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## Ultradeep sequencing reveals HIV-1 diversity and resistance compartmentalization

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► **To cite this version:**

Eleni Giatsou, Basma Abdi, Isabelle Plu, Nathalie Désiré, Romain Palich, et al.. Ultradeep sequencing reveals HIV-1 diversity and resistance compartmentalization. *AIDS. Official journal of the international AIDS Society*, 2020, 34 (11), pp.1609-1614. 10.1097/QAD.0000000000002616 . hal-02996294

**HAL Id: hal-02996294**

**<https://hal.sorbonne-universite.fr/hal-02996294>**

Submitted on 9 Nov 2020

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1 **Title:** Ultradeep sequencing reveals HIV-1 diversity and resistance compartmentalization  
2 during HIV-encephalopathy

3

4 **Short title:** HIV-compartmentalization in the CNS

5

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26 **ABSTRACT**

27 **Objectives:** To examine viral diversity and resistance mutations in different brain areas in  
28 cases of HIV-encephalopathy.

29 **Design:** Twelve post-mortem brain areas from 3 cases of possible or certain HIV-  
30 encephalopathy were analyzed.

31 **Methods:** After amplification of the reverse transcriptase and the V3 loop region of the gp120  
32 protein, ultradeep sequencing was performed with Illumina® technology. Phylogenetic  
33 analysis was performed with Fastree v2.1 using the generalized time-reversible (GTR) model.  
34 Identification of resistant viral variants was performed on Geneious software, according to  
35 HIV-1 genotypic drug resistance interpretation's algorithms, 2018 administered by the French  
36 Agency for Research on AIDS and Viral Hepatitis.

37 **Results:** Phylogenetic analysis revealed significant inter-regional and intra-regional diversity  
38 reflecting persistent HIV-1 viral replication in the different brain areas. Although some  
39 cerebral regions shared HIV-variants, most of them harbored a specific HIV-subpopulation  
40 reflecting HIV compartmentalization in the central nervous system. Furthermore, proportion  
41 and distribution of resistance mutations to Nucleoside and Non-Nucleoside Reverse  
42 Transcriptase Inhibitors differed among different brain areas of the same case suggesting that  
43 penetration of antiretroviral treatment may differ from one compartment to another.

44 **Conclusions:** This study, performed with a powerful sequencing technique, confirmed HIV  
45 compartmentalization in the central nervous system already shown by classical sequencing,  
46 suggesting that there are several reservoirs within the brain.

47

48 **Keywords:** HIV-encephalitis; compartmentalization; viral diversity; resistance mutation;  
49 ultradeep sequencing

50

51 **INTRODUCTION**

52           The Human Immunodeficiency Virus (HIV) enters the brain causing HIV-  
53 encephalopathy. The multinucleated giant cell (MGC), formed by cell-to-cell fusion of  
54 infected macrophages with microglia is the hallmark of this disease [1].

55 HIV-persistence in the CNS is principally due to weak penetration of antiretroviral drugs  
56 through the blood-brain-barrier [2,3]. Sanger-sequencing has shown independent evolution of  
57 drug resistance mutations to Nucleoside and Non-Nucleoside Reverse Transcriptase Inhibitors  
58 (NRTIs/NNRTIs) and protease inhibitors (PI) in different brain areas suggesting that  
59 differential drug penetration may occur among them [4]. Ultra-deep sequencing (UDS)  
60 detects minority variants that represent up to 1% of the HIV-1 population and that were  
61 incriminated for systemic therapeutic failure in treatment naïve patients [5–8]. Moreover,  
62 phylogenetic studies based on Sanger-sequencing determined brain-specific variants [9–11].  
63 Analysis of the envelope gene in either Sanger or Single Molecule Real Time (SMRT)  
64 sequencing showed viral strains within the CNS evolving independently in different brain  
65 areas in patients who died from HIV-encephalopathy [12]. More specifically, uniquely  
66 divergent viral strains were identified in frontal, occipital, parietal, temporal lobes and basal  
67 ganglia [12–14].

68           In this study, we used UDS to describe HIV-diversity in the CNS by sequencing the  
69 reverse transcriptase (RT) gene and the hypervariable V3 loop region of the HIV-1 gp120  
70 envelope protein, to detect minority resistant variants and to identify HIV-1 tropism in  
71 specimens derived from different brain areas in three HIV+ cases of HIV encephalopathy.

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74 **METHODS**

75 Twelve post-mortem brain tissues from 3 HIV-positive cases of probable or certain HIV-  
76 encephalopathy were provided by the Raymond Escourolle Neuropathology laboratory of the  
77 Pitié-Salpêtrière Hospital. The twelve tissues represented temporal and frontal lobe, caudate  
78 nucleus, thalamus, cerebellum, medulla oblongata, substantia nigra and spinal cord. The first  
79 case (C1) concerned a 48-year-old woman, HIV-positive for ten years, treated by multiple  
80 antiretroviral therapy. The second case (C2) concerned a 38-year-old man, HIV-positive for  
81 nine years, never treated. Concerning the third case (C3), a 29-year-old woman, she received  
82 treatment but no data was available on duration and date of HIV-diagnosis. Information on  
83 clinical course, specific HIV treatment history and biological parameters was limited, as the  
84 majority of medical records have been destroyed (Supplementary Table 1).

85

86 After DNA/RNA extraction, HIV proviral-DNA was quantified with Generic HIV DNA Cell<sup>®</sup>  
87 kit (Biocentric<sup>®</sup>). RT (RT1 and RT2) and V3 loop regions were amplified by nested PCR  
88 (Supplementary Table 2) and sequencing was performed by Illumina<sup>®</sup> MiSeq (paired-end,  
89 2x300bp).

