

High HIV-1 genetic diversity in Cuba

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Background: HIV-1 subtype B is largely predominant in the Caribbean, although other subtypes have been recently identified in Cuba.

Objectives: To examine HIV-1 genetic diversity in Cuba.

Methods: The study enrolled 105 HIV-1-infected individuals, 93 of whom had acquired the infection in Cuba. DNA from peripheral blood mononuclear cells was used for polymerase chain reaction amplification and sequencing of *pol* (protease–reverse transcriptase) and *env* (V3 region) segments. Phylogenetic trees were constructed using the neighbour-joining method. Intersubtype recombination was analysed by bootscanning.

Results: Of the samples, 50 (48%) were of subtype B and 55 (52%) of diverse non-B subtypes and recombinant forms. Among non-B viruses, 12 were non-recombinant, belonging to six subtypes (C, D, F1, G, H and J), the most frequent of which was subtype G (n = 5). The remaining 43 (78%) non-B viruses were recombinant, with 14 different forms, the two most common of which were D^{pol}/A^{env} (n = 21) and U(unknown)^{pol}/H^{env} (n = 7), which grouped in respective monophyletic clusters. Twelve recombinant viruses were mosaics of different genetic forms circulating in Cuba. Overall, 21 genetic forms were identified, with all known HIV-1 group M subtypes present in Cuba, either as non-recombinant viruses or as segments of recombinant forms. Non-B subtype viruses were predominant among heterosexuals (72%) and B subtype viruses among homo- or bisexuals (63%).

Conclusion: An extraordinarily high diversity of HIV-1 genetic forms, unparalleled in the Americas and comparable to that found in Central Africa, is present in Cuba.

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AIDS 2002, **16**:1643–1653

Keywords: HIV-1, Cuba, subtypes, recombinant forms, molecular epidemiology

Introduction

In the Americas, subtype B is largely predominant among HIV-1 genetic forms, although in Brazil, Argentina and Uruguay, substantial proportions of infections are caused by F subtype or BF recombinant

viruses [1–4]. Apart from these three countries, infections with non-B genetic forms are unusual; however, recently, it was reported that *env* subtypes A, C and H had been identified in 5 of 11 samples in Cuba [5]. The identification of diverse non-B subtypes in Cuba is not unexpected, considering that large numbers of

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Received: 10 August 2002; revised: 1 October 2001; accepted: 21 March 2002.

Cuban military and civilian personnel had been stationed in the 1970s and 1980s in Angola [6], a country neighbouring the Democratic Republic of Congo (DRC), where the highest group M diversity is found [7], and that many of the early cases of HIV infection in Cuba were detected among these individuals [8]. The presence of numerous Cuban aid workers in several sub-Saharan African countries might also have contributed to the introduction of diverse HIV genetic forms of African origin [8,9]. In a large-scale survey carried out between 1986 and 1989, in which more than 5 million individuals were tested, 122 (28%) of 434 HIV-seropositive infections detected were directly attributable to the presence of Cuban military and civilian personnel in Africa [10].

Cuba has the lowest HIV prevalence in the Americas, with an estimated number of infections of 1950 at the end of 1999 for a population of 11.2 million inhabitants [11]. This corresponds to a 0.03% prevalence in adults, which contrasts with a prevalence of 2% in the Caribbean area considered globally (ranging from 0.7% in Jamaica to 5.2% in Haiti), which is only second to that of sub-Saharan Africa [12]. However, a sharp increase in HIV infections in Cuba has recently been reported [13]. Transmission in most cases is by either hetero- or homosexual exposure [11]. Earlier in the epidemic, the controversial policy of mandatory quarantine of all HIV-infected individuals in sanatoria was a matter of lively debate in medical journals [10,14,15]. This policy was modified in 1993, when staying in a sanatorium became voluntary.

To examine the distribution of HIV genetic forms, as well as the prevalence of drug resistance-associated mutations in Cuba [16], a study was conducted under the provisions of UNAIDS, in which segments of *pol* (protease-reverse transcriptase) and *env* of 105 HIV-infected individuals were analysed. The results revealed the presence of a high proportion of non-B subtype viruses, and a high diversity of genetic forms, unprecedented in the Americas and comparable to that of Central African countries.

Methods

Study subjects

The number of HIV-1-infected individuals enrolled was 105, of whom 74 were men, 30 were women and one was a perinatally infected child. Risk categories were: 60 homo- or bisexual, 43 heterosexual, one accidental exposure and one perinatal transmission. Places of residence were 54 Havana City, 21 Villa Clara province, 19 Matanzas province and 10 were distributed among six other provinces (Granma, Camagüey, Pinar del Río, Ciego de Ávila, Cienfuegos and

Sancti Spiritus). 93 individuals were reported to having contracted HIV in Cuba, two in North America (one in the United States and one in Canada) and 10 in Africa (four in DRC, three in Angola, two in Ethiopia and one in Zambia). All samples were collected in 1999.

Sample preparation, amplification and sequencing

Peripheral blood mononuclear cells were separated by centrifugation on Ficoll-Hypaque gradient. Samples were prepared for polymerase chain reaction (PCR) by cell lysis and digestion with proteinase K, as described [17]. A lysate of 2×10^5 cells was used for each PCR. Amplification of *pol* and *env* (C2-V3-C3) segments was done by nested PCR, using primers and thermocycling profiles as described [18,19]. Amplification was checked by electrophoresis in an agarose gel with ethidium bromide staining. After enzymatic removal of dNTP and primers remaining in solution [20], purified PCR products were directly sequenced using ABI Prism BigDye Terminator Cycle Sequencing kit and ABI 377 sequencer (Applied Biosystems, Foster City, California, USA). Electrophoretogram sequences were corrected with BioEdit (Tom Hall, <http://www.mbio.ncsu.edu/BioEdit/bioedit.html>).

