Direct Detection of Proviral *gag* Segment of Human Immunodeficiency Virus in Peripheral Blood Lymphocytes by Colorimetric PCR Assay as a Clinical Laboratory Tool Applied to Different At-Risk Populations

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We used a colorimetric polymerase chain reaction (PCR)-based assay in kit form to detect directly human immunodeficiency virus type 1 (HIV-1) proviral *gag* sequences in peripheral blood cells from 68 healthy blood donors, 51 subjects at risk for HIV infection, 122 patients with HIV-1 infection, 11 patients with indeterminate Western blot (immunoblot) results, 4 blood donors HIV-1 positive by enzyme immunoassay, and 13 children born to HIV-1-seropositive mothers. The results obtained in the blood donors and HIV-1-infected patients demonstrated the high degree of diagnostic specificity and sensitivity of the PCR method. HIV-1 infection was excluded in 10 of the 11 patients with indeterminate Western blot results and in all four enzyme immunoassay-positive blood donors. A diagnosis of HIV infection was ruled out by negative PCR results in 5 of 13 children from seropositive mothers, which excluded vertical transmission of the infection in these cases; these children were younger than 3 months and had positive serological results. Two at-risk patients with negative serological results had positive PCR results. All results were confirmed by conventional PCR. In conclusion, colorimetric PCR, which is commercially available in kit form, is an easy and reliable technique that can be used to detect proviral HIV-1 genomes in blood cells, and despite the limitations owing to HIV genome variability, it is useful in the clinical setting for the diagnosis of HIV infection in selected categories of patients.

Screening for human immunodeficiency virus (HIV) infection currently relies on the detection of specific antibodies to viral proteins. Serological assays for the detection of HIV, the enzyme-immunoassay (EIA) for screening for HIV, and Western blot (immunoblot) analysis for confirmation of the presence of HIV antibodies are highly sensitive and specific (7, 9). However, HIV-specific antibodies are detectable, on average, 3 months after infection with the virus (21) and, in rare cases, not until 35 months after infection (11). During the period between infection and the appearance of antibodies, HIV-infected subjects with negative serological tests may transmit the virus to others through blood donations or sexual contacts.

Moreover, Western blot results are indeterminate in a significant number of cases (19), and HIV infection status is difficult to determine in newborns of seropositive mothers in which, at birth, maternal antibodies are transferred to the child's circulation in the absence of HIV infection. In such cases, other serological assays of long-term follow-up samples or assays for the direct detection of HIV are required to evaluate HIV infection (19).

Given the drawbacks of serological assays, attempts have been made to detect HIV proviral DNA directly in peripheral

blood mononuclear cells to identify HIV infection before seroconversion and both in individuals with indeterminate serological results and in newborns of seropositive mothers (22). However, the direct detection of HIV may be complicated by such factors as the very small number of cells harboring HIV, the small number of proviral genome copies per infected cell, and the low level of transcriptional activity of the viral genome, particularly in the first phase of the infection (16). The reference method for the direct detection of HIV is virus culture, which, however, is time-consuming and expensive, and results may vary between different laboratories (6). The use of *in situ* hybridization for the detection of HIV nucleic acid in peripheral blood lymphocytes and Southern analysis of HIV proviral DNA extracted from blood cells have frequently shown low levels of diagnostic sensitivity (22). Similarly, the identification of viral p24 nucleocapsid antigens by specific monoclonal or polyclonal antibodies also has a low level of diagnostic sensitivity, particularly in patients at the early stages of infection (6).

More recently, techniques of DNA amplification by PCR have been successfully applied to the specific detection of HIV proviral DNA (1, 4, 15). In the study described here we applied a PCR-based colorimetric method (24) to detect the HIV-1 proviral genome in circulating lymphocytes from 269 subjects with HIV-1 infection or at risk for this infection to evaluate the clinical reliability of this PCR procedure in commercially available kit form in the diagnosis of HIV infection in different at-risk groups of patients. For results that were not clear-cut,

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TABLE 1. Subjects examined by PCR-based colorimetric assay of proviral HIV *gag* sequences in blood lymphocytes

Subject characteristic	No. of subjects
Blood donors.....	68
At risk of HIV infection.....	51
HIV-infected individuals ^a	122
Patients with indeterminate Western blot result.....	11
EIA-positive and Western blot-negative blood donors...	4
Children of seropositive mothers	13

^a CD4, >500/ml, *n* = 18; CD4, 200 to 500/ml, *n* = 31; CD4, <200/ml, *n* = 73.

we applied the conventional PCR procedure using various pairs of *gag* primers.

