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Highly Sensitive Method for Amplification of Human Immunodeficiency Virus Type 2 DNA

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We evaluated a new human immunodeficiency virus type 2 (HIV-2) DNA amplification strategy based on peripheral blood mononuclear cell long PCR (XL PCR) followed by nested PCR amplification. The primers used were located in the highly conserved long terminal repeat and in the *pol* regions of the genome. Five primer pairs corresponding to different regions of the HIV-2 *env* gene were used in the nested step. Samples from 42 patients were tested, which yielded positive amplification with at least two primer pairs in 40 (95%) samples. A primer pair (EB2/EB5) located on the V3 region succeeded in amplifying proviral DNA in 40 samples.

The worldwide spread of human immunodeficiency virus type 1 (HIV-1) contrasts with the limited circulation of HIV-2, which is mainly restricted to West Africa (5). Most cases of HIV-2 infection diagnosed outside Africa occurred in France and Portugal (8). The vertical and heterosexual HIV-2 transmission rates are lower than those for HIV-1 (10, 12). The incubation period of HIV-2 is longer than that of HIV-1 (1). The HIV-2 reduced replicative capacity supports these epidemiological and clinical pieces of evidence that HIV-2 is less pathogenic than HIV-1 (15), even at the AIDS stage (11). Phylogenetic studies have shown a relatively wide diversity of HIV-2 lentiviruses, and six viral subtypes have been described (4, 6).

Together with the low replicative capacity, viral variability contributes to the inefficiency of direct virological diagnosis. HIV-2 is rarely isolated from peripheral blood mononuclear cells (PBMCs) (15), and the detection rates of proviral DNA by PCR amplification vary between 50 and 80% (7, 9) according to clinical and immunological status. A highly sensitive test is badly needed for diagnosis, mainly in HIV-2 vertical infection screening and dual HIV-1-HIV-2 seropositivity differentiation. We describe a new PCR strategy based on long PCR (XL PCR) followed by different nesting steps to improve the sensitivity of HIV-2 DNA detection.

Patients. The study group was composed of 41 patients included in the French National HIV-2 Cohort and one recent HIV-2 seroconverter. These patients (20 males and 22 females) originated from various countries in West Africa (Burkina Faso, $n = 1$; Cape Verde Islands, $n = 4$; Congo, $n = 2$; Gambia, $n = 1$; Guinea-Bissau, $n = 5$; Ivory Coast, $n = 10$; Mali, $n = 6$; Senegal, $n = 4$), North Africa (Morocco, $n = 1$), and Europe (France, $n = 7$; Poland, $n = 1$). According to the Centers for Disease Control and Prevention (CDC) classification (3), 34 patients were in stage A and 8 were in stage C. Thirteen patients had $CD4^+$ cell counts above 500×10^6/liter, 14 patients had counts between 200×10^6 and 500×10^6/liter, and 15 patients had counts below 200×10^6/liter. Ten patients received monotherapy, 5 were on bitherapy, and 1 was on tritherapy.

Samples. Whole blood was collected from all of the patients, and PBMCs were isolated by Ficoll-Hypaque density gradient centrifugation. DNA was extracted with phenol-chloroform, precipitated with ethanol, and quantified spectrophotometrically. Cellular and plasma viral cultures were carried out with each sample, and quantitative cellular viremia and plasma viremia were determined as previously described (15).

XL PCR. HIV-2 DNA in fresh PBMCs was amplified by nested PCR with first-round XL PCR. The Gene Amp XL PCR kit is optimized to produce high yields of long (XL) PCR products. The enzyme is designated to amplify target DNA sequences ranging from 5 kb to more than 40 kb. The assay was performed according to the manufacturer's instructions (Perkin-Elmer, Applied Biosystems, Alameda, Calif.). We designed the following primer pairs for use in the first amplification step: PFD1 (5′)/long terminal repeat (LTR9574) (3′) and LTR1 (5′)/LTR2 (3′) (Table 1). One microgram of genomic DNA was subjected to the first-round amplification. The PCR conditions consisted of initial denaturation at 94°C for 1 min, followed by 16 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 1 min, and extension at 68°C for 5 min. The subsequent 24 cycles were carried out under the same conditions with incremental lengthening of the extension time (15 s per cycle) and a last extension cycle at 68°C for 10 min. HLA-DQα was routinely amplified as a PCR efficacy control.