Analysis of sequences

Sequences were aligned with reference sequences using Clustal X [21], with manual adjustments, considering protein sequences. Neighbour-joining phylogenetic trees, based on Kimura's two-parameter distances, with consistency of tree topologies assessed by bootstrapping, were constructed using Clustal X and viewed with Treeview (Rod Page, <http://taxonomy.zoology.gla.ac.uk/rod/treeview.html>). Intersubtype recombination in *pol* was analysed by bootscanning using Simplot 2.5 (Stuart Ray, <http://www.med.jhu.edu/deptmed/sray/download/>). Bootstrap support for sequence clusters of 70% or higher was considered significant [22]. Homologies with GenBank sequences were searched using BLAST Search (NCBI, <http://www.ncbi.nlm.nih.gov/BLAST>). To exclude the possibility of PCR-mediated artefacts, intersubtype breakpoints were confirmed in duplicate PCR carried out separately.

Statistical analysis

Significance of differences in prevalences of B and non-B subtype infections among groups with different epidemiologic characteristics was analysed with the χ^2 test with Yate's correction using Sigma software.

Results

Phylogenetic analysis of *pol* sequences

In the phylogenetic neighbour-joining tree of *pol*

sequences (Fig. 1a,c), 50 sequences grouped with subtype B reference viruses and 55 were of non-B subtypes. Of these, 28 grouped with subtype D, seven with subtype G, three with subtype C, one each with subtypes F1, H and J and 14 branched apart from subtype reference sequences. Of the last 14 samples, nine formed a monophyletic cluster supported by 100% bootstrap value. Bootscan analysis using Simplot software (Fig. 2a) indicated that these nine sequences did

not appear to be recombinants of known subtypes, although phylogenetic trees of partial *pol* segments suggested that different segments might be distantly related to G and F subtype viruses. Consequently, *pol* sequences of this cluster are referred to as U, meaning unclassified or unknown subtype. The remaining five sequences not grouping with subtype references were intersubtype recombinant (see below). *pol* sequences of Cuba branching with subtype D, except CU20, clus-