90

91 **Viral diversity.** Geneious Prime software (Biomatters Ltd, Auckland, NZ) was used to keep  
92 reads with a Q-score > 30 and longer than 200bp and to pair forward and reverse reads to  
93 form complete RT1, RT2 and V3 regions. Sequences in 100% agreement were grouped to  
94 form consensus sequences (CS). Second round was performed with sequences in 99%  
95 agreement then 98% and finally 97% as previously described [15]. Then, multiple alignments  
96 of all CS and HXB2 reference genome was performed using Mafft Software v7 [16].  
97 Phylogenetic analysis was performed using approximately-maximum-likelihood method with  
98 FastTree v2.1 using generalized time-reversible (GTR) model on both all CS (HIV\_RT1\_CS

99 and HIV\_V3\_CS) and CS after cleaning viral CS found less than 100 times in each brain area  
100 (HIV\_RT1\_CS100 and HIV\_V3\_CS100).

101 To compare, Sanger sequencing was also performed according to the ANRS (French Agency  
102 for HIV research and Hepatitis) technique (<http://www.hivfrenchresistance.org/>). Multiple  
103 alignment of nucleotide sequences was performed with Mafft [16] and phylogenetic analysis  
104 with PhyML using GTR model and 1000 bootstrap resampling.

105 Finally, HIV-1 tropism was determined with geno2pheno  
106 (<https://coreceptor.geno2pheno.org/>) according to the recommendations of the European  
107 Consensus Group on clinical management of HIV-1 tropism testing (10% FPR).

108

109 **Single-nucleotide polymorphisms (SNPs).** Cleaned reads of RT1/RT2 issued from UDS (see  
110 previous paragraph) were mapped against HXB2 that carried annotations for the RT to  
111 identify SNPs (synonymous and non-synonymous SNPs, the coverage and the number of  
112 reads carrying polymorphism). The minimum variant frequency was set at 1%. Finally, HIV-1  
113 genotypic drug resistance interpretation's algorithms, 2018  
114 (<http://www.hivfrenchresistance.org/table.html>) administered by the French Agency for  
115 Research on AIDS and Viral Hepatitis were used to identify resistance mutations.

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## 118 **RESULTS**

119 HIV proviral-DNA load was only detected in C2 temporal lobe and medulla oblongata  
120 specimen as well as in all C3 specimens mentioned in increasing order: cerebellum (23  
121 copies/  $10^6$  cells), medulla oblongata (31 copies/  $10^6$  cells), temporal lobe (91 copies/  $10^6$   
122 cells), substantia nigra (92 copies/  $10^6$  cells), caudate nucleus (130 copies/  $10^6$  cells), and  
123 frontal lobe (544 copies/  $10^6$  cells) (**Table 1**).

124

125 Phylogenetic trees were generated using both HIV\_RT1\_CS (Supplementary Figure 1)  
126 and HIV\_RT1\_CS100 (**Figure 1**) and viral diversity is depicted in Table 1.

127 Viral diversity of RT1 for C1 was very high varying from 1086 to 3453 different viral CS in  
128 each brain area. Viral variants isolated from temporal lobe, caudate nucleus and spinal cord  
129 clustered independently (**Figure 1A**). However, a small part of viral strains derived from  
130 caudate nucleus and spinal cord was intermingled with 15 common CS between the two areas  
131 (0.6% and 0.4% of their CS, respectively). Viral diversity of C2 was also very high in  
132 temporal lobe region and medulla oblongata (3259 and 3189 respectively) with a clear  
133 separation of viral population between the two compartments and only 3 common CS  
134 (0.09%). Considering HIV\_RT1\_CS100, no viral population was shared between C2's  
135 compartments (**Figure 1C**). Finally, for C3, viral variants isolated from substantia nigra  
136 clustered independently (both HIV\_RT1\_CS and HIV\_RT\_CS100) from caudate nucleus,  
137 cerebellum and frontal lobe variants. However, among the last 3 brain areas, 527 viral CS  
138 were shared (20% of frontal CS, 21.5% of caudate nucleus CS and 15% of cerebellum CS)  
139 (**Figure 1D**).

140 By Sanger sequencing similar results were found, specifically sequences from C1 and C2  
141 clustered independently. However, in C3, sequences from cerebellum, caudate nucleus and  
142 frontal lobe clustered together and these results may explain the more important proportion of  
143 common CS obtained by UDS between these 3 brain areas (Supplementary Figure 2).

144

145 The analysis of the V3 loop region for C1 also showed about thousand different viral  
146 CS per brain area with a limited number of them common between temporal lobe and spinal  
147 cord (**Figure 1B**). HIV-1 tropism was analyzed with HIV\_V3\_CS100: 94% (72/77) of spinal  
148 cord CS100 and 96% (126/131) of temporal lobe CS100 were predicted to use the CCR5 co-

149 receptor. The remaining CS100 of the two brain areas were undetermined and none was  
150 predicted to use CXCR4 co-receptor (Supplementary Table 3).

151

152 SNPs not conferring resistance to NRTIs/ NNRTIs were found for all samples  
153 amplified for RT1. They were carried by either majority or minority variants depending on  
154 brain area and they reflected the viral diversity previously found (Supplementary Table 4).

