Improved detection of HIV-2 proviral DNA in dually seroreactive individuals by PCR

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Objective: To improve the detection rate of HIV-2 proviral DNA in primary uncultured peripheral blood mononuclear cells (PBMC) of HIV-2-seroreactive and HIV-1–HIV-2 dually seroreactive individuals.

Materials and methods: Two newly designed HIV-2 PCR primer pairs in the long terminal repeat (LTR) *gag* and *gag–pol* regions and a previously described *env* and LTR HIV-2 PCR primer pairs were tested on samples from 66 confirmed HIV-2-seropositive individuals (The Gambia, 40; Côte d'Ivoire, 17; Guinea-Bissau, nine), 209 dually seroreactive individuals (The Gambia, 82; Côte d'Ivoire, 127), 24 genetically characterized isolated HIV-1 strains (group M subtypes A–H and group O), one simian immunodeficiency virus (SIV) strain cpz, 10 HIV-2 isolates (subtype A, B and unidentified), two SIV_{sm} isolates, and 10 seronegative samples.

Results: All HIV-2 primers evaluated showed 100% specificity since there was no amplification observed with 24 HIV-1, one SIV_{cpz} and 10 seronegative samples. One single copy of the HIV-2 genome could be detected with all outer primer pairs as well as all inner primer pairs on one PCR round used. Sensitivity of primers (at least one of the four primer pairs was positive) to HIV-2-seropositive samples was 100% (all nine) in Guinea-Bissau, 71% (12/17) in Côte d'Ivoire, 100% (all 20) in Gambian AIDS patients, and 85% (17/20) in Gambian pregnant women. Doubling the PBMC of dually seroreactive individuals from 7.5×10^4 to 1.5×10^5 in the PCR revealed the presence of both HIV-1 and 2 proviral DNA in 72% (92/127) in Côte d'Ivoire and 72% (59/82) in The Gambia. By doubling the number of PBMC, HIV-2 detection in dually seroreactive individuals by PCR was increased from 65 to 77% in Côte d'Ivoire and from 67 to 83% in The Gambia.

Conclusions: The use of 1.5×10^5 primary uncultured PBMC and the newly designed HIV-2 primer pairs allowed us to document the highest percentage (72%) ever reported of HIV-1–HIV-2 dual infections amongst HIV-1–HIV-2 dually seroreactive individuals in Côte d'Ivoire and The Gambia. Improved detection of HIV-2 proviral DNA, rather than exposure to both viruses, infection with only one virus, or infection with a unique third virus containing epitopes common to both HIV-1 and HIV-2, contributes to a more accurate monitoring of the prevalence of HIV-1–HIV-2 dual infections.

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Introduction

Dual seroreactivity to HIV-1 and HIV-2 is common in West Africa (Côte d'Ivoire, Ghana, Guinea-Bissau, Senegal, The Gambia) and in India [1–13]. In addition, HIV-2-infected subjects have been described in other countries, mainly in African immigrants, although infection in native individuals has also been reported [3,14,15]. Although HIV-1 and HIV-2 are both present in those areas, their spread has been unequal. Recent studies have shown that HIV-2 is perinatally and sexually less transmissible than HIV-1. Although HIV-2 AIDS appears to be similar to HIV-1-induced AIDS, the rate of disease progression is much slower than that of HIV-1 [1,16,17]. It is still controversial whether HIV-2 infection protects against subsequent HIV-1 infection [18–21]. The diagnosis of cases with serological reactivity to both viruses may be a problem in geographical regions where infection with both HIV-1 and HIV-2 exists. Previous studies have shown the presence of both viruses in one individual by PCR and virus isolation [5,22–25]. It is not known what proportion of dually seroreactives are due to (i) a mixed infection, (ii) a broad immune response against infection with a single serotype of HIV-1 or HIV-2, (iii) an infection with a unique third virus containing epitopes common to both viruses, or (iv) exposure to both viruses but infection with only one. It is clear from these studies that dual infection is possible, but it remains to be shown whether this is the explanation for all dual serological reactivity.

