Virus Genes
DOI 10.1007/s11262-013-0894-2

Longitudinal HIV-1 gp120-C2V3C3 phylogenetic surveillance and tropism evolution in patients under HAART

Franco A. Moretti · Manuel Gómez-Carrillo · Jorge F. Quarleri

Received: 30 October 2012 / Accepted: 18 February 2013

Abstract This 8-year longitudinal study was aimed to analyze the HIV-1 gp120-C2V3C3 sequence dynamics, their phylogenetic relationships and tropism evolution in patients under HAART. Such viral analysis comprised two compartments: plasma and PBMC. Fifty gp120-C2V3C3 genomic sequences were characterized from 16 patients: 41 from plasma when viremia was measurable and 9 from PBMCs if plasma viral load was undetectable. The vast majority of HIV isolates (43 out of 50) were ascribed to BF subtype, irrespective of the compartment (plasma or mononuclear-cells) analyzed. A statistically well-supported clustering phenomenon was observed for each patient sampling data. Each cluster comprised viral sequences from both compartments analyzed. In the vast majority of cases, the viral sequences obtained along active production periods were intermingled with those identified from proviral sequences. A substantial stability of co-receptor tropism for the HIV BF subtype was observed over an 8-year followup.

Keywords HIV · gp120 · V3loop · Tropism

Under the extreme conditions induced by an effective highly active antiretroviral therapy (HAART), the genetic behavior of the human immunodeficiency virus (HIV) may change due to a rapid adaptation to different selective pressures. The unknown outcome of the resulting viremia represents an important obstacle to cure HIV infection in HAART-treated individuals [1].

The HIV-1 envelope gene (*env*) encodes the surface glycoprotein 120 (gp120) and transmembrane glycoprotein 41 (gp41), and it is the site of major viral genetic diversity [2]. The C2-V3 region of *env* encodes an important target for immune responses, determines co-receptor specificity, and exhibits a high degree of phylogenetically informative variability [3, 4]. The third hypervariable domain (V3) of gp120 is a cysteine-bound loop structure composed of 35 amino acids (296–330, HXB2 reference sequence, GenBank accession # K03455) and plays a key role in viral entry into susceptible cells by interacting with the CD4 receptor and the CCR5 or CXCR4 co-receptors [5].

In terms of compartmentalization, previous studies have shown that either R5 or X4 viruses may be found in both circulating and proviral forms, but there is an apparent shift toward a more abundant representation of X4 populations in PBMC than in plasma [6].

This study was aimed to analyze the dynamics of intra-patient HIV population over time among viral strains from HAART-treated patients, their phylogenetic relatedness and viral characteristics such as cellular tropism and *env* gene mutational events, both in plasma and PBMC. In order to achieve this, 16 HIV+ patients under HAART were selected from a large cohort of HIV-infected patients followed up at the Infectious Diseases Unit of the Fernandez Hospital (Buenos Aires, Argentina) able to be retrospectively and longitudinally studied for at least a period of 8 years as well as exhibiting HIV–RNA or proviral DNA capable to be analyzed in at least two consecutive samples. All individuals participating in this study provided a written consent, previously evaluated and approved by a local ethics

F. A. Moretti · M. Gómez-Carrillo · J. F. Quarleri (✉)
Instituto de Investigaciones Biomédicas en Retrovirus y Sida (INBIRS), Universidad de Buenos Aires, Paraguay 2155 piso 11, C1121ABG Buenos Aires, Argentina
e-mail: quarleri@fmed.uba.ar

F. A. Moretti · M. Gómez-Carrillo · J. F. Quarleri
CONICET, Buenos Aires, Argentina

Published online: 06 March 2013

committee, according to the ethical principles of the 1975 Declaration of Helsinki.

Fifty-four plasma and PBMC were separated directly after collection from whole blood by centrifugation on a Ficoll–Hypaque density gradient. PBMC pellet (1×10^6 cells) and plasma (200 µl) were aliquoted in Trizol reagent and Trizol LS (GIBCO, Life Technologies, USA), respectively and stored at −80 °C until use. Total RNA and genomic DNA were isolated following the manufacturer's instructions.

