

Detection of the B^{''}-GWGR variant in the southernmost region of Brazil: unveiling the complexity of the human immunodeficiency virus-1 subtype B epidemic

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Typical human immunodeficiency virus-1 subtype B (HIV-1B) sequences present a GPGR signature at the tip of the variable region 3 (V3) loop; however, unusual motifs harbouring a GWGR signature have also been isolated. Although epidemiological studies have detected this variant in approximately 17-50% of the total infections in Brazil, the prevalence of B^{''}-GWGR in the southernmost region of Brazil is not yet clear. This study aimed to investigate the C2-V3 molecular diversity of the HIV-1B epidemic in southernmost Brazil. HIV-1 seropositive patients were analysed at two distinct time points in the state of Rio Grande do Sul (RS98 and RS08) and at one time point in the state of Santa Catarina (SC08). Phylogenetic analysis classified 46 individuals in the RS98 group as HIV-1B and their molecular signatures were as follows: 26% B^{''}-GWGR, 54% B-GPGR and 20% other motifs. In the RS08 group, HIV-1B was present in 32 samples: 22% B^{''}-GWGR, 59% B-GPGR and 19% other motifs. In the SC08 group, 32 HIV-1B samples were found: 28% B^{''}-GWGR, 59% B-GPGR and 13% other motifs. No association could be established between the HIV-1B V3 signatures and exposure categories in the HIV-1B epidemic in RS. However, B-GPGR seemed to be related to heterosexual individuals in the SC08 group. Our results suggest that the established B^{''}-GWGR epidemics in both cities have similar patterns, which is likely due to their geographical proximity and cultural relationship.

Key words: HIV - subtype B - B^{''}-GWGR - southernmost Brazil - molecular epidemiology

The third hypervariable region 3 (V3) of the human immunodeficiency virus-1 (HIV-1) gp120 protein consists of 35 amino acids and plays an important role in viral infection by promoting the interaction between the virus and its co-receptor in the host membrane (Hwang et al. 1991). Despite the recognised potential of HIV-1 to escape from neutralising antibodies through the extensive variability of its viral envelope glycoproteins, especially gp120, it is well known that the four amino acids at the tip of the V3 loop are subjected to strong purifying selection pressure due to their functional importance (Kwong et al. 2002, Liu et al. 2008). Although the vast majority of HIV-1 sequences present a GPGX signature at this position regardless of subtype, unusual patterns have been reported around the world (Shimizu et al. 1992, Brown et al. 1996, Kim et al. 1999, Leal & Villanova 2010).

Subtype B is the most geographically widespread variant of HIV-1 (HIV-1B) (Hemelaar 2012). The pandemic form of subtype B, which is prevalent in European, American and Asian countries, is typically characterised as having a GPGR motif (B-GPGR) at the tip of the V3 loop. However, several molecular studies have shown that various genetically and antigenically distinct V3 motifs, which are diversified particularly at the second position of the tetramer, co-circulate in the HIV-1B epidemic (Shimizu et al. 1992, Morgado et al. 1994, Candotti et al. 1999, Kim et al. 1999, Leal et al. 2008, Franca et al. 2011). In particular, some strains have been found to harbour an alternative signature in which the second residue of the tetrapeptide, proline, is substituted with tryptophan (B^{''}-GWGR) (Potts et al. 1993, Casseb et al. 1998, 2002, Morgado et al. 1998, Santoro-Lopes et al. 2000, Brito et al. 2006, Araujo et al. 2010, Franca et al. 2011). Clinical studies support the hypothesis that the B^{''}-GWGR motif is correlated with slower disease progression in infected patients when compared with those infected with the B-GPGR variant (Santoro-Lopes et al. 2000, Brito et al. 2006, Araujo et al. 2010).

Brazil is accepted as the epicentre of the B^{''}-GWGR epidemic (Diaz et al. 2008, Pinto et al. 2008). While viruses presenting this motif are sporadically observed in other countries, several studies in Brazil have found this variant at frequencies ranging from 17-50% (Morgado et

doi: 10.1590/0074-0276108062013010

Financial support: FAPESC, CNPq, FIOCRUZ

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Received 1 April 2013

Accepted 26 June 2013

al. 1994, Casseb et al. 1998, Araujo et al. 2010, Franca et al. 2011). Despite these results, recent findings about the temporal trends of the B"-GWGR epidemic in this country suggest a decline in the prevalence of this variant (Araujo et al. 2010, Franca et al. 2011). Several studies have highlighted the complexity of the HIV-1 epidemic in the southernmost region of Brazil, where subtype C, subtype B and several recombinant forms have been detected (Soares et al. 2003, Santos et al. 2006, de Medeiros et al. 2011, Gräf et al. 2011, Gräf & Pinto 2013), but no studies have attempted to detect B"-GWGR in this region. The current extent of the B"-GWGR epidemic in Brazil remains an unresolved question. Thus, the present study aims to investigate the molecular diversity of the HIV-1 subtype B epidemic in distinct exposure categories in the southernmost region of Brazil.