Amongst the six subtypes of HIV-2 (subtypes A–F) reported to date [26,27], virus isolation has failed for subtypes C, D, E and F from Liberia and Sierra Leone. They have been subtyped by using primary uncultured peripheral blood mononuclear cells (PBMC). Moreover, only 13 strains (10 subtype A, three subtype B) have been fully sequenced [28].

In previous studies in Côte d'Ivoire, the overall prevalence of dual infection, defined as the simultaneous presence of proviral HIV-1 and HIV-2 DNA in primary uncultured PBMC of dually seropositive samples, varied from 33% (12 out of 36) up to 62% (21 out of 34) [5,22–24]. Previously, we have shown in a limited number of dual seropositive patients in Côte d'Ivoire that the detection rate of proviral HIV-2 DNA can be increased up to 71% (five out of seven) by coculturing the patient's PBMC with PBMC of healthy HIV-seronegative donors during a period of 3 weeks. This suggests that the proviral DNA of HIV-2 may be present in very low copy numbers in primary uncultured PBMC of dually seropositive individuals, and only after stimulation of the PBMC by cocultivation is enough HIV-2 proviral DNA formed to be detectable by PCR [22]. Based on nested PCR and Southern blotting, 26 (77%) out of 34 dually seropositive individuals in Senegal were infected with both HIV-1 and HIV-2 [29].

Differences in the serological diagnosis as well as in the PCR-based techniques used to document HIV-1 and HIV-2 infection might explain the different prevalences of dual infections among dually seroreactive individuals. In this study, we tested dually seroreactive individuals from Côte d'Ivoire and The Gambia by PCR using newly designed HIV-2 primers and doubling the number of primary uncultured PBMC to improve the HIV-2 proviral DNA detection rate.

Materials and methods

Samples

PBMC of 66 samples were obtained from HIV-2-seroreactive individuals living in The Gambia [20 symptomatic (HIV-related diseases were observed) and 20 asymptomatic healthy pregnant women], Côte d'Ivoire (17 asymptomatic) and Guinea-Bissau (eight asymptomatic and one symptomatic) for the evaluation of HIV-2 PCR sensitivity. No detailed clinical stage information was available for samples from Guinea-Bissau, Côte d'Ivoire and The Gambia. To evaluate the specificity of DNA extracted from cultured PBMC, 24 HIV-1 isolates belonging to all *env* subtypes (three each of subtypes A, B, C, D, E and F, and two each of subtypes G, H, and O) and one simian immunodeficiency virus (SIV) strain cpz were used as well as 10 seronegative individuals. The genotypic characterization methods (sequence analysis/heteroduplex mobility assay) of HIV-1 strains used in this study have been previously published [30,31]. DNA extracted from cultured PBMC of 10 HIV-2 isolates [two subtype A (VI495, VI884) [32], one subtype B (VI1011) [32], and seven unknown] and two SIV_{sm} [33] were used. Finally, primary uncultured PBMC from 82 dually seroreactive individuals from The Gambia (47 asymptomatic and 35 symptomatic) and from 127 dually seroreactive female sex workers from Côte d'Ivoire (116 asymptomatic and 11 symptomatic) were evaluated by all HIV-2 primer pairs. Dual seroreactivity in samples from The Gambia and Côte d'Ivoire were serologically confirmed by the use of Peptilav 1-2 (Sanofi Diagnostics Pasteur, Marnes-la-Coquette, France). This supplemental test is a line immunoassay using HIV-1 and HIV-2-specific peptides. These antigens are applied as parallel lines on a nylon strip. Each strip also contains a control line. In this assay, a serum sample was considered dually seroreactive when the intensities of the two peptides were equal to each other and stronger than or equal to the intensity of the control line. This assay was performed according to the instructions of the manufacturer, as previously described [34].

Cell lysate

The primary uncultured PBMC used in this study were obtained by Ficoll-isopaque density gradient centrifugation of 10–20 ml heparinized blood and stored in liquid nitrogen in 90% fetal calf serum and 10% dimethylsulphoxide until use. The thawed cells were washed twice in phosphate-buffered saline and counted. They were resuspended in lysis buffer at a concentration of 6×10^6 cells/ml and proteinase-K-treated before use in PCR, as previously described [35].