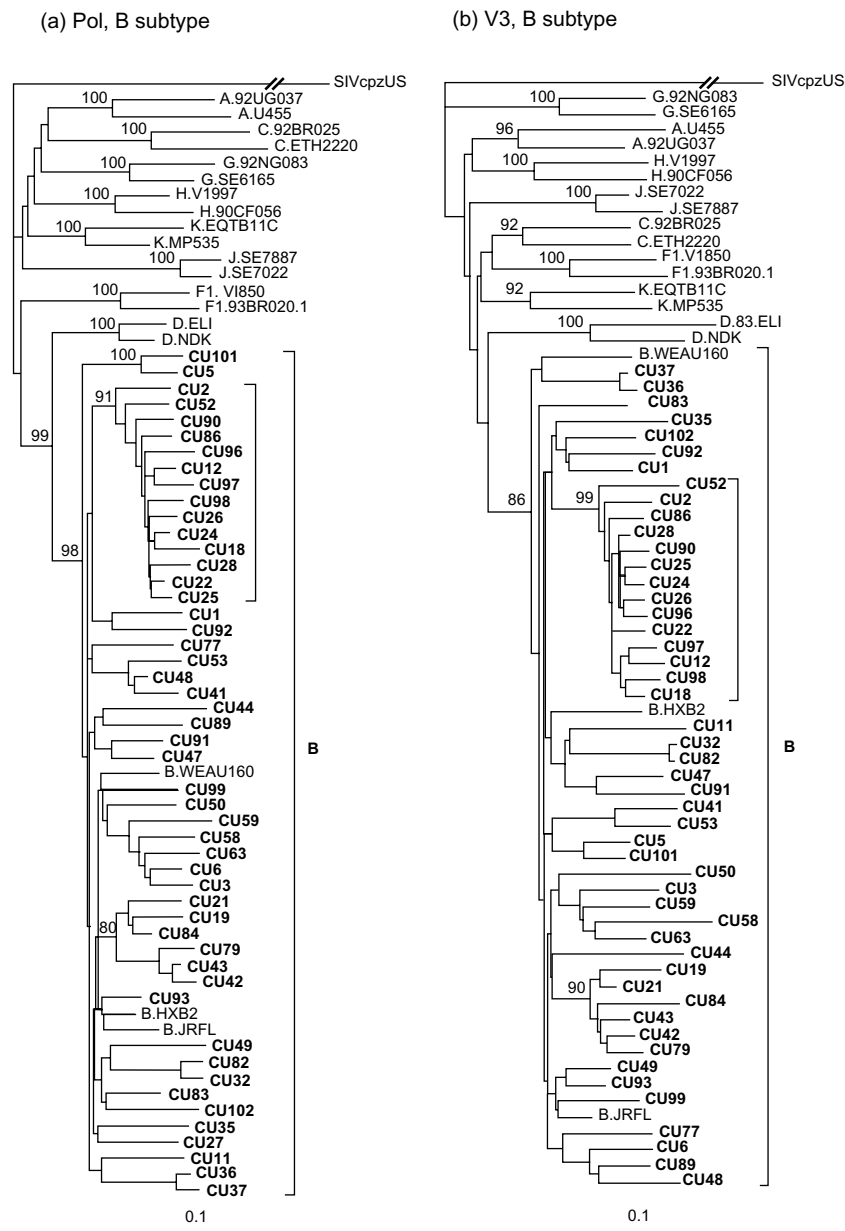


Fig 1. Phylogenetic neighbour-joining trees of *pol* and V3 region sequences of B subtype (a,b) and non-B subtype (c,d) viruses of Cuba. Viruses of Cuba are shown in boldface. Asterisks denote *pol* sequences that were identified as recombinant upon bootscan analysis. Bootstrap values 70% or higher of key nodes are shown. Relevant clusters are signalled with brackets. Subtypes or CRF with which viruses of Cuba cluster are indicated on the right of the corresponding brackets. U denotes a cluster of viruses of unknown subtype in *pol*. MAL-like denotes viruses with *pol* sequences clustering with the African complex (ADKU) recombinant isolate MAL.

tered with each other in the phylogenetic tree, although bootstrap support of this group did not reach significant values. Further analysis by bootscanning revealed that five of the sequences of this cluster (CU17, CU45, CU54, CU57 and CU70) were intersubtype recombinant (see below). When these recombinants were excluded from the analysis, the bootstrap value supporting the cluster formed by the remaining sequences of the group was 92%. The bootstrap value supporting branching of this cluster with subtype D references increased to 76% after excluding CU20 and the reference isolate 84ZR085.

To examine intersubtype recombination, *pol* sequences were analysed by bootscanning, which revealed that 12 were recombinant (Fig. 2d-l), with subtypes (in 5'-3' order) as follows (U denotes unknown subtype): three UK (CU33, CU34 and CU55, which grouped in the phylogenetic tree with the African MAL isolate [23-25]), two GBGB (CU100 and CU103), one BU (CU13), one UB (CU66), two GD (CU45 and CU57, which exhibit different crossover points), one DB (CU54), one BD (CU17) and one DUD (CU70). In all cases, grouping of different segments with different reference sequences was supported by significant boot-

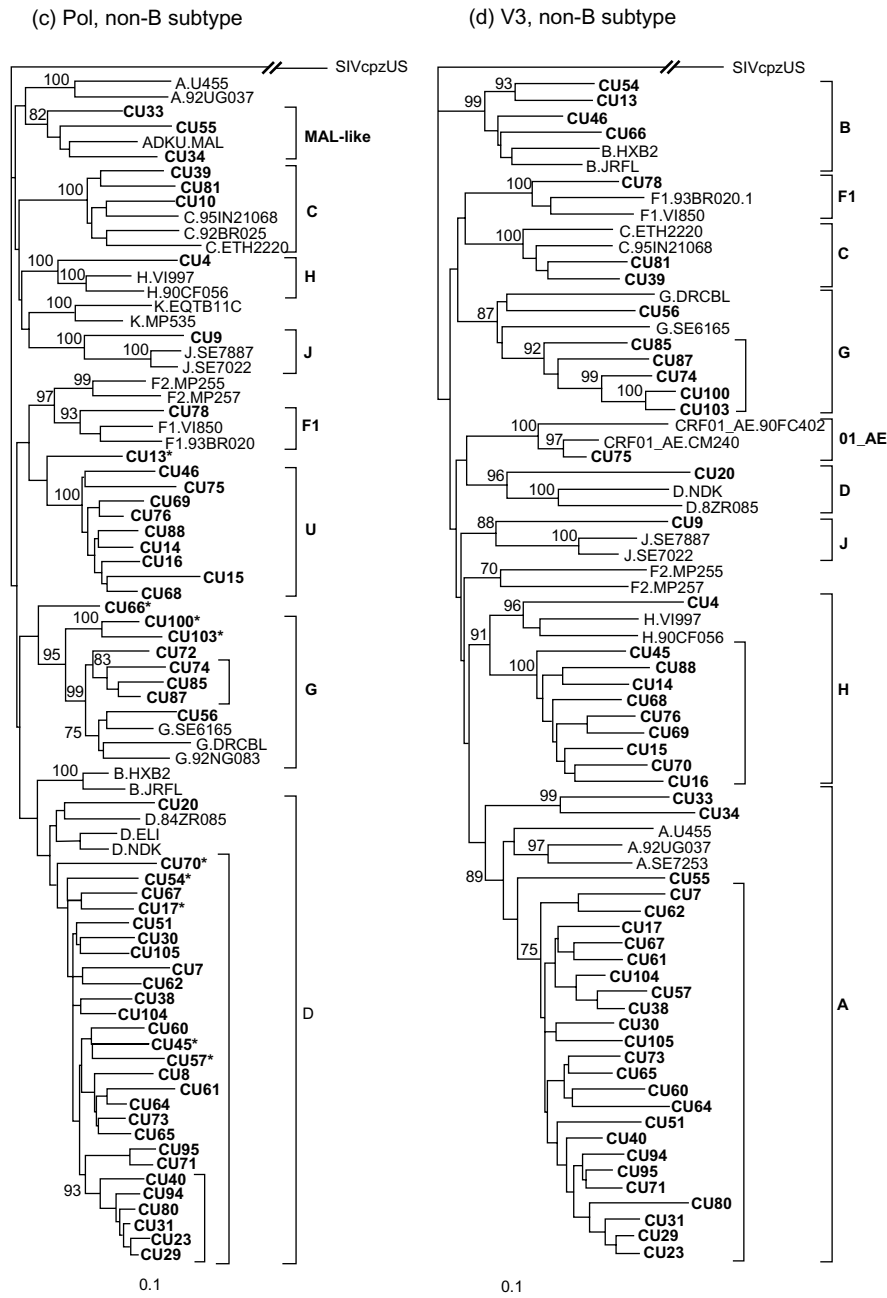


Fig 1. (continued).