155 SNPs conferring resistance to ZDV, ABC, TDF/ FTC (NRTIs) and ETR (NNRTIs) if  
156 associated to other mutations of the RT gene were found: specifically, M41L conferring  
157 resistance to NRTIs and V90L and V106I to the NNRTI (**Table 1**). In C1, the majority of  
158 caudate nucleus's and spinal cord's variants harbored M41L (98, 4% each) and V90L (96.7%  
159 and 97.6% respectively) not found in temporal lobe. In C2, no resistance mutations were  
160 identified in neither temporal lobe nor medulla oblongata. In C3, minority variants in caudate  
161 nucleus and substantia nigra carried V90I (16.2% and 1.6% respectively). However, V106I  
162 was carried only by 1% of variants in caudate nucleus and M41L only by 2.3% of variants in  
163 substantia nigra. Finally, no resistance mutations were identified in neither frontal lobe nor  
164 cerebellum.

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## 167 **DISCUSSION**

168 The CNS is an important viral reservoir of HIV and can be particularly difficult to  
169 target as a consequence of limited drug penetration [17,18]. This study is the first to use  
170 Illumina® technology to describe viral diversity and to analyze resistance mutations to  
171 NRTIs/NNRTIs in diverse areas of the CNS in three cases diagnosed with probable or certain  
172 HIV-encephalopathy. These cases concern a woman and a man with a long HIV disease



173 course, either treated or not respectively, as well as a treated woman for whom disease  
174 duration is unknown.

175 Firstly, we found that HIV proviral-DNA load varied both among different cases and among  
176 different brain areas of a single case as previously reported for HIV-RNA load in different  
177 brain regions of HIV+ cases [19]. However, the highest rates were not necessarily found in  
178 the same areas among studies and such discordance may be expected because DNA load  
179 reflects the size of the viral reservoir and not cell-free replicating virus [20]. In C1, although  
180 HIV proviral-DNA load was undetectable, sequencing and viral diversity analysis were  
181 effective in 3 of the 4 samples. This discrepancy may be explained by a higher sensibility of  
182 nested PCR compared to real-time PCR or the use of different primers between the two  
183 techniques.

184 Although RT1 sequencing for the C1's and C2's cerebellum and C2's thalamus specimens,  
185 RT2 for all cases and V3 for C2 and C3 failed, our results of viral diversity and tropism were  
186 consistent with those previously obtained by SMRT on an HIV+/cART+ case diagnosed with  
187 HIV encephalopathy. Indeed, the authors showed by sequencing full-length envelope gene  
188 that frontal lobe sequences clustered independently of occipital and parietal lobes and all of  
189 them were predicted to use CCR5 co-receptor while most non-brain sequences were predicted  
190 to use CXCR4 co-receptor. In our study, the majority of brain areas harbored a distinct HIV-  
191 subpopulation and those with effective V3 sequencing showed that strains used CCR5 co-  
192 receptor. While some variants isolated from caudate nucleus were intermixed to various  
193 degrees with sequences from spinal cord, frontal lobe or cerebellum region, brainstem  
194 (substantia nigra and medulla oblongata) harbored a specific subpopulation in C2 and C3.  
195 Overall, our results confirm previous evidence by Sanger-sequencing that several HIV-  
196 reservoirs exist within the CNS [13,14] and prove a high intra-regional and inter-regional  
197 viral diversity just like a study based on SMRT [12], reflecting persistent viral replication in

198 the CNS. Compartmentalization is evident in all of our three cases regardless of treatment  
199 status. However, in C2, who received no treatment, there is a clear separation of viral  
200 population between the two compartments examined, while in C1 and C3 who received  
201 treatment, we note some common viral strains between two regions.

202 HIV-1 resistance mutations to antiretroviral drugs were reported to be regionally distributed  
203 in diverse areas of the brain by classical sequencing (15). Our study, detecting minority  
204 variants up to 1% by UDS, found similar results with different distribution of resistance  
205 mutations among brain areas of the same case. These results suggest that selection pressure  
206 may vary across brain compartments and that antiretroviral treatment does not penetrate  
207 equally all of them. Finally, resistance mutations were expected in C1 and C3 who received  
208 treatment unlike C2 for whom no mutation was found.

209

210 In conclusion, this study shows significant inter-regional and intra-regional viral  
211 diversity and confirms HIV-compartmentalization in different brain areas already shown by  
212 studies based on classical sequencing suggesting that there are several reservoirs within the  
213 CNS.

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225 **ACKNOWLEDGEMENTS**

226

227 **Funding.** This work was funded by the *Agence Nationale de Recherche sur le SIDA et les*  
228 *Hépatites Virales* (ANRS) AC43 “Next-generation sequencing and resistance” working  
229 group.

230

231 **Authors contributions.** Conception and design of the study: IP, VC, DS, AGM; acquisition  
232 and analysis of the data: EG, BA, ND, RP, AJ; drafting of significant portion of the  
233 manuscript or figures: EG, AJ. All the authors read, corrected and approved the final  
234 manuscript.

235

236 **Conflicts of interest.** No conflicts of interest to disclose.

237

238 **Meeting presentation.** This work was presented as an e-poster at the Conference on  
239 Retroviruses and Opportunistic Infections (CROI), in March 2020 (poster no 425).

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325 **Table 1:** HIV-1 reservoir quantification, viral diversity and resistance mutations among the  
 326 different brain areas of cases 1, 2 and 3.