Primers

The primer pairs were selected by comparing the recently published HIV-2 sequences in the HIV-2/SIV database provided by the Los Alamos National Laboratory [28] and the computer software OLIGO (Research Genetics, USA). The sequences of the newly designed as well as previously published *env* [23] and long terminal repeat (LTR) primers [36] used in this study are shown in Table 1. The HIV-1 primers used have been described previously [35].

PCR

All PCR were performed on 7.5×10^4 PBMC in a total volume of 50 µl. The reaction mixture contained 50 mmol/l KCl, 10 mmol/l Tris-HCl pH 8.3, 1.25 mmol/l $MgCl_2$, 0.2 mmol/l of each dNTP, 1.25 U *Taq* polymerase (Perkin Elmer, Zaventem, Belgium) and 0.5 µmol/l of each primer. First-round cycling conditions for all primer pairs were 2 min at 95°C followed by 35 cycles for 30 sec at 94°C, 30 sec at 45°C, and 1 min at 72°C in a thermal DNA cycler (9600R, Perkin Elmer). In the last round, the extension was lengthened to 7 min. The nested PCR was performed using 1 µl of the amplified DNA from the first-round PCR in a fresh 25 µl reaction buffer under the following conditions: 35 cycles of 30 sec at 94°C, 30 sec at 55°C (all primer pairs), and 1 min at 72°C. Although annealing temperatures between 52°C and 63°C were calculated for the primer pairs used, annealing temperatures were fixed at 45°C for the first PCR and at 55°C for nested PCR for all primer pairs. This strategy allows for a higher sensitivity in the first round and a high specificity in the second round. DNA input was doubled (1.5×10^5 cells) in the newly designed PCR protocol. Ten microlitres of amplified product were loaded onto a 2% agarose gel. After electrophoresis and staining with ethidium bromide, the amplified fragment was visualized by transillumination at 254 nm.

The β-globin gene was amplified in a single PCR assay for 35 cycles under the same conditions as above with an annealing temperature of 55°C to assure the DNA quality of the cell lysate [35]. Cell lysates that were negative in PCR with β-globin primers were excluded from the study (n = 3).

Statistical analysis

For each of the different HIV-2 primers, the detection rates of HIV-2 proviral DNA in primary uncultured PBMC of dual and HIV-2-seropositive individuals were compared between low and high cell inputs using the McNemar's χ^2 test.

Results

Specificity of the HIV-2 nested PCR

All primers evaluated showed 100% specificity since no amplification was observed when 22 HIV-1 group M, two HIV-1 group O, one SIV_{cpz}, and 10 seronegative samples were tested.

Sensitivity of the HIV-2 nested PCR

The analytical sensitivity of the reaction was measured by diluting a pGH123 clone of HIV-2_{GH-1} (kindly

Table 1. HIV-2 nested primer sets.

Primer	Sequences (5′–3′)	Position (ROD)	Remarks
LTR			
H2L100	GCTGGCAGATTGAGCCCTG	18–35	OF
H2L200	AAGGGTCCTAACAGACCAGGG	243–262	OR
H2L101	CAGCACTAGCAGGTAGAGCCTGGG	49–71	IF
H2L201	GGCGGCGACTAGGAGAGATGG	191–210	IR
LTR–*gag*			
ishi 01	AGAGGCTGGCAGATTGAGC	14–32	OF
ishi 02	CAAGACGGAGTTTCTCGCG	569–551	OR
ishi I1	GAGGTTCTCTCCAGCACTAGCA	38–59	IF
ishi I2	TAGGAGCACTCCGTCGTGGT	403–384	IR
gag–pol			
*pol*1297F	GGGGAAAGAAGCCCCGCAACTT	1858–1879	OF
*pol*812R	GCAAATGTGGGGGTATTATAAGGATT	2540–2565	OR
*pol*348F	CAGAGGACTTGCTGCACCTCAATTCT	2068–2093	IF
*pol*599R	TTTCTGCCAAAAATGTTGATTGGGGT	2321–2346	IR
env			
SE24	GGGATAGTGCAGCAACAGCAACAGC	7782–7806	OF
SE25bis	GGGGAAGAGAAAACAGGCCTATAGC	8263–8287	OR
SE27bis	AAAAACCTCCAGGCAAGAGTCACTGCTA	7863–7890	IF
SE28	ACCCATGGTACAGTAGTGTGGCAGAC	7949–7975	IR