Unfortunately, in several patients, data on HAART scheme, T CD4-cell counts or clinical stage at baseline were missing. Hence, in order to define the viral production, viral load was determined (VERSANT HIV-1 RNA 3.0 Assay Siemens, lower limit of detection 50 copies/ml). It is known that those patients who are measurably viremic, most of the virus is produced by recently infected cells, generally thought to be activated CD4+ T cells [7, 8]. PBMC proviral DNA was characterized in those sampling times where the plasma viral load was undetectable (<50 copies/ml). In patients on HAART, HIV-1 persistence is evidenced not only by the latent reservoir in resting CD4+ T cells but also by free virus in the plasma considered as "residual replication" [9]. Given the short half-life of free virus [8], this residual viremia indicates active virus production. This virus production may reflect low-level ongoing replication that continues despite HAART and/or release of virus from latently infected cells that become activated or from other stable cellular reservoirs [10].

The HIV-*env* (covering C2-V3-C3 domains of gp120) genomic region was amplified by RT (for RNA samples) and nested-PCR (for both RNA and DNA samples) following a protocol reported previously [11], generating 320 bp amplicons (from nucleotide 763 to 1083, according to HXB2 numbering). In order to avoid false-positive results due to carryover contamination, Kwok and Higuchi rules were strictly followed [12]. Moreover, positive and negative controls were included in all extractions and amplification reactions, and samples from each individual were handled separately. In addition, RNA eluates were treated with DNAse to avoid possible proviral DNA contamination.

Since all participants were on HAART, the detection of genomic RNA and/or proviral DNA was limited. HIV-RNA was detected in 41 out of 54 plasma samples. Proviral DNA in PBMC was measurable in 9 out of 13 sampling times with undetectable viral RNA. Three out of 16 patients (patients 2, 6, and 8) only one HIV-*env* sequence is available because no further viral amplification was obtained from plasma or PBMCs. For these patients it is not possible to analyze the viral evolution and therefore they were excluded for the final analysis.

The gp120-C2V3C3 PCR products originated from viral RNA and proviral DNA were directly sequenced using the Big Dye Terminator Kit in an ABI Prism 3100 DNA automated sequencer (Applied Biosystems, CA, USA). The sequences obtained were aligned using the Mafft program [13] and then manual edits were performed to keep codon correspondence.

The phylogenetic inference by maximum likelihood (ML) method was performed. One hundred and seventeen reference sequences belonging to the more representative subtypes worldwide were obtained from Los Alamos Data Base (http://www.hiv.lanl.gov/content/sequence/HIV) to be used as out-groups in tree devising.

The most appropriate substitution model was selected with hierarchical likelihood ratio testing (jModelTest 3.7) [14] and used to estimate the phylogenetic relationships among the gp120-C2V3C3 sequences with a ML method by heuristic searches. A non-parametric bootstrap analysis of 100 replicates, implemented in the PhyML v3.0 program, was performed [15]. The number of reference sequences was reduced as well as the tree was rooted for illustration purposes and drawn using Dendroscope v2.7.4 [16].

The determination of the HIV subtype was based on the *env* region using Blast (www.ncbi.nlm.nih.gov/BLAST), NCBI genotyping tool (http://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi) and REGA software (UKZN, South Africa, University of Oxford, UK, University of Pretoria, South Africa and, Katholieke Universiteit Leuven, Belgium) [17, 18]. The three typing methods agreed with the subtype assignment.

Figure 1 represents the phylogenetic relationships inferred by ML method. The dendrogram topology identifies highly bootstrap supported (>89 %) patient-defined HIV clusters included in two HIV subtypes: B and BF recombinant, as previously reported in Argentinean HIV-infected individuals [19–22]. The vast majority (43 out of 54) of the HIV isolates were ascribed to BF subtype; such predominance was exhibited at both compartments analyzed (plasma: 35/47; PBMC: 8/9). Although no compartment-specific viral variants evolving in a particular site were found, two isolates (01-2007; 07-2004) were classified outside the patient-related cluster inferring a plausible dissimilar origin. Considering that both patients are intravenous drug users, they are at risk of superinfection [23]. As well, such phenomenon could reflect the biphasic reduction in plasma HIV-1 after initiation of HAART where the first one was originated in activated CD4+ T cells and then by another population of infected cells with longer half-life [24, 25]. Likewise, ongoing viral evolution could be assumed that despite under HAART, the replication is still present. However, most study fails to detect such evolution taking into account dissimilar patient adherence [10, 26, 27].