	CASE 1		CASE 2	CASE 3	
	<b>Temporal lobe</b>			<b>Frontal lobe</b>	
<b>HIV proviral DNA</b> (copies/10 <sup>6</sup> cells)	<40		91	544	
<b>HIV_RT1_CS</b> No No shared (%)	1086 1 (0.09) with CN		3259 3 (0.09) with MO	2624 527 (20.1) with CN and Cer 55 (2.1) only with CN 83 (3.2) only with Cer	
<b>HIV_RT1_CS100</b> No No shared (%)	2 0 (0)		146 0 (0)	30 7 (23.3) with CN and Cer 3 (10) only with Cer	
<b>Resistance mutation</b> Nucleotide substitution (position) coverage % reads carrying mutation No of reads carrying mutation	0 - - - -		0 - - - -	0 - - - -	
	<b>Caudate nucleus</b>		<b>Thalamus</b>	<b>Caudate nucleus</b>	
<b>HIV proviral DNA</b> (copies/10 <sup>6</sup> cells)	<40		<40	130	
<b>HIV_RT1_CS</b> No No shared (%)	2590 15 (0.6) with SC 1 (0.04) with TL		Not amplified	2451 527 (21.5) with FL and SN 234 (9.5) only with Cer 55 (2.2) only with FL	
<b>HIV_RT1_CS100</b> No No shared (%)	141 2 (1.4) with SC		Not amplified	60 7 (11.7) with FL and Cer 9 (15) only with Cer	
<b>Resistance mutation</b>	<b>M41 L</b>	<b>V90 I</b>	Not amplified	<b>V90 I</b>	<b>V106 I</b>
Nucleotide substitution (position)	A-C (586)	G-A (733)		G-A (733)	G-A (781)
coverage	289 139	552 684		328 900	328 913
% reads carrying mutation	98,4	96,7		16,2	1
No of reads carrying mutation	284 513	534 445		53 282	3 289
	<b>Cerebellum</b>				
<b>HIV proviral DNA</b> (copies/10 <sup>6</sup> cells)	<40		<40	23	
<b>HIV_RT1_CS</b> No No shared (%)	Not amplified		Not amplified	3526 527 (14.9) with FL and CN 234 (6.6) only with CN 83 (2.4) only with FL	
<b>HIV_RT1_CS100</b> No No shared (%)	Not amplified		Not amplified	73 7 (9.6) with FL and CN 9 (12.3) only with CN 3 (4.1) only with FL	
<b>Resistance mutation</b> Nucleotide substitution (position) coverage % reads carrying mutation No of reads carrying mutation	Not amplified		Not amplified	0 - - - -	
	<b>Spinal cord</b>		<b>Medulla oblongata</b>	<b>Substantia nigra</b>	
<b>HIV proviral DNA</b> (copies/10 <sup>6</sup> cells)	<40		31	92	
<b>HIV_RT1_CS</b> No No shared (%)	3453 15 (0.4) with CN		3189 3 (0.09) with TL	1650 0 (0)	
<b>HIV_RT1_CS100</b> No No shared (%)	158 2 (1.3) with CN		72 0 (0)	15 0 (0)	
<b>Resistance mutation</b>	<b>M41 L</b>	<b>V90 I</b>	0	<b>M41 L</b>	<b>V90 I</b>
Nucleotide substitution (position)	A-C (586)	G-A (733)	-	A-C (586)	G-A (733)
coverage	384 658	618 797	-	62 637	100 327
% reads carrying mutation	98,4	97,6	-	2,3	1,6
No of reads carrying mutation	378 503	603 946	-	1 441	1 605

327

328 *Cer: cerebellum; CN: caudate nucleus; CS: consensus sequences; DNA: desoxyribonucleic acid; FL: frontal*  
329 *lobe; HIV: human immunodeficiency virus; MO: medulla oblongata; No: number; SC: spinal cord; SN:*  
330 *substantia nigra; TL: temporal lobe; -: not applicable; No: number*  
331 *HIV\_RT1\_CS: all cleaned consensus sequences of RT1 fragment; HIV\_RT1\_CS100: all cleaned consensus*  
332 *sequences of RT1 fragment after filtering out consensus sequences found less than 100 times*  
333 *In case 1, the majority of caudate nucleus's and spinal cord's variants shared the same resistance mutations*  
334 *M41L and V90I. M41L: the substitution of methionine for leucine in position 41 of RT1 confers resistance to*  
335 *ZDV, ABC and TDF/FTC (NRTIs) on condition that this substitution is associated to two others specific*  
336 *mutations within the RT gene. V90I: The substitution of valine for isoleucine in position 90 of RT1 confers*  
337 *resistance to ETR (NNRTI) only if two others mutations are presents within the RT gene. No resistance mutation*  
338 *was identified in any of brain areas studied in case 2 (temporal lobe and medulla oblongata). Case 3 presented*  
339 *resistance mutations only in caudate nucleus and substantia nigra. V106I: The substitution of valine for*  
340 *isoleucine in position 106 confers resistance to ETR only if associated to two others specific mutations of the RT*  
341 *gene.*  
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354 **Figure 1: Approximately maximum-likelihood phylogenetic trees constructed with**  
355 **Fastree (2.1) of RT1 consensus viral sequences issued from the different brain areas.**  
356 Phylogenetic trees were inferred with viral consensus after filtered out those found less than  
357 100 times (HIV\_RT1\_CS100 or HIV\_V3\_CS100). **A.** RT1 of C1, **B.** V3 of C1, **C.** RT1 of  
358 C2, **D.** RT1 of C3.