SUBJECTS, MATERIALS AND METHODS

The present study investigated 278 samples from three distinct groups of HIV-1-positive individuals from various outpatient clinics in the southernmost region of Brazil. The first group (RS98) contained 83 blood samples that were obtained from HIV-1-positive individuals recruited from a health reference centre in Porto Alegre, the capital city of the state of Rio Grande do Sul, in 1998. The second group (RS08) was comprised of 97 samples from HIV-infected individuals that were collected in Porto Alegre between 2005-2008. The third group (SC08) was composed of blood samples that were collected from 98 HIV-positive patients at follow-up appointments at a reference centre in the city of Florianópolis, the capital city of the state of Santa Catarina, between 2004-2008. The demographic data of the patients included in this study were extracted from clinical records or were obtained through direct interview. All of the individuals from Porto Alegre were antiretroviral treatment-naïve, while the individuals from Florianópolis were either naïve or under antiretroviral therapy at the time of blood collection. This study was approved by the ethical committees of the institutions involved and all patients provided written informed consent.

DNA was extracted from 200 µL of each whole blood sample using a QIAamp DNA kit (Qiagen, CA, USA) according to the manufacturer's protocol. The partial C2-V3 region of the *env* gene (nucleotides 6921-7283, relative to strain HXB2) was amplified by polymerase chain reaction (PCR) using nested primers as previously described (Delwart et al. 1993). The products were purified using a PureLink PCR Purification kit (Invitrogen, CA, USA) according to the manufacturer's directions. The purified DNA was sequenced using the ABI BigDye Terminator v.3.1 Cycle Sequencing Ready Reaction kit (Applied Biosystems, CA, USA) and processed with an automated ABI 3130xl Genetic Analyzer (Applied Biosystems). The sequences were edited and then aligned with reference sequences retrieved from the Los Alamos Sequence Database using CLUSTALX (Larkin et al. 2007).

Subtypes were assigned based on maximum likelihood (ML) phylogenetic reconstruction conducted on MEGA 5 software under the GTR+G+I model of nucleotide substitution (Tamura et al. 2011). A bootstrap test

with 1,000 replicates was used to estimate the confidence of the branching patterns of the phylogenetic tree. The sequences were also submitted to the REGA Subtyping Tool of the BioAfrica Database to corroborate the subtypes assigned by the ML analysis.

The molecular signatures of the HIV-1 subtype B V3 loop were identified through visual inspection from amino acid positions 15-18 (nucleotides 7158-7169, relative to strain HXB2). The typical subtype B signature was identified as B-GPGR, whereas viruses harbouring the alternative W (tryptophan) signature were assigned as B"-GWGR. Assuming that the epidemiological, serological and clinical differences observed between the B and B" signatures in previous studies are accurate (Hendry et al. 1996, Santoro-Lopes et al. 2000, Casseb et al. 2002, Brito et al. 2006, Leal et al. 2008, Pinto et al. 2008), sequences presenting related motifs that retained the P or W at second position of the tetrapeptide (XPXX and XWXX) were also considered as B-GPGR or B"-GWGR, respectively, because these most likely evolved from an ancestral sequence containing one of these motifs (Diaz et al. 2008). Sequences depicting an amino acid other than W or P at position 16 of the V3 loop were evaluated separately.

Statistical comparisons between and within groups were made using Pearson's χ^2 -test and Fisher's exact test when appropriate. The statistical analyses were performed using WinPepi v.11.22 (Abramson 2004) and the significance level was set at $p < 0.05$. Due to their low frequency, the sequences harbouring amino acids other than P or W in the second position of the V3 loop were excluded from the statistical analyses.