HIV-1 is believed to establish reservoirs in cryptic cellular lineages, which shelter the virus from immune and therapeutic control when suppressed by HAART [28–31], and creates a bottleneck in viral evolution, selecting specific viral variants capable of maintaining a residual or null replication. This stage could be characterized by low levels

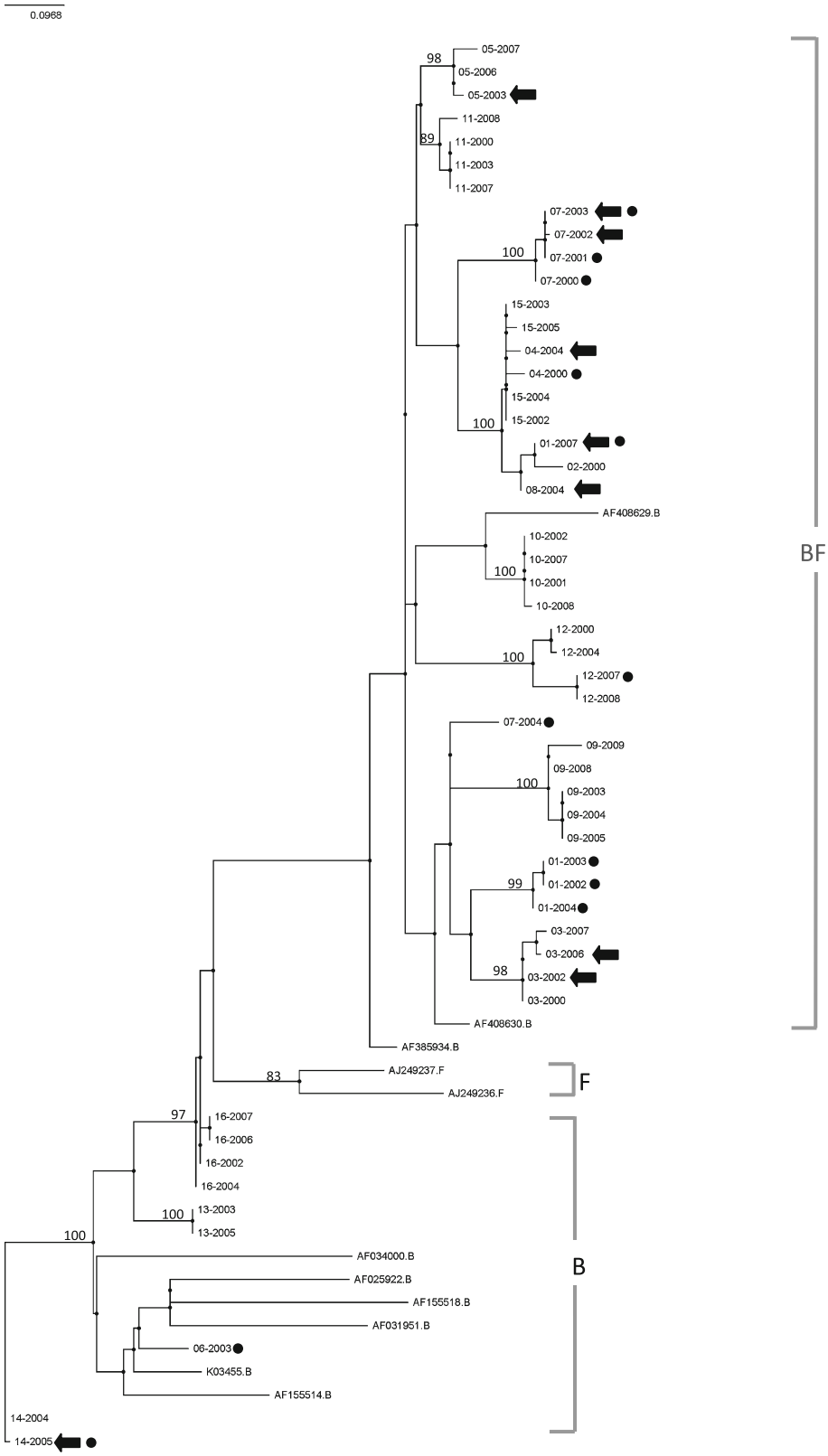

Fig. 1 Maximum likelihood dendrogram of HIV-*env* sequences characterized into the B, F, and BF_recombinant subtypes (identified by lateral brackets). Reference HIV sequences from each subtype were included (GenBank accession number followed by the known subtype assignment). *Black arrows* indicate the nucleotide sequences characterized from PBMC. *Black circles* indicate HIV isolates exhibiting predicted X4-tropism. Only significant bootstrap values are given on branches. Branch lengths are proportional to the number of nucleotide substitutions per aligned site (*bar* 0.1 substitutions)