MATERIALS AND METHODS

Patients. The patients enrolled in the study were from the Istituto di Clinica delle Malattie Infettive, Università Cattolica del Sacro Cuore, Rome, the Dipartimento di Biopatologia Umana, Centro Trasfusionale, Università "La Sapienza", Rome, and the Dipartimento di Pediatria and the Istituto di Malattie Infettive, Facoltà di Medicina, Università "Federico II," Naples. Samples were analyzed in the Laboratorio di Virologia, Istituto Superiore di Sanità, Rome, or in CEINGE-Biotecnologie Avanzate, Dipartimento di Biochimica e Biotecnologie Mediche and Dipartimento di Patologia Cellulare e Molecolare, Università "Federico II," Naples. Blood samples drawn by venipuncture from 269 HIV-1-infected and noninfected subjects were assayed in the study; the subjects were classified as follows: (i) 68 HIV-seronegative, healthy blood donors; (ii) 51 subjects with behavior that put them at risk for HIV infection, i.e., those who engage in intravenous drug use, male-to-male sex, and sex with HIV-positive partners; (iii) 122 patients with documented HIV type 1 (HIV-1) infection; (iv) 11 subjects with indeterminate results by Western blot assay for HIV-1 sub-grouped into those at low risk and those at high risk for HIV infection; (v) 4 blood donors repeatedly positive by the HIV-1 antibody EIA; and (vi) 13 children of seropositive mothers (Table 1).

Serological analysis. Blood specimens from patients were assayed for HIV-1 antibody reactivity by using various registered EIA kits: HIV-1/HIV-2 EIA Recombinant (Abbott, North Chicago, Ill.), HIV-mixt (Organon Teknika, Boxtel, The Netherlands), and Cobas Core anti-HIV-1/HIV-2 EIA (Roche Diagnostic Systems, Milan, Italy); samples reactive by EIA were confirmed by Western blot assay (Sorin, Turin, Italy), which was considered positive when at least one anti-*gag*- or anti-*pol*-specific and two anti-*env*-specific bands were present. When Western blot reactivity did not meet the criteria for positivity, the test result was considered indeterminate. When no reactivity to any protein was detected on the strip the test result was considered negative.

DNA extraction from samples. EDTA or sodium citrate blood specimens were treated as follows. Four 0.5-ml aliquots of each blood sample were independently mixed with 1 ml of lysis buffer (Amplicor HIV-1; Roche Diagnostic Systems) and were then centrifuged to pellet the lymphocytes and other mononucleated blood cells. The pellets were then washed twice with lysis buffer to remove erythrocyte debris and hemoglobin. Total DNA was extracted by adding to each mononuclear pellet 200 μ l of extraction buffer containing 10 mM Tris HCl, 50 mM KCl, 2 mM MgCl₂, 0.5% Nonidet P-40, and 50 μ g of proteinase K per ml; the extracted DNA solutions were then incubated at 60°C for 30 min, and proteinase K was finally inactivated at 100°C for 30 min.

PCR-based colorimetric assay (method A). A 50- μ l aliquot of the DNA was extracted as described above and was used for PCR amplification. The primers SK462 and SK431 (24), the latter of which was biotinylated at the 5' end, were included in the amplification buffer provided with the Amplicor kit; the buffer also contained deoxyribonucleotides (dATP, dGTP, dCTP, and dUTP), *Taq* polymerase, and the uracil-*N*-glycosylase (UNG) enzyme. The latter catalyzes the cleavage of uracil bases from the deoxyribose moieties of the preamplified chain. When the chain is heated in the first PCR step, it breaks at uracil positions, thus avoiding carryover contamination. The enzyme is inactivated at temperatures above 55°C, hence, during the first PCR denaturation step. Since PCR products from a previously amplified DNA always contain uracil as a substitute for thymine, whereas the natural target sequence does not, incorporation of UNG in the preamplification mixture prevents contamination from the carryover of preamplified products. The DNA was amplified in a GeneAmp 9600 thermal cycler (Perkin-Elmer Cetus, Norwalk, Conn.) in a 100- μ l reaction mixture during 35 cycles as follows: 10 s at 60°C, 10 s at 55°C, and 10 s at 72°C for the first 5 cycles and then 10 s at 90°C, 10 s at 60°C, and 10 s at 72°C for the remaining 30 cycles. The samples were then stored at 72°C until they were denatured by adding 100 μ l of the denaturing solution provided with the kit.