RESULTS

Phylogenetic analysis of group RS98 revealed the co-circulation of four HIV-1 subtypes: B (55%), C (39%), F1 (5%) and A (1%). Of the 46 HIV-1 subtype B samples in RS98, 54% were typed as B-GPGR, 26% as B"-GWGR and 20% presented an amino acid other than W or P at position 16 of the V3 loop (Tables I, II). In this study, B"-GWGR viruses accounted for 15% of all of the HIV infections evaluated in Porto Alegre in 1998. Based on their medical records, the RS98 HIV-1B-infected patients were categorised according to the route of probable infection: heterosexual (HET) (50%), men who have sex with men (MSM) (44%), people who inject drugs (4%) and blood transfusion (2%). Their classification according to gender was 70% male and 30% female (Table I). The HIV-1B signatures according to the patients' gender and exposure category are shown in Table I. No association could be established between the subtype B molecular signatures and the exposure category or gender.

Of the 97 HIV-1 individuals in group RS08, 33% were subtyped as B and 67% were subtyped as C. The subtype B V3 loop signatures were as follows: 59% B-GPGR, 22% B"-GWGR and 19% other motifs (Tables I, II). In total, B"-GWGR was observed in 8% of all of the HIV-1 infections evaluated in Porto Alegre between 2005-2008. Regarding the exposure categories of the subtype B samples, group RS08 showed (53%) HET (6 males and 11 females) and (31%) MSM individuals. The exposure

category was not determined for five individuals. No statistically significant associations between the subtype B signatures and the exposure category or gender were observed for RS08. After verifying the homogeneity of the gender and number of the individuals in each exposure category, statistical comparison based on Pearson's χ^2 -test revealed no temporal differences between the RS98 and RS08 groups regarding the subtype B epidemic.

In group SC08, subtype B was detected in 32 samples with a differential distribution of the molecular signatures: 59% of the samples presented the B-GPGR motif, 28% presented the B^{''}-GWGR motif and 13% presented other motifs (Tables I, II). The remaining 68% samples were assigned as subtype C. Epidemiologically, the B^{''}-GWGR variant was found to be responsible for approximately 9% of the total infections in Florianópolis. Regarding the risk factors for HIV-1B infection, 72% of the patients were HET (15 females and 8 males) and 28% were MSM. Similar distributions of the HIV-1B molecular signatures were observed in Porto Alegre (RS08) and Florianópolis (SC08) during the same time period. A statistically significant association ($p = 0.035$) was observed between the HET exposure category and the B-GPGR variant in the SC08 group.

Nineteen samples presented 13 different motifs that were unrelated to the B-GPGR or B^{''}-GWGR motifs in this analysis (9 samples from RS98, 6 samples from RS08 and 4 from SC08) (Table II). In addition, six motifs that were related to the B-GPGR motif and two motifs

that were related to the B^{''}-GWGR motif were detected. Three samples from group RS98 exhibited unique motifs: GRGA, GRGR and RRGG.

DISCUSSION

Now present in at least 23 countries around the world (Pinto et al. 2008, Leal & Villanova 2010), the B^{''}-GWGR variant of HIV-1 seems to have originated in Brazil (Diaz et al. 2008, Pinto et al. 2008, Leal & Villanova 2010) and since its isolation in the early 1990s, it has been widely studied in this country (Potts et al. 1993, Louwagie et al. 1994, Casseb et al. 1998, 2002, Morgado et al. 1998, Santoro-Lopes et al. 2000, Brito et al. 2006, Pinto et al. 2008, Araujo et al. 2010, Franca et al. 2011). However, the vast majority of studies concerning the B^{''}-GWGR motif have focused on the study of the HIV-1 epidemic in southeastern Brazil, where the prevalence of subtype B is extremely high. In addition, information about the B^{''}-GWGR epidemic in other regions of Brazil is scarce. This is the first report of the circulation of B^{''}-GWGR viruses in the southernmost region of Brazil.

The current study shows that B^{''}-GWGR motif-containing viruses play an important role in the HIV-1 subtype B epidemic in southernmost Brazil (Table I). Our molecular analysis of HIV-1 blood samples from Porto Alegre showed that the B^{''}-GWGR motif was present in approximately 24% of the subtype B samples. These results revealed no significant difference in the distribution of molecular signatures within the subtype B

TABLE I
Human immunodeficiency virus-1 subtype B variable region 3 loop motifs frequencies according to patient's gender and exposure category