Table 1 Sampling times, HIV-1 viral load and gp120-C2V3C3 main features

Patient	Year	Viral load[a]	Plasma Subtype[b]	PBMC	Plasma Tropism[c]	PBMC	V3 stem substitutions[d]		
							I309	R313	F315
1	2002	5,860	BF	n.a.	X4	n.a.	–	–	–
	2003	11,842	BF	n.a.	X4	n.a.	–	–	–
	2004	1,122	BF	n.a.	X4	n.a.	–	–	–
	2007	<50	n.a.	BF	n.a.	R5	–	–	–
2	2000	8,727	BF	n.a.	R5	n.a.	–	–	I
	2007	<50	n.a.	n.a.	n.a.	n.a.	–	–	–
3	2000	213,128	BF	n.a.	R5	n.a.	M	–	–
	2002	<50	n.a.	BF	n.a.	R5	M	–	–
	2006	<50	n.a.	BF	n.a.	R5	M	–	–
	2007	3,535	BF	n.a.	R5	n.a.	M	–	–
4	2000	735	BF	n.a.	R5	n.a.	–	–	I
	2004	<50	n.a.	BF	n.a.	R5	–	–	–
5	2003	<50	n.a.	BF	n.a.	R5	–	–	–
	2006	142,839	BF	n.a.	R5	n.a.	–	–	–
	2007	13,742	BF	n.a.	R5	n.a.	–	–	–
6	2003	113,338	B	n.a.	X4	n.a.	–	–	–
	2004	<50	n.a.	n.a.	n.a.	n.a.	–	–	–
	2007	<50	n.a.	n.a.	n.a.	n.a.	–	–	–
7	2000	320,565	BF	n.a.	X4	n.a.	–	–	–
	2001	>500,000	BF	n.a.	X4	n.a.	–	–	I
	2002	<50	n.a.	BF	n.a.	X4	–	–	I
	2003	<50	n.a.	BF	n.a.	X4	–	–	I
	2004	245	BF	n.a.	X4	n.a.	–	–	–
8	2004	<50	n.a.	BF	n.a.	R5	–	–	–
	2005	<50	n.a.	n.a.	n.a.	n.a.	–	–	–
9	2003	239,349	BF	n.a.	R5	n.a.	–	–	–
	2004	>500,000	BF	n.a.	R5	n.a.	–	–	–
	2005	3,690	BF	n.a.	R5	n.a.	–	–	–
	2008	1,084	BF	n.a.	R5	n.a.	–	–	–
	2009	3,662	BF	n.a.	R5	n.a.	–	–	–
10	2001	12,657	BF	n.a.	R5	n.a.	–	–	–
	2002	37,876	BF	n.a.	R5	n.a.	–	–	–
	2007	1,410	BF	n.a.	R5	n.a.	–	–	–
	2008	216	BF	n.a.	R5	n.a.	–	–	–
11	2000	3,894	BF	n.a.	R5	n.a.	–	–	–
	2003	9,058	BF	n.a.	R5	n.a.	–	–	–
	2007	52,912	BF	n.a.	R5	n.a.	–	–	–
	2008	5,330	BF	n.a.	R5	n.a.	–	–	–
12	2000	50,556	BF	n.a.	R5	n.a.	L	H	–
	2004	30,425	BF	n.a.	R5	n.a.	L	H	–
	2007	>500,000	BF	n.a.	X4	n.a.	L	H	–
	2008	465,642	BF	n.a.	R5	n.a.	F	H	–
13	2003	251	BF	n.a.	R5	n.a.	–	–	–
	2005	22,105	BF	n.a.	R5	n.a.	–	–	–
14	2004	290,721	B	n.a.	R5	n.a.	L	–	–
	2005	<50	n.a.	B	n.a.	X4	L	–	–