Nested PCR. Different pairs of primers were used for nested PCR (Table 1). The nested PCR procedure consisted of amplification of 5 μl of the first XL PCR product in a final volume of 100 μl with 10 μl of PCR buffer, 200 μM (each) deoxynucleoside triphosphates, 30 pmol of internal primers, and 2 U of *Taq* polymerase. The PCR conditions were as follows: 40 amplification cycles with denaturation at 94°C for 5 min, followed by 38 cycles (30 s at 94°C, 30 s at 55°C, and 30 s at 72°C), and a last extension cycle at 72°C for 7 min. The five primer pairs used in the nested PCR were EB0/EB6, EB2/EB5, EB7/ENVE, ENVA/ENVB, and ENVF/ENVG (Table 1). DNA from negative cells (PBMCs from healthy blood donors) was introduced into each reaction. Nested PCR products were analyzed on a 1.4% agarose gel with ethidium bromide staining.

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TABLE 1. Oligonucleotide sequences for XL and nested PCR amplification of HIV-2 proviral DNA

Primer	Position	Orientation	Nucleotide sequence	PCR type	Source or reference
PFD1	4729–4749	Sense	5′-GGTCTATTWCAGAGAAGGCAG-3′	XL	In house
LTR9574	9574–9592	Antisense	5′-TGGTGAGAGTCTAGCAGGG-3′	XL	Berry et al. (2)
LTR1	75–96	Sense	5′-TTCCCTGCTRGACTCTCACCAG-3′	XL	In house
LTR2	8935–8953	Antisense	5′-ACATCCCTTCCAGTCCCCC-3′	XL	In house
EB0	6300–6324	Sense	5′-ATACAGTGCTTRCCAGACAATGATG-3′	Nested	In house
EB6	6945–6963	Antisense	5′-CCATTRAAGCCAAACCAWGT-3′	Nested	In house
EB2	6783–6804	Sense	5′-TCATGTGAYAARCAYTATTGGG-3′	Nested	In house
EB5	7310–7329	Antisense	5′-CTCCTCTGCAGTTAGTCCAC-3′	Nested	In house
EB7	7149–7165	Sense	5′-CCYAGGCAAGCATGGTG-3′	Nested	In house
ENVE	7920–7939	Antisense	5′-GCACATCCCCATGAATTTAG-3′	Nested	In house
ENVA	7691–7723	Sense	5′-GCTAGGGTTCTTGGGTTTTCTCGC(AG)ACAGCAGG-3′	Nested	Gao et al. (6)
ENVB	8415–8425	Antisense	5′-CAAGAGGCGTATCAGCTGGCGGATCAGGAA-3′	Nested	Gao et al. (6)
ENVF	8265–8291	Sense	5′-TATAGGCCWGTTTTCTCTTCCCCYCCC-3′	Nested	In house
ENVG	8557–8580	Antisense	5′-CTTCTTGGATCCACTCGCACCCAT-3′	Nested	In house

The virus was isolated from PBMCs of 25 of the 42 patients (60%). The frequency of virus isolation from PBMCs correlated negatively ($P < 0.05$) with the $CD4^+$ cell count. Cultures were positive for 2 of 13 (15%) patients with $CD4^+$ counts of $>500 \times 10^6$/liter and for none of those receiving antiretroviral treatment. For the 14 patients with $CD4^+$ counts ranging from 200×10^6 to 500×10^6/liter, cultures were positive for 11 (78%) patients, 2 of whom were receiving antiretroviral treatment. Cultures were positive for 12 (80%) of the 15 patients with $CD4^+$ cell counts below 200×10^6/liter, all of whom were receiving either one ($n = 8$) or two ($n = 4$) antiretroviral drugs. Of the 25 patients with a positive HIV-2 culture, 19 were symptom free, and 6 were in stage C of the CDC classification (Table 2).

The mean $CD4^+$ count in the 17 patients with negative cultures was 637×10^6/liter (range, 139×10^6 to $1{,}515 \times 10^6$/liter); 15 patients were symptom free (stage A), and 2 were in stage C. Fifteen of the 17 patients with negative cultures had never received antiretroviral therapy. The virus was isolated from the plasma of 4 of 41 (10%) patients, all of whom had $CD4^+$ cell counts below 200×10^6/liter (mean, 75×10^6/liter; range, 13×10^6 to 155×10^6/liter). Two of these patients were symptom free (stage A3), and the other two were at stage C. All four patients were on monotherapy (Table 2).

The PCR strategy consisted of first-round amplification with primer pair PFD1/LTR9574. When the nested PCR was negative with these primers, a new XL PCR procedure was performed with LTR1/LTR2.