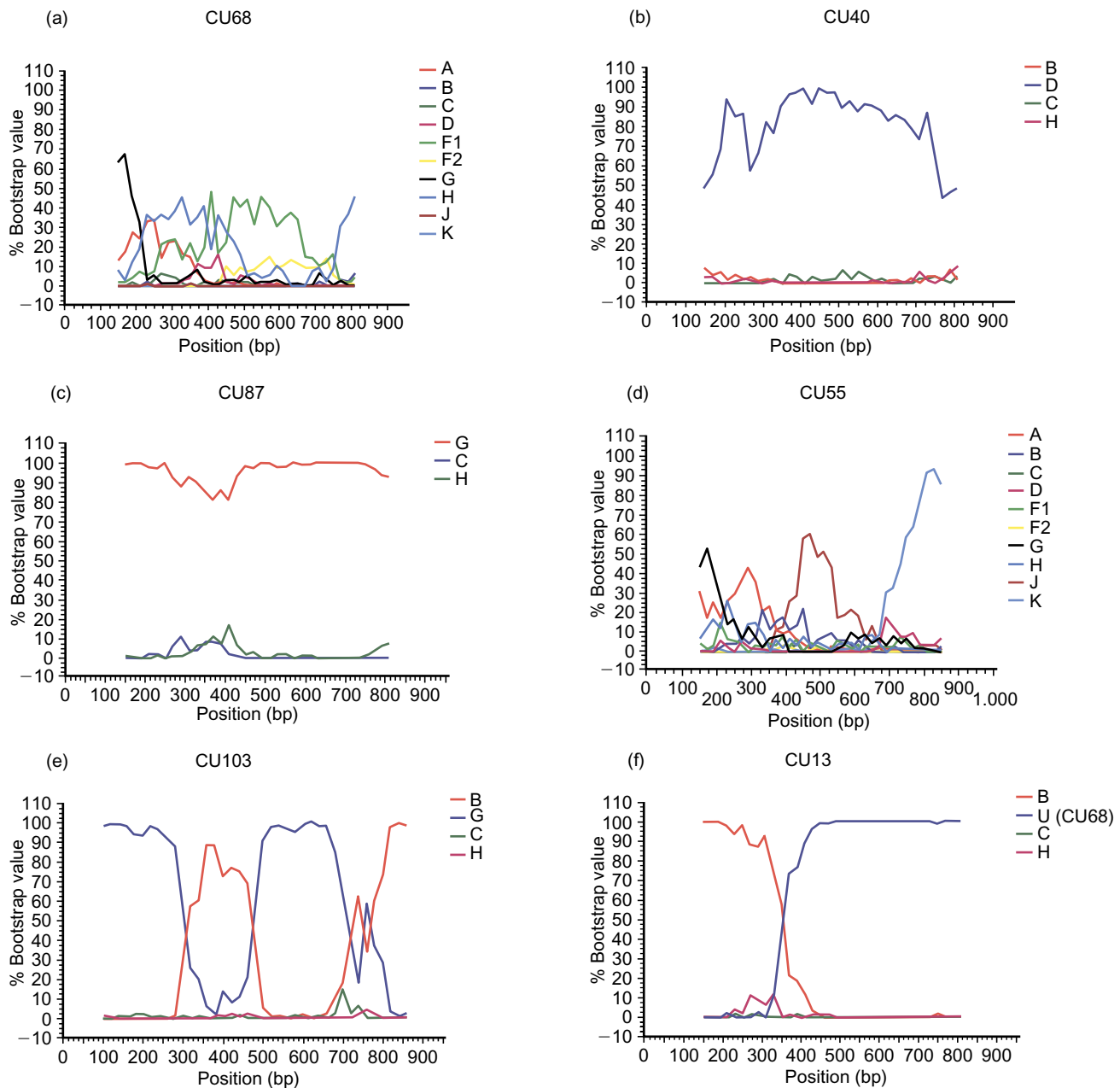


Fig. 2. Bootscan plots of *pol* sequences of Cuba. Horizontal axis represents the position of the midpoint of the window from nucleotide (nt) 1 of protease, and the vertical axis represents bootstrap values supporting branching with reference sequences. Windows of 300 nt were used, except for CU45 (180 nt) and CU103 (200 nt), advancing in steps of 20 nt. Trees were constructed with the neighbour-joining algorithm using Kimura's two-parameter distances, with transversion to transition ratio set to 2. Subtype reference isolates used were U455 (A), JRFL (B), ETH2220 (C), NDK (D), 93BR020.1 (F1), MP255 (F2), DRCBL (G), 90CF056 (H), SE7022 (J), and MP535 (K). D and G subtype references used for analysis of recombinant sequences were D and G subtype *pol* sequences of viruses from Cuban isolates CU87 and CU40, respectively, which clustered uniformly with database D and G subtype reference isolates in the analysed segment, as shown in (b) and (c). In bootscans of CU13, CU66, and CU70, the *pol* sequence of CU68 from the U cluster of Cuba was used as reference.