Table 1 continued

Patient	Year	Viral load[a]	Plasma Subtype[b]	PBMC	Plasma Tropism[c]	PBMC	V3 stem substitutions[d]		
							I309	R313	F315
15	2002	33,677	BF	n.a.	R5	n.a.	–	–	–
	2003	19,455	BF	n.a.	R5	n.a.	–	–	–
	2004	31,461	BF	n.a.	R5	n.a.	–	–	–
	2005	61,552	BF	n.a.	R5	n.a.	–	–	–
16	2002	9,203	B	n.a.	R5	n.a.	–	Q	–
	2004	>500,000	B	n.a.	R5	n.a.	–	Q	–
	2006	10,057	B	n.a.	R5	n.a.	–	Q	–
	2007	9,058	B	n.a.	R5	n.a.	–	Q	–

n.a. not available data, – indicates no amino acid substitution

[a] Viral Load was measured using VERSANT HIV-1 RNA 3.0 Assay Siemens

[b] Subtype was determined using Blast, NCBI genotyping tool and REGA software

[c] Co-receptor tropism was analyzed using Geno2Pheno under optimized cutoffs based on analysis of clinical data from MOTIVATE (10 % FPR)

[d] Amino acid substitutions at V3 stem are shown. The I307 did not exhibit any amino acid change among HIV isolates from this study

of viral replication that proceeds from these reservoirs [32], and which source is only detectable as provirus. Given that the viral RNA could be undetectable under HAART, the proviral HIV appears to be the only "visible face" of the virus.

The viral tropism defined by predicted co-receptor usage (CXCR4 and CCR5) was analyzed using the Geno2Pheno method under optimized cutoffs based on the analysis of clinical data (false-positive rate (FPR) means the probability of classifying an R5-virus falsely as X4 was fixed at 10 %) [33]. Despite this method for HIV tropism assignment is based on a computer prediction, a good correlation with biological assays, such as Trofile™ and ES-Trofile™ was reported [34]. Genotyping and tropism results are shown in Table 1 and 2. Because all patients included in the study were under HAART, we are not able to establish any correlation between the level of HIV production with the HIV subtype and cellular tropism.

A substantial stability of co-receptor tropism for the HIV BF subtype was observed over an 8-year followup. One patient infected with HIV-1 BF subtype (#12) experienced the R5 to X4 tropism switch, and another one (#1) shifted from X4 to R5. Arasteh et al. (2009) argued that neither in vitro nor in vivo data suggest that CCR5 co-receptor inhibition induces a tropism switch from CCR5 to CXCR4 through mutations in the envelope gene [35]. Therefore, the emergence of the CXCR4-tropic virus could be the result of preexisting population outgrowth.

These results increase the limited data about the interpretation of co-receptor tropism for the HIV BF subtype by means of geno2pheno [36]. Determination of HIV-1 co-receptor tropism is a major prerequisite before starting treatment with a CCR5-antagonist. While most of the patients currently under treatment with maraviroc are probably infected with HIV-1 subtype B viruses, recently published data show differences in the distribution of co-receptor tropism in different HIV-1 subtypes [37].

The viral tropism and CD4 affinity could be influenced when mutational events occur on critical amino acid residues in the HIV-V3 stem. When these substitutions affect hydrophobic residues (I307, I309, and F315) the V3 exposure may be altered and consequently, HIV infectivity [38]. Among the patients included in the present study, the longitudinal intra-patient analysis of the V3 stem exhibited the absence of I307 mutation. A low frequency of I309L and F315x (3/16 patients each) was observed and their appearance was patient-related. As the specific substitutions within this hydrophobic motif would enhance the affinity to the CD4 molecule, it may allow the virus to more efficiently infect alternative cellular lineages such as monocyte-derived macrophages, which present low concentration of CD4 molecules on their membrane [38]. Likewise, substitutions at amino acid R313 could reduce the affinity of the V3 loop with key neutralizing antibodies [39], an essential factor in driving viral kinetics. Therefore, this study looked for any substitution at V3-R313 and found that 2 patients exhibited alternative R313Q/H all along the followup. Thus, these findings did not allow us to relate it with any changes in viral kinetics.