359 *Branches are colored according to the tissue origin as follow: red: caudate nucleus; blue (C1*  
360 *and C2: temporal lobe, C3: frontal lobe); green: spinal cord; yellow: brainstem (C2: medulla*  
361 *oblongata and C3: substantia nigra); pink: cerebellum*

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379 **Supplementary Figure 1: Approximately maximum-likelihood phylogenetic trees**  
380 **constructed with Fastree (2.1) of RT1 consensus viral sequences issued from the**  
381 **different brain areas.** Phylogenetic trees were inferred with all viral consensus found  
382 (HIV\_RT1\_CS or HIV\_V3\_CS). **A.** RT1 of C1, **B.** V3 of C1, **C.** RT1 of C2, **D.** RT1 of C3.  
383 *Branches are colored according to the tissue origin as follow: red: caudate nucleus; blue (C1*  
384 *and C2: temporal lobe, C3: frontal lobe); green: spinal cord; yellow: brainstem (C2: medulla*  
385 *oblongata and C3: substantia nigra); pink: cerebellum*

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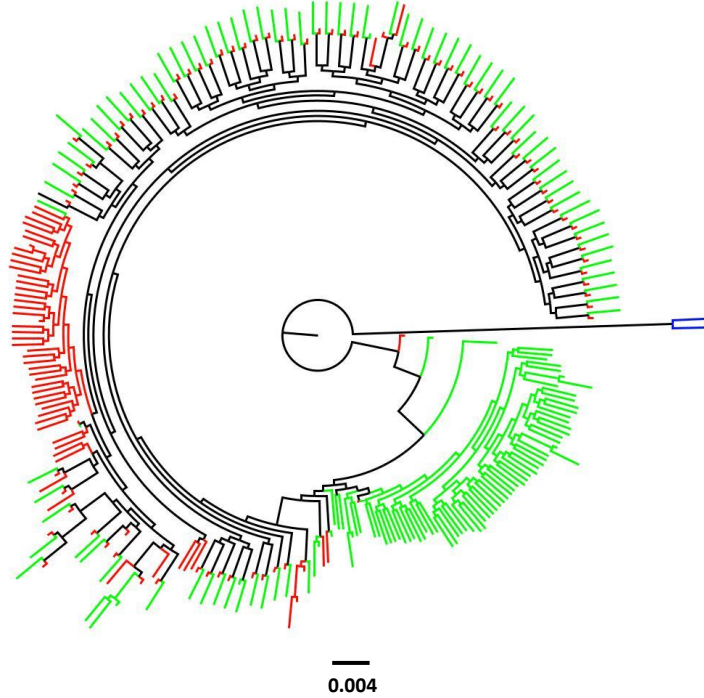
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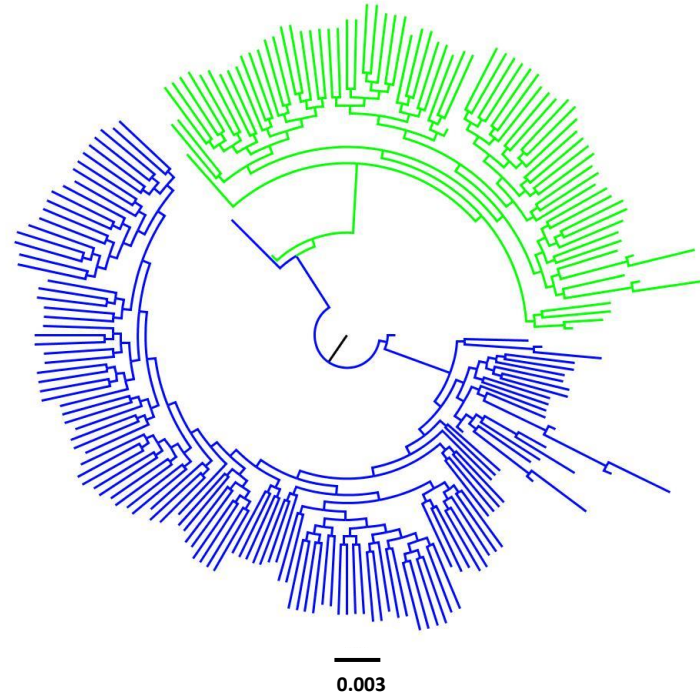