The results of nested PCR with the different primer pairs are presented in Tables 2 and 3. The test was positive with at least one primer pair for 40 of 42 (95%) patients. The results of proviral DNA amplification are presented in Table 2 according to clinical and virological data. Twenty-nine samples, of which 7 were from culture-negative patients, were positive with all five primer pairs. Five samples, of which three were from culture-negative patients, were amplified by four primer pairs. Four samples, one of which was from a culture-negative patient, were positive with three primer pairs. Finally, two samples, both from culture-negative patients, were amplified by only two primer pairs (Table 3).

None of the primer pairs gave a positive reaction with samples from two patients. Both patients were symptom free (stage A) and previously untreated. Both patients were negative for cellular and plasma viremias, and the $CD4^+$ cell counts were 705×10^6 and 700×10^6/liter.

Positive amplification of HLA-DQα confirmed the accuracy of the PCR approach.

HIV-2 genetic diversity and the reduced replicative capacity of the virus, reflected by low cellular and plasma virus loads (2, 15), pose problems for proviral DNA detection in PCR-based assays. To improve the capacity of PCR approaches for detection of HIV-2 DNA, we established a new strategy based on

TABLE 2. Correlations between clinical and virological data in 42 HIV-2-infected patients

Patient characteristic (*n*)	No. of patients positive for viremia/no. tested		No. of patients with XL PCR result[a]				
	Cellular	Plasma	5/5	4/5	3/5	2/5	0/5
CDC stage							
A1 (11)	2/11	0/11	5	2	2	1	1
A2 (13)	10/13	0/13	10	1	2	0	0
A3 (10)	7/10	2/10	8	1	0	1	0
C1 (2)	0/2	0/2	0	1	0	0	1
C2 (1)	1/1	0/1	1	0	0	0	0
C3 (5)	5/5	2/5	5	0	0	0	0
Treatment							
Monotherapy (10)	9/10	4/10	10	0	0	0	0
Bitherapy (5)	5/5	0/5	5	0	0	0	0
Tritherapy (1)	0/1	0/1	0	1	0	0	0
Nontreated (26)	11/26	0/26	14	4	4	2	2

[a] XL PCR results are given as number of tests positive out of five primer pairs tested.

TABLE 3. HIV-2-positive amplification by nested PCR with different primer pairs

No. of samples	Result with primer pair[a]:				
	ENVA/ENVB	EB2/EB5	EB7/ENVE	ENVF/ENVG	EB0/EB6
29	+	+	+	+	+
3	+	+	+	−	+
2	+	+	+	+	−
2	+	+	+	−	−
2	+	+	−	+	−
2	−	+	−	+	−

[a] +, HIV-2 positive; −, HIV-2 negative.

the use of XL PCR, followed by a nested PCR step with several primer pairs. This strategy allowed us to bypass HIV-2 diversity, because the primers used in first amplification step correspond to apparently conserved regions in the LTR and *pol* genes (13). The use of different primer pairs in the nested PCR step also improved the detection rate and increased the amount of proviral DNA, which could be very small in clinical samples. With this strategy, we obtained positive results for 95% of the patients (40 of 42). For 73% of the patients (29 of 40), all five primer pairs used in the nested PCR step yielded positive amplification (Table 3).

The primers we designed for use in the nested PCR step were located in different regions of the *env* gene (13) (Table 1). The best results were obtained by using the EB2/EB5 primer pair located in the V3 region of the *env* gene, which gave positive results for 40 of 42 patients. These results confirm that the HIV-2 V3 region is highly conserved, contrary to the HIV-1 V3 region (13). The ENVA/ENVB primer pair (6) amplified proviral DNA in 38 of 42 samples. These primers are located in the conserved gp36 coding region of *env*. EB0/EB6, based on sequences located in the V1 to V2 region of the *env* gene, was the least efficient primer pair (32 of 42 samples), probably because of the known variability of the V1-to-V2 region of the HIV-2 *env* gene.

Grankvist et al. (7) suggested that the low efficiency of PCR amplification of the HIV-2 genome relative to HIV-1 was due to the presence of fewer than 1 infected cell per 100,000. In their study, Grankvist et al. obtained positive nested PCR amplification in 83.7% of samples by using primers originating in the *pol* gene (7), a value similar to that obtained by other study groups (i.e., 84% [14] and 88% [9]). Our strategy, based on the use of XL PCR in the first step and nested PCR with primers from the *env* gene, succeeded in amplifying the proviral DNA in 95% of samples and avoided the use of radioactive probes for the nested step. Furthermore, direct amplification of HIV-2 DNA from fresh PBMCs makes this method widely accessible. Our amplification approach should be useful for HIV-2 diagnosis and follow-up of vertically infected children. This strategy might equally improve the efficiency of the type differentiation in case of dual serological reactivities.