strap values, and results were consistent independently of reference sequences used. Bootscan analysis and phylogenetic trees of partial segments of the amplified sequences indicated that D subtype and U segments of recombinant *pol* sequences of CU13, CU66, CU45,

CU57, CU54, CU17 and CU70 (but not unclassified segments of CU33, CU34 and CU55) grouped with viruses of the D subtype and U *pol* clusters of Cuba, respectively, described above. Similarly, the G subtype segments of CU57 GD recombinant sequence clustered

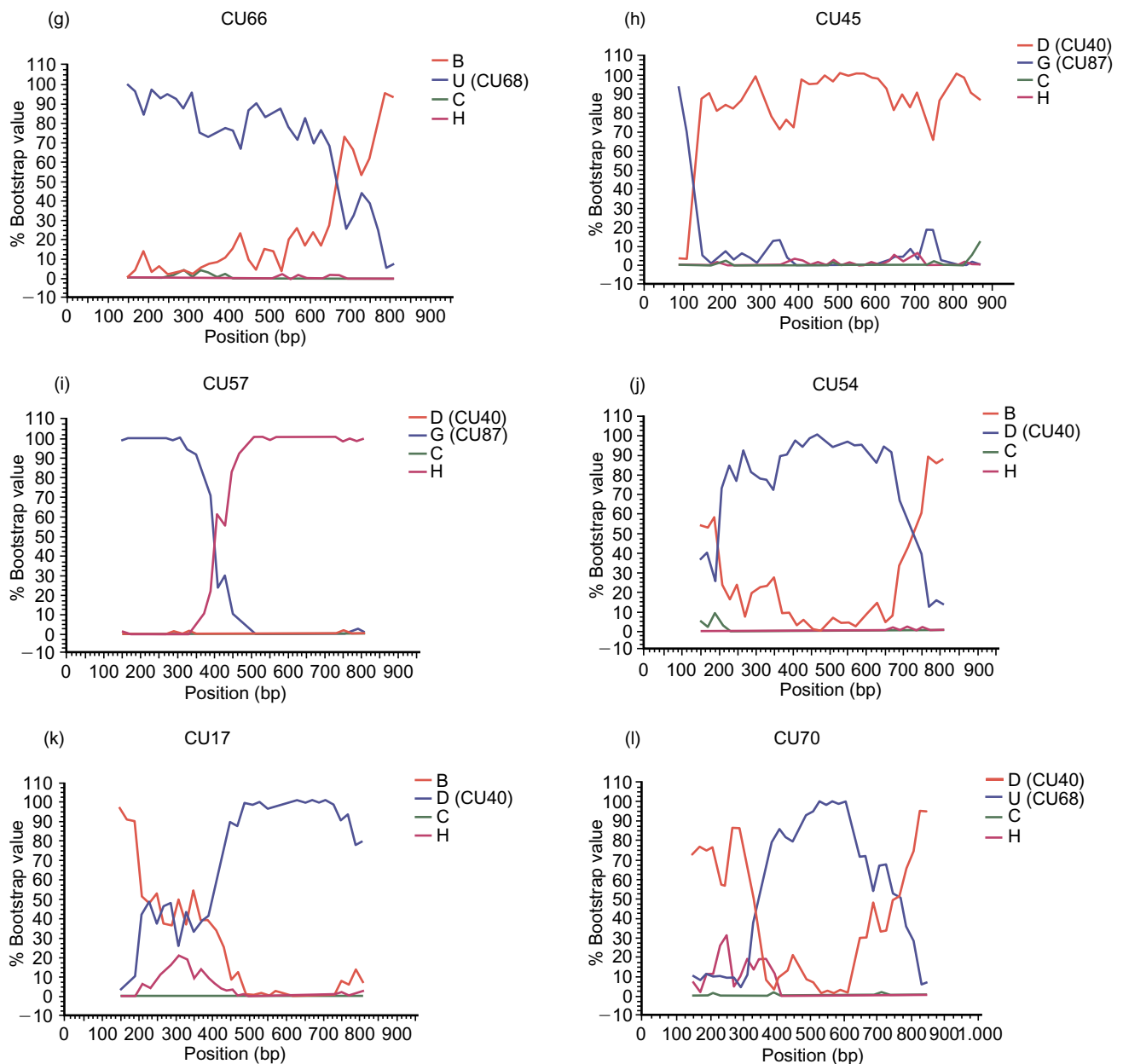


Fig. 2. (continued)

with G subtype *pol* sequences of CU74, CU85 and CU87 (which form a monophyletic group). This indicates that these recombinant were probably generated by recombination of genetic forms circulating in Cuba.

Phylogenetic analysis of V3 sequences

In phylogenetic trees (Fig. 1b,d), sequences of the V3 region grouped with the following subtypes or CRF: A ($n = 26$), B ($n = 54$), C ($n = 2$), D ($n = 1$), F1 ($n = 1$), G ($n = 6$), H ($n = 11$), J ($n = 1$) and CRF01-AE ($n = 1$). In two samples, CU10 and CU72, which

are of subtypes C and G, respectively, in *pol*, the V3 region could not be amplified.