Colorimetric detection of amplified products was carried out in a 96-microwell plate. Twenty-five microliters of amplification mixture and 100 μ l of hybridization buffer, which were included in the kit, were mixed at room temperature in

the microwell plate coated with the SK102-specific oligonucleotide probe (24) that hybridizes with the biotinylated PCR products. The microwell plate was incubated at 37°C for 1 h to allow the hybridization to occur and was then washed five times with 300 μ l of washing solution. One hundred microliters of avidin-horseradish peroxidase solution was then added to each well, and the plate was incubated at 37°C for 10 min to allow avidin binding to biotinylated DNA fragments. The plate was washed again to eliminate excess avidin, and a colorimetric reaction was started in each well by adding a solution containing tetramethylbenzidine and H₂O₂. The former in the presence of H₂O₂ is converted by peroxidase into a colored substance that absorbs at 450 nm. The reaction was stopped by adding 100 μ l of a sulfuric acid solution, and the absorbance was finally measured by a microwell absorbance reader.

The optical density (*A*₄₅₀) measurable by our instrumentations ranged from 0 to a maximum of 2.9. All samples were analyzed in duplicate, and all values exceeding an *A*₄₅₀ of 0.35 were considered positive. This is the cutoff value used in a recent study conducted by this PCR procedure (3). Four positive and four negative controls consisting of 10 copies of the *gag* DNA region inserted into a plasmid vector and 10 copies of the same plasmid without any DNA insert, respectively, were also processed in parallel with each series of samples. In the case of discordant results between duplicate PCRs, i.e., one positive and one negative replicate, as well as between serological and PCR results, the amplification procedure was repeated on a different lymphocyte pellet obtained from an independently extracted blood sample. If the serological and PCR results remained discordant, HIV-1 proviral DNA amplification was performed on yet another lymphocyte pellet by using either the colorimetric-based PCR assay (method A) and a different pair of primers (SK38, SK39) and probe SK19 (5) or a conventional PCR procedure as described below (method B). When possible, follow-up samples were taken for subsequent analyses.

Conventional *gag* DNA PCR (method B). A *gag* PCR was run in a Gene Amp PCR System 9600 (Perkin-Elmer Cetus) with SK100-SK104 primers (14) or GAG1-GAG2 primers (15). The total incubation volume consisted of 50 μ l with 25 μ l of DNA extracted as described above and 25 μ l of a reaction mixture consisting of 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 200 μ M (each) deoxynucleoside triphosphates, 1.25 U of AmpliTaq DNA polymerase (Perkin-Elmer), and 200 nM (each) primer.

When the SK100 and SK104 primers were used, samples were subjected to 35 cycles of amplification as follows: 1 min of denaturation at 95°C and then primer annealing at 55°C for 1 min and chain extension at 72°C for 1.5 min; during the last cycle the 72°C extension step was prolonged to 10 min. Twenty-five microliters of PCR products was separated by electrophoresis on a 1.5% (wt/vol) agarose gel plate, and then DNA fragments were transferred by capillary blotting onto a Hybond nylon filter (Amersham International, Buckinghamshire, United Kingdom) by conventional Southern transfer technique and fixed by UV irradiation. The SK19 probe (20) was labeled with [γ -³²P]ATP (NEN-Dupont, Milan, Italy) by a T4 polynucleotide kinase reaction. Hybridization was carried out at 42°C for 24 h in 30% formamide, 3 $\times$ SSPE (1 $\times$ SSPE is 0.18 M NaCl, 10 mM NaH₂PO₄, and 1 mM EDTA [pH 7.7]), 5 $\times$ Denhardt's reagent, 0.5% sodium dodecyl sulfate (SDS), and 100 μ g of sonicated salmon sperm DNA per ml. Nonbound probe was removed by two 30-min washings with a solution containing 2 $\times$ SSPE and 0.1% SDS at 42°C. The filter was finally exposed to X-ray film for 24 h.