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405 **Supplementary Figure 2: Maximum-likelihood phylogenetic tree constructed with**  
406 **PhyML of RT1 nucleotide sequences issued from the different brain areas.** *Phylogenetic*  
407 *tree were inferred with nucleotide sequences generated by Sanger sequencing and rooted with*  
408 *HXB2 reference sequence. Branches are colored according to the tissue origin as follow: red:*  
409 *caudate nucleus; blue (C1 and C2: temporal lobe, C3: frontal lobe); green: spinal cord;*  
410 *yellow: brainstem (C2: medulla oblongata and C3: substantia nigra); pink: cerebellum*  
411 *Nodes presenting a branch support > 70% (bootstrap analysis with 1000 replicates) are*  
412 *indicated by an asterisk.*  
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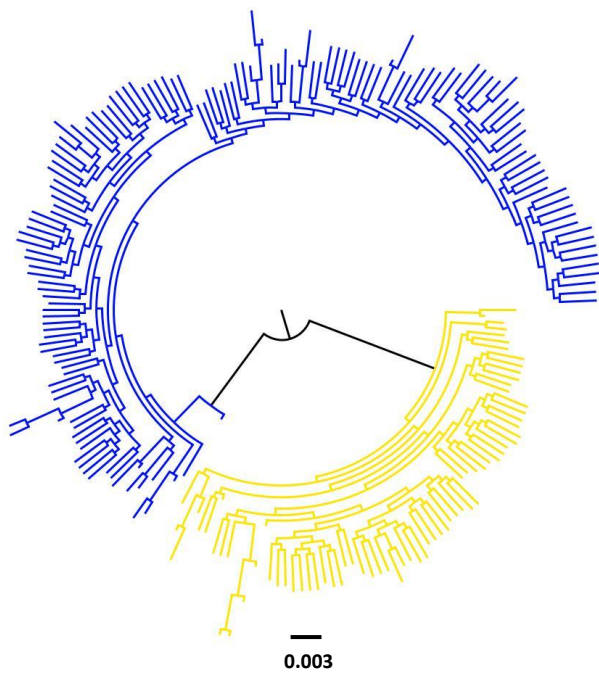
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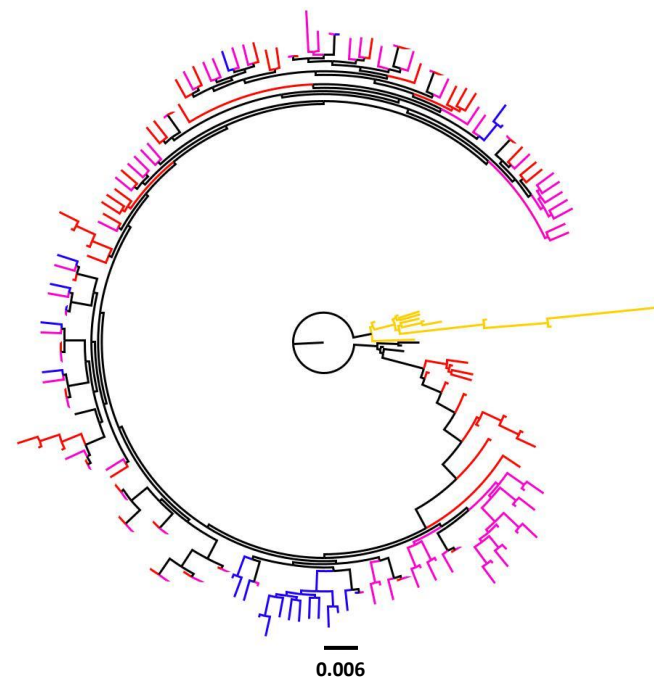
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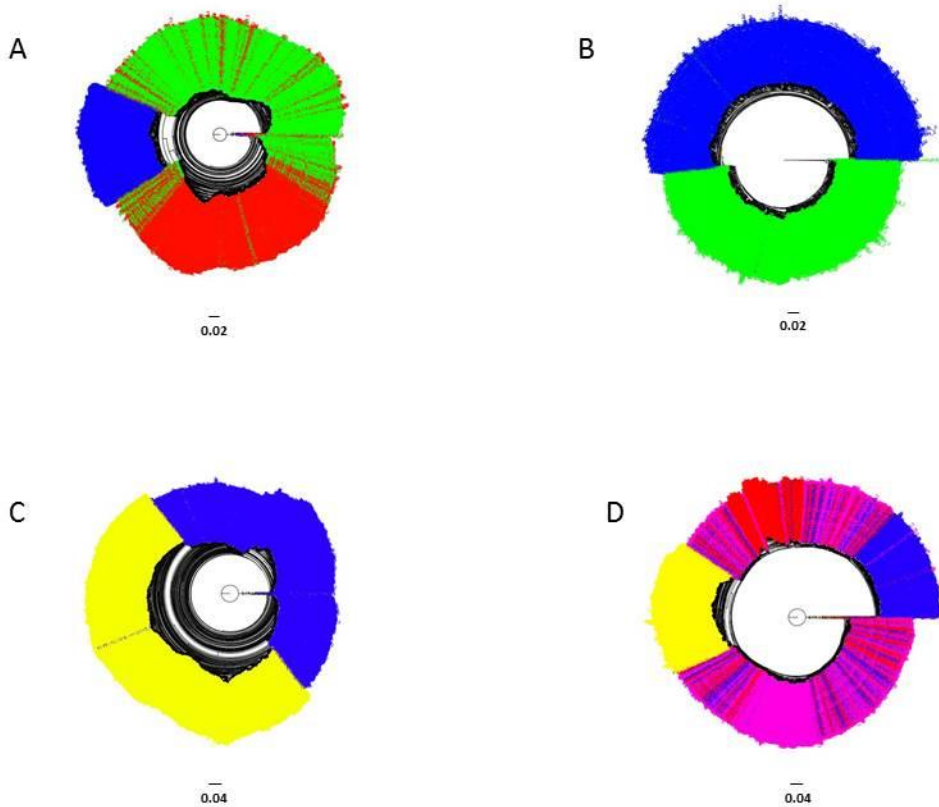
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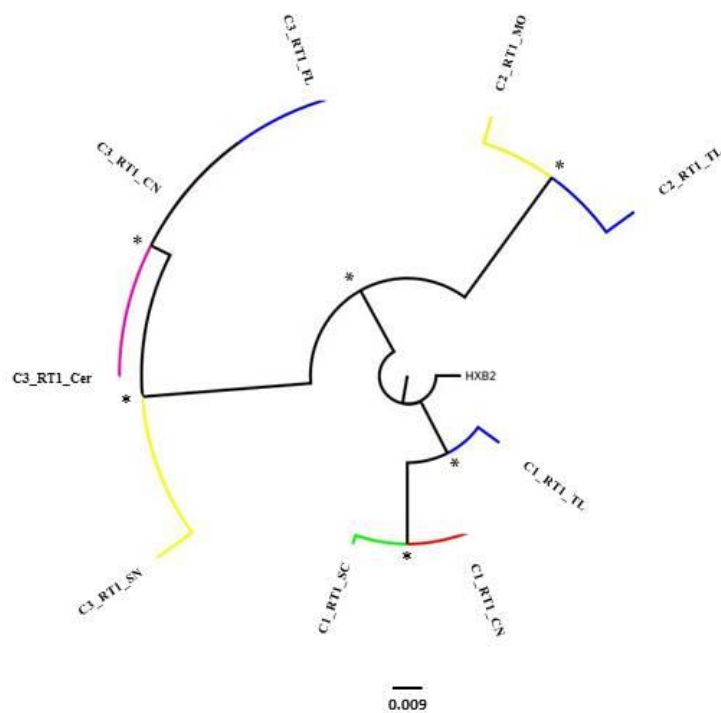
D