Subtypes in V3 were coincident with those in *pol* for all viruses that were of non-recombinant subtypes B, C, F1, G, H and J in *pol*. Viruses of the Cuban D subtype *pol* cluster, except CU8, were of subtype A in V3, forming in this segment a monophyletic group, which also included viruses with recombinant *pol* sequences CU17 (BD in *pol*) and CU57 (GD in *pol*). Viruses forming the U cluster in *pol*, except CU46 and CU75, were of subtype H in V3, forming in this segment a monophyletic group, which also included

CU8 (D in *pol*), CU45 (GD in *pol*) and CU70 (DUD in *pol*). CU46 grouped with subtype B, and CU75 with CRF01-AE in V3. The three viruses that were MAL-like in *pol* grouped with subtype A reference sequences in V3, similarly to the isolate X327 of Spain [26], but different from both MAL [23–25] and the partly MAL-like virus NOGIL3 of Norway [27], which are of subtypes D and H, respectively, in *env*. Subtypes in *pol* and V3 of all viruses are shown in Tables 1 and 2, with distribution of genetic forms in Cuba represented graphically in Fig. 3. Overall, 43 (78%) of the 55 non-B subtype viruses were recombinant. The number of genetic forms identified was 21, including seven non-recombinant subtypes and 14 recombinant forms.

Table 1. Distribution of non-recombinant HIV-1 genetic forms in Cuba.

Number of samples	Subtypes (<i>pol</i> and <i>env</i> V3)
50	B
5	G
3	C
1	D
1	F1
1	H
1	J

Subtypes in *pol* (protease–reverse transcriptase) and *env* V3 region of HIV-1 viruses from Cuba are shown, with the number of samples of each genetic form, from a total of 105 analysed, shown on the left column. Two viruses, with *pol* subtypes G and C, respectively, that could not be amplified in V3 are included here with non-recombinant viruses.

Table 2. Distribution of recombinant HIV-1 genetic forms in Cuba.

Number of samples	Subtypes	
	<i>pol</i> (5'–3')	V3
21	D	A
7	U	H
1	BD	A
1	DB	B
1	DUD	H
1	D	H
1	U	B
1	BU	B
1	UB	B
1	U	CRF01_AE
1	GD	A
1	GD	H
2	GBGB	G
3	UK	A

Subtypes in *pol* (protease–reverse transcriptase) and *env* V3 region of HIV-1 viruses from Cuba are shown, with the number of samples of each genetic form, from a total of 105 analysed, shown on the left column. (Two viruses, with *pol* subtypes G and C, respectively, that could not be amplified in V3 are included in Table 1. Alternating subtype segments in *pol* of recombinant viruses are placed in 5'–3' order. U, unclassified segment.

Phylogenetic relations with database sequences

Homologies of non-B subtype sequences with sequences deposited in GenBank were searched using the BLAST algorithm. No sequences homologous to those of the U *pol* cluster grouping with these in phylogenetic trees were found in the database. There were 10 database H subtype sequences that grouped with the Cuban U/H viruses in V3, with significant bootstrap values (80%), seven of which were from the DRC (Fig. 4). One subtype A sequence from the Republic of Congo grouped with the Cuban D/A recombinants in V3. *Pol* sequences of these African viruses are not available; therefore, it is not known if they are phylogenetically related to the U/H and D/A recombinants of Cuba, respectively, in *pol*.

Pol sequences of CU33, CU34 and CU55 clustered with MAL isolate and with MAL-like *pol* sequences of NOGIL3 of Norway [27] and X327 of Spain [26]. In V3, CU33 and CU34 grouped with X327 and with two other subtype A sequences of the Republic of Congo (Fig. 4). *Pol* sequences of these two African viruses are not available and, therefore, it is not known if they are also MAL-like in this segment.

Viruses of subtypes A and H in *env*, previously reported in Cuba [5], grouped with D/A and U/H recombinant viruses, respectively, of our study (Fig. 4).

Genetic–epidemiological correlations

There were differences in the prevalence of non-B subtype viruses between groups defined by gender, risk category and place of infection. Non-B subtype viruses (including recombinants) were more frequent among heterosexuals (72%) than among homo/bisexuals (37%) ($P < 0.001$), and among women (73%) than among men (44%) ($P < 0.05$). However, the prevalence of non-B subtype viruses among heterosexual men (69%) did not differ significantly from that in women.

Nine of ten infections (90%) reported to having been acquired in Africa were with non-B viruses. Notably, seven were non-recombinant. Subtypes of non-B viruses acquired in Africa were: three C (two acquired in Ethiopia and one in Zambia), one F1 (Angola), one G (DRC), one H (Angola), one J (Angola) and two MAL-like in *pol* and subtype A in *env* (both acquired in DRC). Among infections acquired in Cuba, 46 (50%) of 92 were with viruses of non-B subtypes.