When GAG1-GAG2 primers were used, a nested PCR was performed with MH1A-MH2A as inner primers (13). PCR cycle profiles were as follows: a first round of 35 cycles at 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min; the last elongation step at 72°C was run for 7 min; the second PCR round was done with MH1A-MH2A primers and consisted of 25 cycles at 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min; the last elongation step at 72°C was prolonged to 7 min. PCR products were analyzed by gel electrophoresis on an ethidium bromide-stained 2% agarose gel.

RESULTS

Reproducibility. As a preliminary evaluation of the diagnostic performances of the colorimetric PCR assay, the reproducibility of the assay was determined. Twenty-four positive and 24 negative controls, consisting of 10 copies of the *gag* DNA region inserted into a plasmid vector and 10 copies of the same plasmid without any DNA insert, respectively, were amplified in the same run to measure the intra-assay precision. The same 20 negative and 20 positive controls were assayed in 10 runs to calculate the interassay precision (Fig. 1). The mean absorbance values of the negative controls were 0.086 for the intra-assay repetitions and 0.074 for the interassay repetitions; no samples had absorbance values greater than the *A*₄₅₀ cutoff value of 0.35. The means and the coefficient of variation for positive controls were not calculated because most of the values obtained exceeded an *A*₄₅₀ of 2.9, the maximum absorbance detectable by our instrumentation; however, no *A*₄₅₀

FIG. 1. Intra-assay (A and B) and interassay (C and D) precisions of colorimetric PCR procedure evaluated on negative (A and C) and positive (B and D) control samples. Bars correspond to each individual sample.

value was less than 2.4 among the intra-assay determinations or 2.1 among the inter-assay controls.

Diagnostic specificity. To evaluate the diagnostic specificity of the colorimetric PCR-based assay, blood samples from 68 HIV-1-seronegative blood donors were assayed (Fig. 2); all of the samples tested showed negative PCR results (100% diagnostic specificity); the values obtained, expressed as a ratio of sample absorbance to cutoff absorbance, ranged from 0.052 to 0.725.

Colorimetric PCR analysis was negative for the samples from four EIA-reactive and Western blot-negative blood donors (data not shown).

EIA and colorimetric PCR results for samples from 51 subjects at risk for HIV infection are as follows. Two of the 51 samples were reactive by EIA and were also confirmed to be positive by PCR. Among the 49 samples nonreactive by EIA and Western blotting, 2 were repeatedly PCR positive. When the two samples were tested by method A with SK38-SK39 primers and the SK19 probe, one sample was confirmed to be positive, whereas the other had an indeterminate result, resulting in discordance between replicates (data not shown). The latter sample (coded sample R71) was further analyzed by

PCR by method B (Fig. 3). A faint 290-bp band suggested a very low number of HIV DNA copies in the cell lysate. Unfortunately, no other sequential samples were available, nor were the patients available for clinical follow-up.

Colorimetric PCR analysis of samples from 122 HIV-1-infected patients seropositive by both EIA and Western blotting confirmed HIV-1 infection in all but one of the patients, for whom the test was repeatedly indeterminate. The patient was an asymptomatic homosexual male with a CD4 count of $>500/\mu\text{l}$. The colorimetric test performed with SK38 and SK39 on a different pellet was also repeatedly indeterminate, resulting in discordance between duplicate samples (data not shown). Samples from this patient repeatedly analyzed by method B were also indeterminate; a faint band corresponding to the expected PCR products was present in some analyses but not in others (data not shown).

Analytical sensitivity. To evaluate the analytical sensitivity of the colorimetric PCR assay, the remaining 121 HIV-1-infected patients were divided into three classes on the basis of their peripheral blood CD4 cell counts (Table 1). There were no significant differences in the sample absorbance/cutoff absorbance ratios among the three subgroups, and more than

FIG. 2. Diagnostic specificity of the colorimetric PCR procedure evaluated on samples from blood donors serologically negative for HIV-1 infection. Bars correspond to each sample, and data are expressed as a ratio between sample absorbance and cutoff absorbance (A_{450} of 0.350). The dashed line indicates the cutoff value of the assay.