LTR, Long terminal repeat; ROD, Los Alamos National Laboratory [28]; OF, outer forward; OR, outer reverse; IF, inner forward; IR, inner reverse.

provided by Prof. A. Adachi, National Institute of Health, Tokyo, Japan). One copy of the genome could be detected with all outer primer pairs as well as all inner primer pairs used in this study. In addition, all isolated HIV-2 (n = 10) and SIV_{sm} (n = 2) samples could be detected by all primer sets used in this study (data not shown). The sensitivity of the nested primers from the LTR, LTR–*gag*, *gag–pol* and *env* regions on samples from symptomatic HIV-2-infected patients in The Gambia was 100% with the higher (1.5×10^5) cell input and 95, 95, 85 and 75%, respectively, with the lower (7.5×10^4) cell input. The sensitivity of these same primer pairs on samples from pregnant women in The Gambia was 80, 85, 80 and 79% in the high input protocol, and 80, 70, 60 and 55% in the low input protocol, respectively. The sensitivity of the nested primers, using LTR, LTR–*gag*, *gag–pol* and *env* primers on samples from Côte d'Ivoire was 71, 59, 44 and 69% in the high input protocol, and 53, 41, 35 and 47% in the low input protocol, respectively. These differences between the high and low input protocols on the Côte d'Ivoire samples were not statistically significant (Table 2). All nine (100%) HIV-2-seroreactive samples from Guinea-Bissau could be detected by nested PCR using LTR primer pairs and eight (89%) out of nine, seven (78%) out of nine, and six (67%) out of nine in the high input protocol could be amplified by LTR–*gag*, *gag–pol* and *env* primer pairs, respectively. Using both *gag–pol* and *env* primers, the high input protocol was significantly more sensitive than the low input protocol for HIV-2-seropositive samples from The Gambia (Table 2).

Detection rate of HIV-2 PCR on samples from dually seroreactive individuals

The detection rate for each of the HIV-2 LTR, LTR–*gag*, *gag–pol* and *env* nested primers on dually seroreactive samples from Côte d'Ivoire was 74, 41, 27 and 41% in the high input protocol, and 57, 35, 21% and not tested for the lower protocol, respectively. These differences were statistically significant (Table 2). The overall detection rate of the LTR, LTR–*gag*, *gag–pol* and *env* nested primers on samples from The Gambia was 74, 70, 64 and 60% in the high and 64, 53, 49 and 100% for the low input protocols, respectively (Table 2). The best detection rate (77%) was observed with the combination of the four primers (LTR, LTR–*gag*, *gag–pol* and *env*) on samples from Côte d'Ivoire, but no advantage with any primer combination was observed on the samples from The Gambia. Overall, 72% of dually seroreactive individuals were both HIV-1 and HIV-2 PCR-positive. A high input protocol significantly improved the detection rate of HIV-2 proviral DNA in dually seroreactive individuals ($P < 0.001$ in LTR, $P < 0.025$ in LTR–*gag*, and $P < 0.025$ in *gag–pol*, from Côte d'Ivoire (Table 2). There were not enough samples from Côte d'Ivoire

Table 2. Sensitivity of HIV-2 primers for monitoring proviral HIV-2 DNA in peripheral blood mononuclear cells of HIV-2 and HIV-1–HIV-2 dually seroreactive individuals originating in Guinea-Bissau, The Gambia and Côte d'Ivoire.