**Supplementary Figure 1: Approximately maximum-likelihood phylogenetic trees constructed with Fasttree (2.1) of RT1 consensus viral sequences issued from the different brain areas. Phylogenetic trees were inferred with all viral consensus found (HIV\_RT1\_CS or HIV\_V3\_CS). A. RT1 of C1, B. V3 of C1, C. RT1 of C2, D. RT1 of C3. Branches are colored according to the tissue origin as follow: red: caudate nucleus; blue (C1 and C2: temporal lobe, C3: frontal lobe); green: spinal cord; yellow: brainstem (C2: medulla oblongata and C3: substantia nigra); pink: cerebellum**



**Supplementary Figure 2: Maximum-likelihood phylogenetic tree constructed with PhyML of RT1 nucleotide sequences issued from the different brain areas.** *Phylogenetic tree were inferred with nucleotide sequences generated by Sanger sequencing and rooted with HXB2 reference sequence. Branches are colored according to the tissue origin as follow: red: caudate nucleus; blue (C1 and C2: temporal lobe, C3: frontal lobe); green: spinal cord; yellow: brainstem (C2: medulla oblongata and C3: substantia nigra); pink: cerebellum* Nodes presenting a branch support >70% (bootstrap analysis with 1000 replicates) are indicated by an asterisk



**Supplementary Table 1: Participants' characteristics and selected brain areas**

Case	Age (years)	HIV diagnosis	Year of death	Death cause	cART	Selected brain areas	Anatomopathology
1	48	1996	2006	Pulmonary Embolism	Yes	Temporal lobe Caudate nucleus Cerebellum Spinal cord	Microglial activation and rare MGC positive for P24 antigen
2	38	2001	2010	Sepsis/ARDS	None	Temporal lobe Thalamus Cerebellum Medulla oblongata	Rare toxoplasma cysts without necrosis associated with numerous microglial nodules
3	29	Unknown	2007	Pulmonary Embolism	Yes	Frontal lobe Caudate nucleus Cerebellum Substantia nigra	Rare toxoplasma cysts without necrosis associated with numerous microglial nodules

*ARDS: Acute respiratory distress syndrome. cART: combination antiretroviral therapy. MGC: multinucleated giant cells; HIV: human immunodeficiency virus; ART: antiretroviral therapy; MGC: multinucleated giant cells*

**Supplementary Table 2:** Experimental conditions for the amplification by nested PCR of RT1, RT2 and V3 region before sequencing on MiSeq Illumina® system.

Amplified fragments		Primers	Direction	Sequences (5'-3')		
RT1	Outer	Forward		TAG TCC TAT TGA RAC TGT ACC AGT		
		Reverse		ATC CTA CAT ACA ART CAT CCA TG		
	Inner	Forward		<b>AAG ACT CGG CAG CAT CTC</b> CAA TGG CCA TTG ACA GAA GAA A		
		Reverse		<b>GCG ATC GTC ACT GTT CTC</b> CAT GGA ATA TTG CTG GTG ATC C		
RT2	Outer	Forward		AGT CTT TTG ATG GGT CAT AAT A		
		Reverse		GGG ARG TYA ATT AGG AAT ACC		
	Inner	Forward		<b>AAG ACT CGG CAG CAT CTC</b> CAG ATG TGG GGA TGC ATA TTT		
		Reverse		<b>GCG ATC GTC ACT GTT CTC</b> CAC TGT ATG TCA TTG ACA GTC CAG		
V3	Outer	Forward		CAG TAC AAT GTA CAC ATG G		
		Reverse		ATG GGA GGG GCA TAC ATT G		
	Inner	Forward		<b>AAG ACT CGG CAG CAT CTC</b> CAT TAC AGT AGA AAA ATT CCC CTC		
		Reverse		<b>GCG ATC GTC ACT GTT CTC</b> CAA ATG GCA GTC TAG CAG AAG		
<b>RT1 or RT2 PROTOCOL AMPLIFICATION</b>				<b>V3 PROTOCOL AMPLIFICATION</b>		
<b>1<sup>st</sup> PCR</b>				<b>1<sup>st</sup> PCR</b>		
RT-PCR	50°C	30 min	RT-PCR	50°C	30 min	
denaturation	94°C	7 min	denaturation	94°C	7 min	
35 cycles	94°C	10 sec	35 cycles	94°C	10 sec	
	55°C	30 sec		53°C	30 sec	
	68°C	1 min		68°C	1 min	
1 cycle	68°C	7 min	1 cycle	68°C	7 min	
<b>Nested PCR</b>				<b>Nested PCR</b>		
denaturation	98°C	1 min	denaturation	98°C	1 min	
3 cycles	98°C : 10 sec ; 66-64°C : 30sec ; 72°C : 15sec		40 cycles	98°C	10 sec	
3 cycles	98°C: 10sec ; 64-62 °C: 30sec ; 72°C: 15sec			60°C	30 sec	
3 cycles	98°C: 10sec ; 62-60 °C: 30sec ; 72°C: 15sec			72°C	20 sec	
30 cycles	98°C: 10sec ; 60 °C: 30sec ; 72°C: 15sec		1 cycle	72°C	2 min	
1 cycle	72°C	7 min		-		

*Min : minute ; sec : second ; PCR : polymerase chain reaction ; RT-PCR: reverse transcriptase PCR*