Within subtype B and D/A recombinant clusters, there were some subclusters, supported by high bootstrap values, of viruses found in individuals sharing epidemiological features. One of these subclusters comprised 14 subtype B viruses (signalled with brackets in Fig. 1), all of which corresponded to homo- or bisexual men, 12 of them from Havana City, representing 46% of subtype B viruses among individuals of this risk

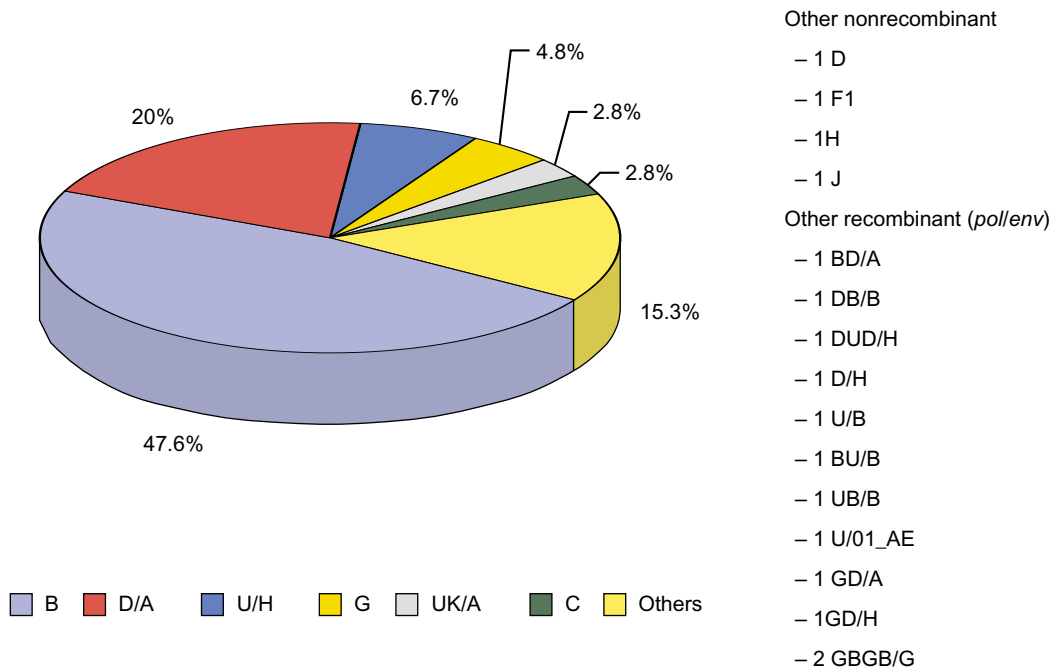


Fig. 3. Graphic depiction of the distribution of HIV-1 genetic forms in Cuba.

category in this city. Within the D/A recombinant cluster, five of the six viruses found in homo- or bisexual men from Havana City formed a subcluster supported by high bootstrap values (signalled with brackets in Fig. 1). Genetic distances in phylogenetic trees (Fig. 1) and dates of diagnosis (1996 or later in all but one of the B subtype subcluster, and 1997 or 1998 in the D/A recombinant subcluster) were consistent with relatively recent outbreaks, each originating from a common source.

Discussion

Here we report the most extensive survey to date on HIV genetic diversity in Cuba, with 105 samples analysed from an estimated HIV-infected population of approximately 2000 individuals. Previous studies were limited to 15 individuals in 1995 [28] and a recently published study of 11 individuals [5]. In both studies, only the V3 region was analysed. In the first study, it was stated that the predicted V3 amino acid sequences were similar to those of subtype B reference isolates. In the second study, V3 sequences were of subtypes B ($n = 6$), A ($n = 2$), C ($n = 2$) and H ($n = 1$). In our study, two segments of the viral genome, *pol* and *env*, were analysed phylogenetically using neighbour-joining trees and also by bootscanning to detect possible intersubtype recombination. The results show that approximately half (52%) of HIV-1 infections are caused by non-B subtype or recombinant viruses, with a high diversity of genetic forms, including non-

recombinant viruses of seven subtypes (B, C, D, F1, G, H and J) and recombinant viruses of 14 different genetic forms in 78% of the non-B samples, containing segments of subtypes A, B, D, G, H and K and CRF01-AE, as well as unclassified segments. Only one of the recombinant forms, a virus related to a MAL-like isolate reported in Spain (but probably acquired in Africa) [26], has been reported previously. Nevertheless, in spite of this diversity, our results provide phylogenetic and epidemiological evidence of only four genetic forms (B and G subtypes and D^{pol}/A^{env} and U^{pol}/H^{env} recombinants) currently circulating in Cuba. The remaining viruses either were acquired in Africa or were detected in only one or two individuals. D/A recombinants ($n = 21$) and U/H viruses ($n = 7$) grouped in respective monophyletic clusters in both *pol* and *env*, suggesting that they are candidates for recognition as circulating recombinant forms, pending sequencing of full-length genomes [29]. These two recombinant forms might be of African origin, since the parental viruses were not detected in Cuba. In GenBank, no sequences phylogenetically related to the *pol* sequences of the U/H or D/A recombinants of Cuba are found, but there are African viruses with *env* sequences related to these recombinants (Fig. 4). *pol* sequences of these African viruses are not available, which does not allow us to confirm if recombinant viruses related to those circulating in Cuba are present in Africa.