80% of the patients in each subgroup had sample/cutoff absorbance ratios greater than 6 (Fig. 4). In patients with CD4 counts of $10/\mu\text{l}$ or less, absorbance values were clearly higher than the absorbance/cutoff value.

Patients indeterminate by Western blotting. The 11 patients with a repeatedly indeterminate Western blot assay result were divided into two groups: those with high-risk behavior and those with low-risk behavior. The serological and PCR (method A) findings are reported in Table 2. Despite the very large number of patients screened, the numbers of patients in the subgroups of low- and high-risk patients were relatively low because the criterion for their entry into the study was that they had indeterminate results by Western blot analysis. In this context, we selected patients with different clinical and serological features with respect to HIV infection, i.e., at-risk subjects, HIV-1-infected individuals, patients with indeterminate Western blot results, EIA-positive and Western blot-negative blood donors, and children of seropositive mothers. Consequently, some of the subgroups were small. The 11 patients came to our observation because they had at least one reactive EIA. In some cases, EIA reactivity was not confirmed with other commercial kits, while some patients had repeatedly positive EIA results. Therefore, we considered to be EIA neg-

ative those patients with a majority of negative EIAs and EIA positive those patients with repeatedly reactive EIAs. Of the six patients with behaviors that put them at high risk for HIV infection, three were negative by all successive EIAs and consistently had negative PCR results. The PCR assay confirmed an HIV-1 infection in only one of the other three subjects who were positive by the EIAs. Samples from the two patients (patients R87 and R98) with discordant EIAs and PCR test results were analyzed by conventional PCR (method B), and PCR confirmed that both samples were negative (Fig. 3). Samples from five patients with low-risk behavior were negative by the colorimetric PCR (method A).

Children born to seropositive mothers. In the 13 children born to seropositive mothers, serological and colorimetric PCR results coincided in six children (patients 1, 3, 7, 9, 10, and 11 in Table 3). Of these, in one child (patient 1) all results were negative, and in the other five patients all results were positive. None of these five PCR-positive children was younger than 10 months; three children with positive PCR results also showed clinical signs of AIDS. In four other children (patients 5, 6, 8, and 13), all less than 3 months of age, PCR was negative. Additional conventional nested PCR analyses (method B) on different lymphocyte pellets with GAG-1 and GAG-2 as outer primers were negative in all four patients (data not shown). Furthermore, two other children (patients 4 and 12) had indeterminate Western blot patterns and positive EIAs; colorimetric PCR analysis was negative in a 3-month-old child (patient 4) with no clinical signs of AIDS and positive in a 3-year-old child (patient 12) who 1 month later showed clinical symptoms of HIV infection. Finally, patient 2, a 30-month-old child with an indeterminate Western blot result and a positive EIA result, was negative by the colorimetric PCR (method A). Repetition of the colorimetric PCR with SK38-SK39 primers confirmed the negative result; however, 8 months later the child had clinical signs of the disease. Serological tests and PCR analyses on the new samples also gave positive results.

FIG. 3. Conventional PCR results. Lanes R98 and R87, samples from EIA-positive, Western blot-indeterminate, at-risk subjects; lane R71, sample from an EIA-negative, at-risk subject; lanes ctr⁺ and ctr⁻, lysates from human T-cell leukemia virus type III_B-infected and noninfected lymphocytes, respectively; lane mix, PCR mixture without samples.

DISCUSSION

Various PCR procedures for the amplification of proviral DNA and the HIV RNA genome have been described (17,

FIG. 4. Analytical sensitivity of the colorimetric PCR procedure evaluated on samples from patients with documented HIV-1 infection. Patients were divided into three groups according to their peripheral CD4 lymphocyte counts. In each group patients were subdivided according to their colorimetric PCR results, expressed as ratios between sample absorbance and cutoff absorbance (A_{450} of 0.350).