*Universal Adapters necessary for libraries' preparation are represented in bold.*

**Supplementary Table 3:** V3 viral diversity and tropism among two different brain area of case 1

Brain area	HIV_V3_CS		HIV_V3_CS100		HIV1-tropism		
	No of CS	Consensus sequences shared	No of CS	Consensus sequences shared	CCR5 (FPR>10%)	Undetermined (5%<FPR<10%)	CXCR4 (FPR<5%)
Temporal lobe	2086	3 (0.14) with TL	131	0	126 (96%)	5 (6%)	0
Caudate nucleus	Not amplified	-	Not amplified	-	-	-	-
Cerebellum	Not amplified	-	Not amplified	-	-	-	-
Spinal cord	1944	3 (0.15) with SC	77	0	72 (94%)	5 (4%)	0

*CS: consensus sequences; DNA: desoxyribonucleic acid; FPR: false positive rate; HIV: human*

*immunodeficiency virus; No: number; SC: spinal cord; TL: temporal lobe; -: not applicable*

*HIV\_RT1\_CS: all cleaned consensus sequences of RT1 fragment; HIV\_RT1\_CS100: all cleaned consensus sequences of RT1 fragment after filtering out consensus sequences found less than 100 times*



**Supplementary Table 4:** Synonymous and non-synonymous polymorphisms not conferring resistance to Nucleoside and Non-Nucleoside Reverse Transcriptase Inhibitors in different brain areas of cases 1, 2 and 3.

CASE 1								
	TEMPORAL LOBE		CAUDATE NUCLEUS		SPINAL CORD			
Polymorphism	D67 D	K65 K	D67 D	K65 K	D67 D	L74 L		
Nucleotide substitution (position)	C -T (666)	A-G (660)	C-T (666)	A-G (660)	C-T (666)	T-C (685)		
Coverage (number of reads)	105 496	289 746	293 115	379 378	377 542	371 256		
% Reads carrying mutation	98,5	98,1	97	97,6	98,7	1,2		
Number of reads carrying mutation	103 914	284 241	284 322	370 273	372 634	4 455		
CASE 2								
	TEMPORAL LOBE							
Polymorphism	K65 E	D67 D	T69 T	L74 L	V90 V	A98 A	E138 G	
Nucleotide substitution (position)	A-G (658)	C-T (666)	T-G (672)	T-C (685)	T-C (735)	A-G (759)	A-G (878)	
Coverage (number of reads)	374 271	379 027	745 373	745 392	745 393	745 393	371 453	
% Reads carrying mutation	1,2	97,2	9,2	92,7	93,5	94	1,5	
Number of reads carrying mutation	4 491	368 414	68 574	690 978	696 942	700 669	5 572	
	MEDULLA OBLONGATA							
Polymorphism	D67 D	L74 L	V90 V	A98 A	K101 K			
Nucleotide substitution (position)	C-T (666)	T-C (685)	T-C (735)	A-G (759)	A-G (768)			
Coverage (number of reads)	319 846	312 409	488 146	504 794	505 950			
% reads carrying mutation	98,5	1,4	95,1	96,3	1,2			
Number of reads carrying mutation	315 048	4 374	464 227	486 117	6 071			
CASE 3								
	FRONTAL LOBE							
Polymorphism	D67 D	L74 L	A98 A	L100 L	K101 K	K103 K		
Nucleotide substitution (position)	C-T (666)	T-C (685)	A-G (759)	T-C (763)	A-G (768)	A-G (774)		
Coverage (number of reads)	215 992	212 686	364 358	364 746	364 304	361 107		
% Reads carrying mutation	98,5	97,4	96,9	97,5	98,1	97,2		
Number of reads carrying mutation	212 752	207 156	353 063	355 627	357 382	350 996		
	CAUDATE NUCLEUS							
Polymorphism	M41 V	D67 D	T69 T	L74 L	A98 A	L100 L	K101 K	K103 K
Nucleotide substitution (position)	A-G (586)	C-T (666)	T-G (672)	T-C (685)	A-G (759)	T-C (763)	A-G (768)	A-G (774)
Coverage (number of reads)	175 388	176 620	328 900	328 900	328 900	328 900	328 900	328 912
% Reads carrying mutation	1,5	98,1	7,4	94,1	96	96,8	97,2	96,2
Number of reads carrying mutation	2 631	173 264	24 339	309 495	315 744	318 375	319 691	316 413
	CEREBELLUM							
Polymorphism	D67 D	L74 L	A98 A	L100 L	K101 K (768)		K103 K (774)	
Nucleotide substitution (position)	C-T (666)	T-C (685)	A-G (759)	T-C (763)	A-G (768)		A-G (774)	
Coverage (number of reads)	286 874	282 159	486 300	486 737	486 197		482 086	
% Reads carrying mutation	98,8	97,9	97,3	98	98,4		97,5	
Number of reads carrying mutation	283 431	276 234	473 170	477 002	478 418		470 034	
	SUBSTANTIA NIGRA							
Polymorphism	K65 K	D67 D	L74 L	A98 A	L100 L	K101 K	K103 K	Y115 Y
Nucleotide substitution (position)	A-G (660)	C-T (666)	T-C (685)	A-G (759)	T-C (763)	A-G (768)	A-G (774)	T-C (810)
Coverage (number of reads)	61 055	60 803	60 047	101 772	101 687	101 451	100 209	55 050
% Reads carrying mutation	1,5	97,5	95,7	94,9	96,2	88,9	95,6	88,5
Number of reads carrying mutation	916	59 283	57 465	96 582	97 823	90 190	95 800	48 719