Further research on viral dynamics of residual populations at the envelope level will be necessary to better understand the specific roles of this region and its domains and help to identify and characterize the main source of residual viremia, which might contribute to persistent immune dysfunction in some patients, despite HAART. Understanding specific strains with persistence implications

Table 2 Deduced V3 loop sequences alignment at the different time-points for each patient and their false-positive ratio (FPR) calculated by geno2pheno[co-receptor]

	10 20 30	FPR* (%)
	....|....|....|....|....|....|....|	
Consensus	**CTRPNXNTRKSIQIGPGRAFYATGDIIGDIRKAHC**	
01-2002	**.....N......PL.L.......EN.....**	8.6
01-2003	**.....N......PL.L.......EN.....**	9.0
01-2004	**.....N....X.PL.L.......EN.....**	5.0
01-2007	**.....N.....**	49.2
02-2000	**.....N.............I.T.....**	9.9
03-2000	**.............M.....**	62.5
03-2002	**.....N.......M.....**	44.2
03-2006	**..G..N.......M.....**	44.2
03-2007	**..K..N.......M.Q.................L.**	12.0
04-2000	**.....N.....................V..R...**	23.0
04-2004	**.....N.....**	49.2
05-2003	**.....N.....**	49.2
05-2006	**.....N.....**	49.2
05-2007	**.....N.....................N.....**	54.4
06-2003	**.....N......N......W....E.....**	1.7
07-2000	**.A...N......S..L...........N.....**	5.0
07-2001	**.A...N......S..L...I........N.....**	5.0
07-2002	**.A..DN......S..L...I........N.....**	3.5
07-2003	**.A...N......S..L...I........N.....**	3.5
07-2004	**.A...N......X....X....XXX...N.....**	1.7
08-2004	**.....N.....**	49.2
09-2003	**.....N.X....H.X......X..X...N.....**	31.3
09-2004	**.....N......H....X...T..X...N.....**	36.5
09-2005	**....XN..X.G.H.X..X...T..X...N.....**	21.2
09-2008	**.....N....G.H.X..X...T..X...N.....**	30.1
09-2009	**.....N....G.H....A...T..E...X.....**	48.9
10-2001	**.....N......H.....**	32.4
10-2002	**.....N......H.....**	34.9
10-2007	**.....N......H.............X.....**	34.9
10-2008	**.....N......H.....X...X.....**	11.4
11-2000	**.....N....X.P......X.X.....**	26.9
11-2003	**.....N......P......X........X..X...**	73.4
11-2007	**.....N......P......X........X..X...**	74.9
11-2008	**.....N.X....X......X....X...X..X...**	18.3
12-2000	**.....N......XL...H..XT..X...X.....**	28.2
12-2004	**.....N......HL...H..HT..S.....**	13.2
12-2007	**.....N......HX...H..HTX.S.....**	6.7
12-2008	**.....N.....XHF...XX.HT..S.....**	14.8
13-2003	**.....N......H.................Q...**	52.1
13-2005	**.....N......H.................Q...**	52.1
14-2004	**....SN..X...HL.X..X..X..AVX...X..X.**	10.9
14-2005	**....SN......HL.L........AV.....**	8.2
15-2002	**.....N.....**	49.2
15-2003	**.....N.....................X....**	49.2
15-2004	**.....N.....**	49.2
15-2005	**.....N.................X.....**	43.0
16-2002	**.....N...T..H....Q.....**	90.3
16-2004	**X....N...T..H....Q.........X..Q...**	95.7
16-2007	**.....N...T..H....Q.....**	90.3
16-2006	**.....N...T..H....Q.....**	90.3

* FPR is the probability of classifying an R5-virus falsely as X4

may be a starting point in developing approaches for viral eradication.

Sequence accession numbers are JQ068869 to JQ068927.

Acknowledgments This work was partly supported by grants from Universidad de Buenos Aires (UBA 2010-2012). We thank Ryan Luu (California State University, Long Beach, USA) for valuable comments and proofreading the manuscript and Sergio Mazzini for his assistance during manuscript preparation.