25–27). In the case of proviral DNA amplification, both actively replicating and quiescent viruses harbored in blood cells can be detected, whereas with RNA amplification only actively replicating viruses are detected and, therefore, diagnostic sensitivity is low (10, 18). These techniques are not routinely applied to the clinical setting: the wide variability of the HIV genome at both the intra- and the interindividual levels (23) could affect the stability of primer-template complexes, and consequently, highly conserved regions of the HIV genome must be used as PCR templates to obtain a high degree of diagnostic sensitivity. In addition, the low number of blood cells harboring the HIV genome can result in small numbers of amplified products on a background of highly complex human genomic DNA, and as a consequence, there is a risk that nonspecific products may be amplified. Furthermore, because of the high degree of sensitivity of PCR, extreme care is required in order to avoid false-positive results from carryover contaminations.

We used a PCR-based colorimetric assay (24) (method A) which exploits the fast and standardized DNA extraction from samples and the introduction of the UNG enzyme to avoid false-positive results from carryover contamination of previously amplified nucleic acid sequences by the same assay. Our

results for the negative controls, i.e., nonspecific DNA molecules, and HIV-1-seronegative healthy blood donors confirm that the technique has a high degree of specificity.

In addition, the colorimetric technique is reliable for the amplification of low numbers of proviral *gag* gene copies. A recent international collaborative study that assessed a reference standard for HIV-1 PCR by the same colorimetric PCR assay evaluated in the present study (3) demonstrated that it is possible to detect one molecule of HIV DNA and that samples containing 10 or more viral copies were reproducibly positive. In our experiments the colorimetric PCR showed good reliability for the amplification of as few as 10 copies of proviral *gag* DNA copies and for this type of amplification in patients with CD4 counts as low as 10/µl or less.

The high level of diagnostic sensitivity that we obtained in patients with documented HIV-1 infection (121 of 122 patients confirmed to be infected by colorimetric PCR; sensitivity,

TABLE 2. Colorimetric PCR (method A) of samples from patients at high and low risk for HIV infection and with indeterminate Western blot result compared with EIA result

Subject and EIA result	No. of subjects	
	PCR positive	PCR negative
High risk		
Positive	1	2
Negative	0	3
Low risk		
Positive	0	0
Negative	0	5

TABLE 3. Colorimetric PCR (method A) for the diagnosis of HIV infection in children of seropositive mothers

Patient no.	Age	EIA result	Western blot assay result	PCR assay result
1	18mo	Negative	Negative	Negative
2	30mo(sample1)	Positive	Indeterminate	Negative
	38mo(sample2)	Positive	Positive	Positive
3	24mo	Positive	Positive	Positive
4	3mo	Positive	Indeterminate	Negative
5	1mo	Positive	Positive	Negative
6	1mo	Positive	Positive	Negative
7	7 yr	Positive	Positive	Positive
8	3mo	Positive	Positive	Negative
9	10mo	Positive	Positive	Positive
10	3 yr	Positive	Positive	Positive
11	4 yr	Positive	Positive	Positive
12	3 yr	Positive	Indeterminate	Positive
13	3mo	Positive	Positive	Negative

99.2%) indirectly confirms the fact that the 104-bp *gag* gene sequence amplified by the SK462 and SK431 primer pair used in the colorimetric PCR procedure is a highly conserved region of the HIV genome. Hence, genomic variability within the population examined has little, if any, effect on the results obtained by this technique.

The analysis of samples from patients with low numbers of circulating lymphocytes harboring HIV proviral DNA, i.e., samples containing fewer than 10 *gag* gene copies, could give nonreproducible PCR results and discordance between replicate samples (3) as a stochastic effect of PCR amplification of extremely low numbers of template molecules. This phenomenon could be one of the factors accounting for the discordance between PCR replicates in the homosexual male with documented serological reactivity and a peripheral CD4 count greater than 500/ μ l (Table 1). However, genome variability may account for the discordant PCR results obtained with different pairs of primers.