REFERENCES

1. **Ancelle, R., O. Bletry, A. C. Baglin, F. Brun-Vézinet, M. A. Rey, and P. Godeau.** 1987. Long incubation period for HIV-2 infection. Lancet **i:**688–689.
2. **Berry, N., K. Ariyoshi, O. Jobe, P. T. Ngum, T. Corrah, A. Wilkins, H. Whittle, and R. Tedder.** 1994. HIV type 2 proviral load measured by quantitative polymerase chain reaction correlates with CD4+ lymphopenia in HIV type 2-infected individuals. AIDS Res. Hum. Retroviruses **10:**1031–1037.
3. **Centers for Disease Control.** 1992. 1993 revised classification system for HIV infection and expanded surveillance case definition for AIDS among adolescents and adults. Morbid. Mortal. Weekly Rep. **41:**1–19.
4. **Chen, Z., A. Luckay, D. L. Sodora, P. Telfer, P. Reed, A. Gettie, J. M. Kanu, R. F. Sadek, J. A. Yee, D. D. Ho, L. Zhang, and P. A. Marx.** 1997. Human immunodeficiency virus type 2 (HIV-2) seroprevalence and characterization of a distinct HIV-2 genetic subtype from the natural range of simian immunodeficiency virus-infected sooty mangabeys. J. Virol. **71:**3953–3960.
5. **De Cock, K. M., G. Adjorlolo, E. Ekpini, T. Sibailli, J. Kouadio, M. Maran, K. Brattegaard, K. M. Vetter, R. Doorly, and H. D. Gayle.** 1993. Epidemiology and transmission of HIV-2. Why there is no HIV-2 pandemic? JAMA **270:**2083–2086.
6. **Gao, F., L. Yue, D. L. Robertson, S. C. Hill, H. Hui, R. J. Biggar, A. E. Neequaye, T. M. Whelan, D. D. Ho, G. M. Shaw, P. M. Sharp, and B. H. Hahn.** 1994. Genetic diversity of human immunodeficiency virus type 2: evidence for distinct sequence subtypes with differences in virus biology. J. Virol. **68:**7433–7447.
7. **Grankvist, O., U. Bredberg-Radèn, A. Gustafsson, J. Albert, P. Albino, P. A. Andreasson, A. Nauclér, G. Biberfeld, and G. Wadell.** 1992. Improved detection of HIV-2 DNA in clinical samples using a nested primer-based polymerase chain reaction. J. Acquired Immune Defic. Syndr. **5:**286–293.
8. **Kanki, P. J., and K. M. De Cock.** 1994. Epidemiology and natural history of HIV-2. AIDS **8:**S85–S93.
9. **Loussert-Ajaka, I., F. Simon, I. Farfara, N. Houhou, J. Couto-Fernandez, M. C. Dazza, and F. Brun-Vézinet.** 1994. Comparative study of single and nested PCR for the detection of proviral HIV-2 DNA. Res. Virol. **145:**337–342.
10. **Matheron, S., C. Courpontin, F. Simon, H. Di Maria, H. Balloul, S. Bartzack, D. Dormont, F. Brun-Vézinet, A. G. Saimot, and J. P. Coulaud.** 1990. Vertical transmission of HIV-2. Lancet **335:**1103–1104.
11. **Matheron, S., G. Mendoza-Sassi, F. Simon, R. Olivares, J. P. Coulaud, and F. Brun-Vézinet.** 1997. HIV-1 and HIV-2 AIDS in African patients living in Paris. AIDS **11:**934–936.
12. **Morgan, G., H. A. Wilkins, J. Pepin, O. Jobe, D. Brewester, and H. Whittle.** 1990. AIDS following mother-to-child transmission of HIV-2. AIDS **4:**879–882.
13. **Myers, G., B. Foley, J. W. Mellors, B. Korber, K. T. Jeang, and S. Wain-Hobson.** 1996. Human retroviruses and AIDS 1996. A compilation and analysis of nucleic acid and amino-acid sequences. Los Alamos National Laboratory, Los Alamos, N.Mex.
14. **Respess, R., C. Christopherson, K. Koffi, G.-M. Gershi-Damet, and S. Kwok.** 1993. Development of a PCR-based assay for HIV-2, abstr. WS-A24-2, p. 40. IXth International Conference on AIDS, Berlin, June 6–11.
15. **Simon, F., S. Matheron, C. Tamalet, I. Loussert-Ajaka, S. Bartzack, J. M. Pépin, C. Dever, E. Gamba, C. Elbim, J. A. Gastaut, A. G. Saimot, and F. Brun-Vézinet.** 1993. Cellular and plasma viral load in patients infected with HIV-2. AIDS **7:**1411–1417.