References

1. V. Simon, D.D. Ho, Q. Abdool Karim, HIV/AIDS epidemiology, pathogenesis, prevention, and treatment. Lancet **368**, 489–504 (2006)
2. B. Korber, B. Gaschen, K. Yusim, R. Thakallapally, C. Kesmir et al., Evolutionary and immunological implications of contemporary HIV-1 variation. Br. Med. Bull. **58**, 19–42 (2001)
3. T. Leitner, S. Kumar, J. Albert, Tempo and mode of nucleotide substitutions in gag and *env* gene fragments in human immunodeficiency virus type 1 populations with a known transmission history. J. Virol. **71**, 4761–4770 (1997)
4. J.A. Levy, Pathogenesis of human immunodeficiency virus infection. Microbiol. Rev. **57**, 183–289 (1993)
5. K.H. Roux, K.A. Taylor, AIDS virus envelope spike structure. Curr. Opin. Struct. Biol. **17**, 244–252 (2007)
6. C. Verhofstede, L. Vandekerckhove, V.V. Eygen, E. Demecheleer, I. Vandenbroucke et al., CXCR4-using HIV type 1 variants are more commonly found in peripheral blood mononuclear cell DNA than in plasma RNA. J. Acquir. Immune Defic. Syndr. **50**, 126–136 (2009)
7. W. Cavert, D.W. Notermans, K. Staskus, S.W. Wietgrefe, M. Zupancic et al., Kinetics of response in lymphoid tissues to antiretroviral therapy of HIV-1 infection. Science **276**, 960–964 (1997)
8. D.D. Ho, A.U. Neumann, A.S. Perelson, W. Chen, J.M. Leonard et al., Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. Nature **373**, 123–126 (1995)
9. E. Eisele, R.F. Siliciano, Redefining the Viral Reservoirs that Prevent HIV-1 Eradication. Immunity **37**, 377–388 (2012)
10. J.R. Bailey, A.R. Sedaghat, T. Kieffer, T. Brennan, P.K. Lee et al., Residual human immunodeficiency virus type 1 viremia in some patients on antiretroviral therapy is dominated by a small number of invariant clones rarely found in circulating CD4+ T cells. J. Virol. **80**, 6441–6457 (2006)
11. J. Albert, E.M. Fenyo, Simple, sensitive, and specific detection of human immunodeficiency virus type 1 in clinical specimens by polymerase chain reaction with nested primers. J. Clin. Microbiol. **28**, 1560–1564 (1990)
12. S. Kwok, R. Higuchi, Avoiding false positives with PCR. Nature **339**, 237–238 (1989)
13. K. Katoh, G. Asimenos, H. Toh, Multiple alignment of DNA sequences with MAFFT. Methods Mol. Biol. **537**, 39–64 (2009)
14. D. Posada, jModelTest: phylogenetic model averaging. Mol. Biol. Evol. **25**, 1253–1256 (2008)
15. S. Guindon, O. Gascuel, A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst. Biol. **52**, 696–704 (2003)
16. D.H. Huson, D.C. Richter, C. Rausch, T. Dezulian, M. Franz et al., Dendroscope: an interactive viewer for large phylogenetic trees. BMC Bioinformatics **8**, 460 (2007)
17. L.C. Alcantara, S. Cassol, P. Libin, K. Deforche, O.G. Pybus et al., A standardized framework for accurate, high-throughput genotyping of recombinant and non-recombinant viral sequences. Nucleic Acids Res. **37**, W634–W642 (2009)
18. T. de Oliveira, K. Deforche, S. Cassol, M. Salminen, D. Paraskevis et al., An automated genotyping system for analysis of HIV-1 and other microbial sequences. Bioinformatics **21**, 3797–3800 (2005)
19. M.A. Pando, I.C. Balan, R. Marone, C. Dolezal, C.S. Leu et al., HIV and other sexually transmitted infections among men who have sex with men recruited by RDS in Buenos Aires, Argentina: high HIV and HPV infection. PLoS ONE **7**, e39834 (2012)
20. M.A. Pando, M. Gomez-Carrillo, M. Vignoles, A.E. Rubio, M.S. dos Ramos Farias et al., Incidence of HIV type 1 infection, antiretroviral drug resistance, and molecular characterization in newly diagnosed individuals in Argentina: A Global Fund Project. AIDS Res. Hum. Retroviruses **27**, 17–23 (2011)
21. M.A. Pando, L.M. Eyzaguirre, G. Carrion, S.M. Montano, J.L. Sanchez et al., High genetic variability of HIV-1 in female sex workers from Argentina. Retrovirology **4**, 58 (2007)
22. M.M. Avila, M.A. Pando, G. Carrion, L.M. Peralta, H. Salomon et al., Two HIV-1 epidemics in Argentina: different genetic subtypes associated with different risk groups. J. Acquir. Immune Defic. Syndr. **29**, 422–426 (2002)
23. S. Yerly, S. Jost, M. Monnat, A. Telenti, M. Cavassini et al., HIV-1 co/super-infection in intravenous drug users. AIDS **18**, 1413–1421 (2004)
24. Z. Zhang, T. Schuler, M. Zupancic, S. Wietgrefe, K.A. Staskus et al., Sexual transmission and propagation of SIV and HIV in resting and activated CD4+ T cells. Science **286**, 1353–1357 (1999)
25. J.T. Blackard, HIV compartmentalization: a review on a clinically important phenomenon. Curr. HIV Res. **10**, 133–142 (2012)
26. T.H. Evering, S. Mehandru, P. Racz, K. Tenner-Racz, M.A. Poles et al., Absence of HIV-1 evolution in the gut-associated lymphoid tissue from patients on combination antiviral therapy initiated during primary infection. PLoS Pathog. **8**, e1002506 (2012)
27. T.L. Kieffer, M.M. Finucane, R.E. Nettles, T.C. Quinn, K.W. Broman et al., Genotypic analysis of HIV-1 drug resistance at the limit of detection: virus production without evolution in treated adults with undetectable HIV loads. J. Infect. Dis. **189**, 1452–1465 (2004)
28. C.A. Lopez, M. Vazquez, M.D. Hill, C. Colon Mdel, T. Porrata-Doria et al., Characterization of HIV-1 RNA forms in the plasma of patients undergoing successful HAART. Arch. Virol. **155**, 895–903 (2010)
29. Lopez CA, Vazquez M, Hill MD, Colon Mdel C, Porrata-Doria T, et al. Characterization of HIV-1 RNA forms in the plasma of patients undergoing successful HAART. Arch Virol 155: 895-903
30. J.K. Wong, M. Hezareh, H.F. Gunthard, D.V. Havlir, C.C. Ignacio et al., Recovery of replication-competent HIV despite prolonged suppression of plasma viremia. Science **278**, 1291–1295 (1997)
31. R.M. Lynch, R. Rong, B. Li, T. Shen, W. Honnen et al., Subtype-specific conservation of isoleucine 309 in the envelope V3 domain is linked to immune evasion in subtype C HIV-1 infection. Virology **404**, 59–70 (2010)
32. L. Shen, R.F. Siliciano, Viral reservoirs, residual viremia, and the potential of highly active antiretroviral therapy to eradicate HIV infection. J Allergy Clin Immunol **122**, 22–28 (2008)
33. L.P. Vandekerckhove, A.M. Wensing, R. Kaiser, F. Brun-Vezinet, B. Clotet et al., European guidelines on the clinical management of HIV-1 tropism testing. Lancet Infect Dis **11**, 394–407 (2011)
34. E. Poveda, J. Alcami, R. Paredes, J. Cordoba, F. Gutierrez et al., Genotypic determination of HIV tropism—clinical and methodological recommendations to guide the therapeutic use of CCR5 antagonists. AIDS Rev **12**, 135–148 (2010)