	Group RS98 (n = 46)			Group RS08 (n = 32)			Group SC08 (n = 32)		
	B ^{''} -GWGR and related (n = 12)	B-GPGR and related (n = 25)	Other (n = 9)	B ^{''} -GWGR and related (n = 7)	B-GPGR and related (n = 19)	Other (n = 6)	B ^{''} -GWGR and related (n = 9)	B-GPGR and related (n = 19)	Other (n = 4)
Gender [n (%)]									
Male	8 (67)	17 (68)	7 (78)	4 (57)	11 (58)	3 (5)	7 (78)	7 (37)	3 (75)
Female	4 (33)	8 (32)	2 (22)	2 (29)	7 (37)	2 (33)	2 (22)	12 (63)	1 (25)
ND	-	-	-	1 (14)	1 (5)	1 (17)	-	-	-
Exposure category [n (%)]									
HET	6 (50)	12 (48)	5 (56)	5 (71)	8 (42)	4 (66)	4 (44)	17 (89) ^a	2 (50)
MSM	5 (42)	13 (52)	2 (22)	1 (14)	8 (42)	1 (17)	5 (56)	2 (11)	2 (50)
PWID	1 (8)	-	1 (11)	-	-	-	-	-	-
BT	-	-	1 (11)	-	-	-	-	-	-
ND	-	-	-	1 (14)	3 (16)	1 (17)	-	-	-
Prevalence (within group) (%)	26	54	20	22	59	19	28	59	13

^a: significant association ($p = 0.035$) between heterosexual (HET) individuals and B-GPGR motif infection; BT: blood transfusion; MSM: men who have sex with men; ND: not declared; PWID: people who inject drugs. Values in the brackets are the relative percentages according to gender or exposure category.

epidemic between 1998-2008 and suggest that the epidemic has been stable in this city since the mid-1990s. In contrast, epidemiological studies have shown that the prevalence of B^{''}-GWGR has been decreasing over time in other regions of Brazil (Hendry et al. 1996, Casseb et al. 1998, Brito et al. 2006, Araujo et al. 2010). B^{''}-GWGR viruses were previously estimated (by anti-V3 serologic assay) to account for approximately 27%, 48% and 64% of all HIV infections from samples isolated in the 1990s in the cities of Salvador, Rio de Janeiro and São Paulo, respectively (Hendry et al. 1996). However, more recently, molecular investigations detected this motif in 18%, 23% and 34% of the investigated HIV-infected individuals in the same cities in the years of 2006-2010 (Araujo et al. 2010, Arruda et al. 2011, Pimentel et al. 2011). Nevertheless, the differences observed in the other Brazilian regions could be related to the varied methods used to detect the B^{''}-GWGR variant or could be due to the

sampling of individuals from different exposure categories. In addition, the observed temporal difference in the B^{''}-GWGR epidemic could be the result of the influence of a random effect on the local transmission networks. However, further studies that use molecular analysis to examine representative sample sizes that include individuals from the same gender, exposure category and acquired immune deficiency syndrome (AIDS) progression stage are needed to assess the temporal trends of the B^{''}-GWGR epidemic in Brazil.

The V3 loop motifs of the viruses in the subtype B epidemic in the city of Florianópolis (SC08) were not significantly different from those of the epidemic in Porto Alegre (RS08) in the same time period. Moreover, due to the circulation of non-B subtypes in southernmost Brazil, especially subtype C, the B^{''}-GWGR variant may be evaluated as part of the total epidemic. In this case, B^{''}-GWGR was responsible for 15% and 8% of the HIV-1 infections in the years 1998 and 2005-2008 in Porto Alegre and for 9% of the HIV-1 infections in 2004-2008 in Florianópolis. Nevertheless, these results are not comparable with the results of other related studies because most of the other studies were performed in states where non-B subtypes are more infrequent and subtype B predominates (Santos et al. 2011, Alcalde et al. 2012, Pilotto et al. 2012). Taken together, our results suggest that the HIV-1 subtype B epidemics in Florianópolis and Porto Alegre have a similar pattern (Table I). The cultural relationship and the geographical proximity of Porto Alegre and Florianópolis may have influenced the dynamic of the transmission chains and could explain the similar results within these cities (Bello et al. 2012). However, comparing the entire HIV-1 epidemic in Southern (which consists of subtype C, subtype B and recombinant forms) with the HIV-1 epidemic in other states of Brazil, it seems that B^{''}-GWGR is not as prevalent in the more southern states of Brazil.