Geographical origin of samples tested (no. tested)	HIV-2 LTR		HIV-2 LTR–*gag*		HIV-2 *gag-pol*		HIV-2 *env*		HIV-1 *pol*		HIV-2 PCR final result*		HIV-1 *pol*-positive HIV-2-positive final result†	
	Low	High	Low	High	Low	High	Low	High	Low	High	Low	High	Low	High
No. samples reactive in PCR (% reactive in PCR of total tested)														
HIV-2-seropositive (n = 66)														
Guinea-Bissau (n = 9)	NT	9 (100)	NT	8 (89)	NT	7 (78)	NT	6 (67)	NT	NT	NT	9 (100)	NT	NT
Côte d'Ivoire (n = 17)	9 (53)	12 (71)	7 (41)	10 (59)	6 (35)	7 (44)‡	8 (47)	11 (69)‡	NT	NT	11 (65)	12 (71)	NT	NT
The Gambia														
Patients (n = 20)	19 (95)	20 (100)	19 (95)	20 (100)	17 (85)	19 (100)§	15 (75)	19 (100)§	NT	NT	19 (95)	20 (100)	NT	NT
Pregnant women (n = 20)	16 (80)	16 (80)	14 (70)	17 (85)	12 (60)	16 (80)¶	11 (55)	15 (79)§	NT	NT	16 (80)	17 (85)	NT	NT
Subtotal	35 (88)	36 (90)	33 (83)	37 (93)	29 (73)	35 (90)¶	26 (65)	34 (89)**						
HIV-1–HIV-2 dually seroreactive (n = 209)														
Côte d'Ivoire (n = 127)	73 (57)	94 (74)††	45 (35)	52 (41)**	27 (21)	34 (27)**	NT	52 (41)	NT	120 (94)	82 (65)	98 (77)††	79 (62)	92 (72)
The Gambia (n = 82)	30/47 (64)	61/82 (74)¶	21/40 (53)	38/54 (70)	19/39 (49)	34/53 (64)	4/4 (100)	32/53 (60)	NT	80/82 (98)	26/39 (67)	44/53 (83)	32/47 (68)	59/82 (72)

Statistical difference in the detection rate of HIV-2 proviral DNA between high and low input of cells was determined for each of the HIV-2 primers under evaluation by using the McNemar's χ^2 test. *HIV-2 PCR is considered positive if a positive PCR reaction is observed with at least one of the four HIV-2 primer pairs examined. †Number of samples simultaneously positive in PCR for both HIV-1 and HIV-2. ‡Only 16 out of 17 samples were tested. §Only 19 out of 20 samples were tested. ¶Significant at the $P < 0.05$ level. **Significant at the $P < 0.025$ level. ††Significant at the $P < 0.001$ level. Low, Lower input 7.5×10^4 cells as template; High, higher input 1.5×10^5 cells as template; LTR, long terminal repeat; NT, not tested.

and The Gambia available to be tested with the HIV-2 *env* primers using the low cell input protocol. By high input DNA PCR, HIV-1 proviral DNA could be detected for 94 and 98% in dually seroreactive PBMC from Côte d'Ivoire and The Gambia, respectively.

Discussion

The nested primers in the LTR region that we recently reported [36] are both specific and more sensitive than the previously described primers for the detection of HIV-2 provirus. Increasing the DNA input to 1.5×10^5 cells as PCR template improved the detection rate of HIV-2 PCR considerably in HIV-2, and even more in HIV-1–HIV-2 dually seroreactive individuals. This may be related to the low viral load of HIV-2 in dual infection in asymptomatic individuals [37]. The genetic variability between different HIV-2 subtypes may affect the detection of provirus by PCR. In the database, there are full-length genomic sequences for only 13 HIV-2 isolates of which 10 isolates belong to subtype A and the remainder are subtype B, whereas for subtypes C–F only partial sequences from *gag/pol/env* regions of single isolate are available. The low detection rate of the LTR–*gag*, *gag–pol* and *env* primers on Côte d'Ivoire HIV-2-seropositive and dually seroreactive samples may be explained by sequence variation or different subtypes circulating in this country. In this study, it was not possible to detect HIV-2 proviral DNA if the frequency of cells carrying HIV-2 proviral DNA was less than one per 1.5×10^5 cells. Previous studies have shown that most patients infected with either HIV-1 or HIV-2 have at least one virus per 1×10^5 cells [37,38]. However, in individuals who are already infected with one HIV serotype, pre-existing immunity may operate to suppress viral growth during a second infection [39,40], although the existence of such cross-protection remains controversial.