35. K. Arasteh, H. Stocker, Tropism switch in patients infected with HIV-1 and its clinical implications for the treatment with CCR5-receptor inhibitors. Eur J Med Res **12**, 397–402 (2007)
36. E. Delgado, A. Fernandez-Garcia, Y. Vega, T. Cuevas, M. Pinilla et al., Evaluation of genotypic tropism prediction tests compared with in vitro co-receptor usage in HIV-1 primary isolates of diverse subtypes. J. Antimicrob. Chemother. **67**, 25–31 (2012)
37. M. Obermeier, R. Ehret, T. Berg, P. Braun, K. Korn et al., Genotypic HIV-coreceptor tropism prediction with geno2pheno [coreceptor]: differences depending on HIV-1-subtype. J Int AIDS Soc **15**, 18214 (2012)
38. R.M. Lynch, T. Shen, S. Gnanakaran, C.A. Derdeyn, Appreciating HIV type 1 diversity: subtype differences in *Env*. AIDS Res. Hum. Retroviruses **25**, 237–248 (2009)
39. T. Cardozo, J. Swetnam, A. Pinter, C. Krachmarov, A. Nadas et al., Worldwide distribution of HIV type 1 epitopes recognized by human anti-V3 monoclonal antibodies. AIDS Res. Hum. Retroviruses **25**, 441–450 (2009)