Although many studies have attempted to unravel the origin and prevalence of the B^{''}-GWGR variant, only one study has sought to understand the spreading pattern of this variant and its relationship with the patient's exposure category (Pimentel et al. 2011). A recent study carried out in Rio de Janeiro found that the B^{''}-GWGR variant was most likely introduced into the local epidemic by bisexual individuals (Pimentel et al. 2011). Analysis of the results for the RS98 and RS08 groups revealed no significant association between the exposure category and the V3 loop motif of HIV-1 subtype B. The lack of an association found here may be explained by the complete intermixing of local transmission chains (Almeida et al. 2012). Alternatively, this result may suggest the inexistence of V3 loop motif stratification by exposure category in the initial spread of HIV-1 subtype B in Porto Alegre. Although previous results have found a significant association ($p < 0.05$) between the MSM exposure category and subtype B in the 1990s (Almeida et al. 2012), our results suggest that there is no difference in regard to the V3 motifs in the subtype B epidemic in MSM and HET individuals between 1998-2008. Analysis of group SC08 suggests that the B^{''}-GWGR motif is also not associated with any exposure category. In

TABLE II
Human immunodeficiency virus-1
subtype B variable region 3 (V3) motifs diversity
according to the local of sample collection and year

V3 motif	Studied groups		
	RS98 (n = 46)	RS08 (n = 32)	SC08 (n = 32)
B-(GPGR) and related motifs			
G P G R	19	14	11
G P G K	2	2	1
A P G R	-	1	1
G P G S	2	-	2
G P G Q	1	-	2
A P G S	-	-	1
G P G G	1	2	1
B ^{''} -(GWGR) and related motifs			
G W G R	8	6	7
G W R R	-	1	1
A W G R	4	-	1
Other V3 motifs			
A F G R	-	1	-
G F G R	3	-	1
G L G R	2	1	-
A M G R	-	1	-
G M G R	-	-	1
G Q G R	-	-	1
G S G R	-	1	-
G T G R	1	-	-
G G G R	-	1	1
G V G R	-	1	-
G R G A	1	-	-
G R G R	1	-	-
R R G G	1	-	-

contrast, it seems that the dissemination of B-GPGR in Florianópolis is associated with the HET population. In a previous study based on the *pol* gene, a significant difference in the subtype distribution among distinct exposure categories in the HIV epidemic in Florianópolis was observed (Gräf et al. 2011). Together, these results suggest the existence of limited events of transmission between MSM and HET individuals due to a reduced overlap of the transmission chains in Florianópolis.

A remarkable feature of the HIV-1 epidemic in the southernmost region of Brazil is the co-circulation of subtypes B and C in high proportions (de Medeiros et al. 2011, Gräf et al. 2011, Almeida et al. 2012, Araújo et al. 2012). In addition, this region encompasses the 10 cities with the highest AIDS incidence rates in Brazil (MS 2012). This scenario becomes even more complex with the identification of a co-circulating molecular variant of subtype B that seems to have clinical particularities (Santoro-Lopes et al. 2000, Casseb et al. 2002, Brito et al. 2006, Leal et al. 2008). The GWGR motif seems to increase the avidity of V3 antibodies for the virus and contributes to slower disease progression in comparison to B-GPGR infection (Casseb et al. 2004, Brito et al. 2006). As longer periods of HIV-1 infection are expected for B⁷⁷-GWGR-containing viruses, the chance of HIV-1 transmission to other individuals should be greater. Consequently, an increased number of infections caused by B⁷⁷-GWGR viruses is anticipated over time in Brazil. In contrast, the increase in the avidity of V3 antibodies could contribute to a decrease in the viral load in B⁷⁷-GWGR infected patients, thereby reducing the chances of HIV-1 transmission to new hosts and consequently decreasing the number of infections caused by this variant. The results presented here for the HIV-1 epidemic in the southernmost region of Brazil demonstrate that the number of infections caused by B⁷⁷-GWGR within the subtype B epidemic is not increasing, but is being maintained. Several hypotheses can explain the stabilisation of this epidemic. However, other studies assessing behavioural as well as biological and clinical data will be needed to answer these questions and predict the future of the HIV-1 epidemic in Brazil. Despite the complex HIV-1 epidemic in the southernmost region of Brazil, the frequency of B⁷⁷-GWGR within subtype B viruses is comparable to that found in other Brazilian states. These results add another layer to the already complex HIV epidemic of southernmost Brazil and highlight the importance of surveillance studies in monitoring the dissemination of HIV-1 variants, specifically B⁷⁷-GWGR, which seems to confer a differential clinical prognosis.

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