The HIV-1 genetic diversity found in Cuba has no parallel in the Americas. In other countries of the Caribbean area, infections are almost uniformly with

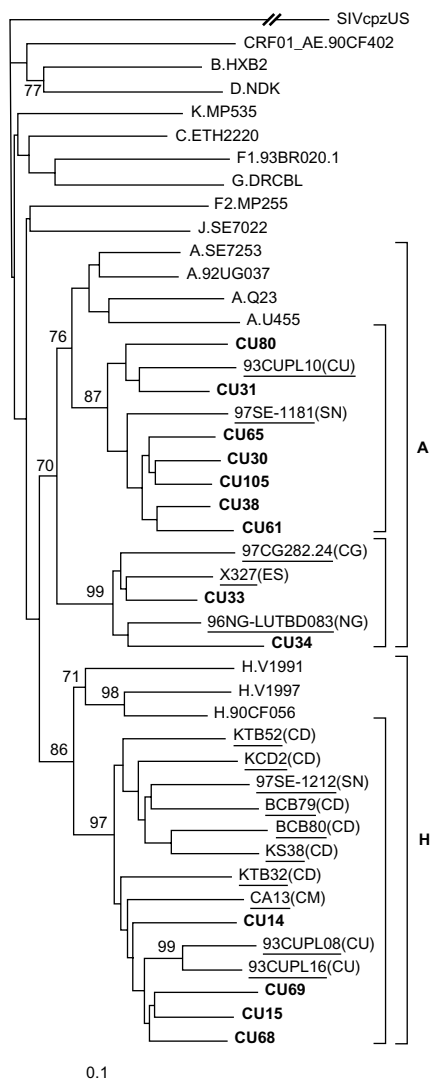


Fig. 4. Neighbour-joining tree of *env* V3 sequences of viruses phylogenetically related to viruses of Cuba. Sequences of Cuba of our study are in boldface, and related database sequences are underlined, identified by the name of the isolate followed by the country two letter code in parentheses. Bootstrap values 70% or greater of key nodes are shown. Relevant clusters are signalled with brackets. Subtypes with which the sequences of Cuba cluster are indicated on the right of corresponding brackets. CD, Democratic Republic of Congo; CG, Republic of Congo; SN, Senegal; NG, Nigeria; ES, Spain; CU, Cuba.

subtype B, although occasional cases of infections with other subtypes have been reported [30–32]. In the American continent, non-B subtype infections are relatively common only in Brazil, Argentina and Uruguay, although these are limited to F and C subtypes and BF recombinants in Brazil [1,2,33,34], BF recombinants in Argentina [3] and F subtype (possibly BF recombinants) and imported CRF01-AE viruses in Uruguay [4,35].

A diversity of HIV-1 genetic forms of a degree comparable to that found in Cuba has only been reported in Central African countries, with the highest group M diversity found in the DRC [8,36]. However, considering that the estimated number of HIV infections in Cuba is only approximately 2000, the HIV genetic diversity found in Cuba, in relative terms, has no parallel in any other country.

Several factors may have contributed to the high HIV genetic diversity in Cuba. Large numbers of Cuban soldiers and civilian personnel were stationed in the 1970s and 1980s in Angola [6,14]. Although there are no published studies on HIV-1 genetic diversity in Angola, it would not be unexpected to find a high HIV-1 genetic diversity in this country, since it is bordering the DRC (in fact, the three infections that were acquired in Angola were of three different subtypes, F1, H and J). Additionally, large numbers of ‘internationalist’ Cuban aid workers, serving in several sub-Saharan African countries, may have also contributed to the introduction of non-B genetic forms [6,10,14]. In our study, only 10 of 105 infections were acquired in Africa, indicating that non-B viruses introduced from Africa have started to circulate in Cuba. A superimposed factor contributing to the generation of HIV genetic diversity in Cuba is recombination [37]. Phylogenetic analyses indicate that 12 infections are with 11 different viruses generated by recombination between the four genetic forms circulating in Cuba. Considering the low prevalence of HIV infections in Cuba, the diversity of recombinants generated locally is disproportionately high. Although there is no direct evidence of this, it could be suspected that recombination of HIV genetic forms would have been facilitated by the former policy of prolonged quarantine of all HIV-infected individuals in selected sanatoria [10,14]. This, together with the scarcity of condoms in Cuba in earlier years [38], could have set conditions favourable for coinfections with multiple genetic forms, with subsequent generation of recombinants.

The finding of a high HIV diversity in Cuba may have implications for vaccine design [39–42] and for the use of tests for viral load determination [43,44] and for detection of drug resistance mutations [45–47]. Although the prevalence of HIV infections in Cuba is the lowest in the Americas, and travel of Cubans outside the country is presently limited, the possibility of exporting some of the multiple genetic forms of Cuba to other countries may not be negligible, considering the expansion of tourism in Cuba in recent years, with a concomitant increase in casual sexual contacts, in relation with declining economic conditions [14,38,48,49].

A recently reported HIV-1 complex recombinant virus from Cameroon (CM53379) sequenced in the full-length

genome [50] clusters with high bootstrap values, both in pol and env, with the U (pol)/H (env) recombinants from Cuba. This supports the Central African ancestry of these Cuban viruses.

In summary, in contrast to the almost uniform predominance of subtype B in other Caribbean countries, a high diversity of HIV-1 genetic forms, unprecedented in the Americas, has been found in Cuba. Some of these genetic forms were imported from Africa and others were generated locally by recombination. The causes of this diversity are related to historical circumstances peculiar to Cuba, which have contributed to make HIV diversity in this country a mosaic of the West (represented by B subtype sequences, presumably of North American origin) and of Africa (represented by multiple non-B subtype and recombinant viruses) grafted into the Caribbean.

Acknowledgments

We thank José Esparza for his contribution to organizing the UNAIDS program under which this study was done, and Francisco Parras for his support of this study.

Sponsorship: This work was financed by Technical Service Agreement HQ/98/457048, UNAIDS, and by grant 98BVII236 of Plan Nacional del SIDA, Ministerio de Sanidad y Consumo, Spain.

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