One of two high-risk patients with no serological reactivity in either the EIA or the Western blot had a repeatedly positive colorimetric PCR result. We thus repeated the PCR using a different pair of primers and obtained discordant results between replicates, whereas a faint band was visible in the conventional PCR (Fig. 3, lane R71; see Results). These results suggested the presence of very low numbers of HIV-1 DNA in blood samples from this patient, possibly at the limits of sensitivity of the colorimetric PCR test. In subjects with at-risk behavior, the clinical significance of the occasional positivity of PCR in the absence of serological reactivity is unclear (12, 14) and PCR positivity should be verified on subsequent samples. However, silent infection in the prolonged absence of serological reactivity was recently demonstrated in 2 of 65 at-risk subjects by viral culturing methods (2). Hence, although follow-up samples from these patients were not available and a single positive PCR result cannot be prognostic of HIV infection, we might speculate that colorimetric PCR can reveal possible silent or early infections in subjects with at-risk behavior.

Five of five subjects with low-risk behavior and nonreactive EIA and indeterminate Western blot results were PCR negative. This indicates that PCR can be a useful tool for the diagnosis of HIV infection in these cases. The assay confirmed HIV-1 infection in only one of the three EIA-positive, Western blot-indeterminate subjects. However, since EIA reactivity in the other two high-risk patients was repeatedly borderline (sample/cutoff absorbance ratios of 1.08 and 1.03, respectively), perhaps we are dealing with either nonspecific serological reactivity or HIV infection with low copies of viral DNA. Again, only follow-up samples could help to discriminate between false-positive EIA reactivity and HIV infection.

Finally, our experience with colorimetric PCR in children born to seropositive mothers demonstrated that PCR performed within the first 3 months of life can contribute to the diagnosis of HIV infection. In fact, maternal antibodies are always detected at birth and can persist for up to 18 months (8). However, in a 30-month-old child, repeated colorimetric PCR-negative results were obtained, in spite of persistent positive serological reactivity. Follow-up samples were positive by the colorimetric PCR assay, while the child began to show clinical signs of AIDS. This is the only patient with false-negative results that we have encountered in our experience with the colorimetric PCR-based method and can perhaps be attributed to the presence of a genomic mutation early on in the disease that prevented the primers from recognizing the template or to the extremely low copy number of HIV proviral genomes in the sample. At more advanced stages of infection,

the enhanced copy number or the presence of increased numbers of HIV variants would probably produce PCR-positive results.

In summary, despite the limits imposed by genome variability and the possibility of an extremely low number of circulating lymphocytes harboring HIV proviral DNA, the colorimetric PCR-based assay (method A) might be useful for (i) screening newborns of seropositive mothers, (ii) resolving the infection status of subjects with indeterminate Western blot and/or with doubtful EIA reactivities, and (iii) detecting HIV DNA in silent or early infections in subjects with high-risk behaviors.