Lee *et al.* [41] have previously reported the development of a high-input PCR which enables analysis of a 10-fold higher amount of cellular DNA per reaction than standard PCR protocols. Simply doubling the amount of DNA in our assay increased the detection rate of HIV-2 proviral detection by PCR considerably. The low detection rate by PCR of HIV-2 proviral DNA in PBMC from healthy asymptomatic HIV-2-seropositive pregnant women was clearly shown in our study compared with a high detection rate of HIV-2 proviral DNA in PBMC from symptomatic patients seropositive to HIV-2. This suggests that the sensitivity of HIV-2 PCR was low because viral load was low in asymptomatic patients [42].

Our study illustrates the urgent need to generate more sequence data on HIV-2 isolates from diverse geographical origins, and to make data more rapidly available to international sequence databanks, which will enable primer sets to be designed more rapidly. A considerable improvement in the detection of HIV-2 proviral DNA in PBMC of HIV-2-seropositive and dually seroreactive individuals has been demonstrated in this study. Using the higher PBMC cell input protocol and HIV-2 LTR PCR, 72% of dually seroreactive individuals tested in Côte d'Ivoire and The Gambia were shown to be HIV-1–HIV-2 dually infected. So far, this is the highest percentage of dual HIV-1 and HIV-2 infections among dually seroreactive individuals ever reported in Côte d'Ivoire and as such the first report for The Gambia, by using PCR for the amplification of HIV-1 and HIV-2 proviral DNA in uncultured PBMC.

Peeters *et al.* [22] reported that HIV-2 detection was increased by first culturing the patient's PBMC. This led us to suggest that the proviral DNA of HIV-2 might be present in very low copy numbers in PBMC of dually seroreactive individuals [22]. George *et al.* [5] demonstrated a lower concordance between PCR and virus culture results, and between PCR and serology due to apparent low sensitivity of the HIV-2 PCR, based on specimens that were only reactive in HIV-2 serology. They also suggested that HIV-2 strains were more variable than HIV-1 strains [5]. Leonard *et al.* [24] concluded that dual seroreactivities are not always due to infection by both viruses by showing data that only four (36%) out of 11 samples were both HIV-1 and HIV-2 PCR-positive in dual serological profiles, and suggested the need for a combination of serological and PCR assays for appraisal of HIV-1–HIV-2 dual infection. According to our data, 72% of dually seroreactive individuals were proven to be dually infected. A low percentage of the detection might be due to HIV-2 sequence variability. Dieng Sarr *et al.* [29] reported that lack of a HIV-2 proviral signal in a dual serological profile was significantly correlated with low CD4+ lymphocyte counts ($P = 0.04$).

In our study, nine (4.3%) samples (seven from Côte d'Ivoire, two from The Gambia) were not detected by HIV-1 *pol* primers. These HIV-1 *pol* primers were previously documented to have a sensitivity of 93% and specificity of 100% when used to amplify proviral HIV-1 DNA from genetically proven HIV-1 isolates representing different subtypes [35]. To study the true proportion of dual infection among dually seroreactive individuals, more studies like this are required whereby new HIV-2 and HIV-1 primers will be designed based on the rapid expansion of the number of newly documented HIV-1 and HIV-2 nucleotide sequences.

The specificity of serodiagnosis for dual infection is still a challenge [34]. Discrepancies between PCR and serology might be due to the different tests and

algorithms that were used in the different studies [5,21–24,29].

The most important conclusions from this study are that mixed infections with HIV-1 and HIV-2 in the same individual are relatively common in Côte d'Ivoire and The Gambia. Improvement of the HIV-2 primers used, as well as doubling the number of PBMC from which HIV DNA was extracted, has allowed us to document mixed infections in 72% of dually seroreactive individuals. This is the highest prevalence of HIV-1 and HIV-2 mixed infections ever documented among dually seroreactive individuals, given that primary uncultured PBMC were used to extract the proviral HIV-1 and HIV-2 DNA. Improved detection of HIV-2 proviral DNA, rather than exposure to both viruses but infection with only one, or infection with a unique third virus containing epitopes common to both HIV-1 and HIV-2, contributes to a more accurate monitoring of the prevalence of HIV-1–HIV-2 dual infections. This study highlights the need of better standardized markers in addition to the sometimes confusing serological markers for documenting the true prevalence of HIV-1 and HIV-2 mixed infections.

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