombinant or presenting this variant in the genome architecture viral is recommended.

5. CONCLUSIONS

In conclusion, this study confirms the high genetic variability of HIV-1 in a group of patient samples diagnosed between April 2013 and April 2014. Analysis of the pol gene in our study allowed to verify the frequency and diversity of recombinant forms, and the displacement of subtype B by CRF20, 23, 24_BG as a predominant variant in the studied population, in comparison to previous studies carried out in Cuba. The results obtained in addition to updating the epidemiological situation to the surveillance program of the Cuban Ministry of Public Health, would reinforce studies on the implications of the genetic diversity of HIV in the epidemic, pathogenesis, vaccine development and the design of new therapeutic strategies.

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