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REFERENCES

- Abbott, M. A., B. J. Poiesz, B. C. Byrne, S. Kwok, J. J. Snisky, and G. D. Ehrlich. 1988. Enzymatic gene amplification: quantitative and qualitative methods for detecting proviral DNA amplified in vitro. *J. Infect. Dis.* **158**: 1158-1169.
- Aiuti, F., F. Ensoli, V. Fiorelli, I. Mezzaroma, E. Pinter, E. Guerra, and A. R. Varani. 1993. Silent HIV infection. *Vaccine* **11**:538-541.
- Bootman, J. S., and P. A. Kitchin. Reference preparations in the standardization of HIV-1 PCR: an international collaborative study. *J. Virol. Methods*, in press.
- Borghi, V., G. Lami, G. Frigieri, M. T. Cecchi, G. Zoboli, M. Tavio, M. Pecorari, N. Mongiardo, A. Cossarizza, and B. De Rienzo. 1993. Polymerase chain reaction in the early diagnosis of HIV-1 infection in high risk subjects. *Microbiologica* **16**:181-184.
- Brandt, C. D., T. A. Rakusan, A. V. Sison, S. H. Josephs, E. S. Saxena, K. D. Herzog, R. H. Parrott, and J. L. Sever. 1992. Detection of human immunodeficiency virus type 1 infection in young pediatric patients by using polymerase chain reaction and biotinylated probes. *J. Clin. Microbiol.* **30**:36-40.
- Burgard, M., M. J. Mayaux, S. Blanche, A. Ferroni, M. L. Guillard-Moscato, M. C. Allemon, N. Ciraru-Vigneron, G. Firtion, C. Floch, and F. Guillot. 1992. The use of viral culture and p24 antigen testing to diagnose human immunodeficiency virus infection in neonates. *N. Engl. J. Med.* **327**:1192-1197.
- Centers for Disease Control. 1989. Interpretation and use of the Western blot assay for serodiagnosis of human immunodeficiency virus type 1 infections. *JAMA* **262**:3465-3466.
- Chadwick, E. G., R. Yague, S. Kwok, J. J. Snisky, D. E. Kellogg, and S. M. Wolinsky. 1989. Enzymatic amplification of the human immunodeficiency virus in peripheral blood mononuclear cells from pediatric patients. *J. Infect. Dis.* **160**:954-959.
- Downie, J. C., R. Howard, B. Bowcock, and A. L. Cunningham. 1989. HIV antibody testing strategy: evaluation of ELISA screening and Western blot profiles in a mixed low risk/high risk patient population. *J. Virol. Methods* **26**:291-303.
- Embretson, J., M. Zupancic, J. Beneke, M. Till, S. M. Wolinsky, J. L. Ribas, A. Burke, and A. T. Haase. 1993. Analysis of human immunodeficiency virus-infected tissues by amplification and in situ hybridization reveals latent and permissive infections at single-cell resolution. *Proc. Natl. Acad. Sci. USA* **90**:357-361.
- Imagawa, D. T., M. H. Lee, S. M. Wolinsky, K. Sano, F. Morales, S. Kwok, J. J. Snisky, P. G. Nishanian, J. Giorgi, and J. L. Fahey. 1989. Human immunodeficiency virus type 1 in homosexual men who remain seronegative for prolonged periods. *N. Engl. J. Med.* **320**:1458-1462.
- Jones, D. S., E. Abrams, C. Y. Ou, S. Nesheim, E. Connor, K. Davenney, P. Thomas, M. Sawyer, K. Krasinski, and M. Bamji. 1993. Lack of detectable human immunodeficiency virus infection in antibody-negative children born to human immunodeficiency virus-infected mothers. *Pediatr. Infect. Dis. J.* **12**:222-227.
- Kaye, S., C. Loveday, and R. S. Tedder. 1991. Storage and preservation of whole blood samples for use in detection of human immunodeficiency virus type-1 by the polymerase chain reaction. *J. Virol. Methods* **35**:217-226.

14. **Knuver-Hopf, J., H. Heinze, B. Lambrecht, H. Mohr, J. Beyer, and H. Schmitt.** 1993. Blood donations indeterminate in HIV-1 Western blot analysed by IgM immunoblot and polymerase chain reaction. *Vox Sang.* **64**:89–93.
15. **Loche, M., and B. Mach.** 1988. Identification of HIV-infected seronegative individuals by direct diagnostic test based on hybridisation to amplified viral DNA. *Lancet* **ii**:418–421.
16. **McCune, J. M.** 1991. HIV 1: the infective process in vivo. *Cell* **64**:351–363.
17. **Nuovo, G. J., M. Margiotta, P. MacConnell, and J. Becker.** 1992. Rapid in situ detection of PCR-amplified HIV-1 DNA. *Diagn. Mol. Pathol.* **1**:98–102.
18. **Patterson, B. K., M. Till, P. Otto, C. Goolsby, M. R. Furtado, L. J. McBride, and S. M. Wolinsky.** 1993. Detection of HIV-1 DNA and messenger RNA in individual cells by PCR-driven in situ hybridization and flow cytometry. *Science* **260**:976–979.
19. **Proffitt, M. R., and N. Yen-Lieberman.** 1993. Laboratory diagnosis of human immunodeficiency virus infection. *Infect. Dis. Clin. N. Am.* **7**:203–219.
20. **Rayfield, M., K. De Cock, W. Heyward, L. Goldstein, J. Krebs, S. Kwok, S. Lee, J. McCormick, J. M. Moreau, K. Odehouri, G. Schochetman, J. J. Sninski, and C. Y. Ou.** 1988. Mixed human immunodeficiency virus (HIV) infection in an individual: demonstration of both HIV type 1 and type 2 proviral sequences by using polymerase chain reaction. *J. Infect. Dis.* **158**:1170–1175.
21. **Simmonds, P., F. A. L. Lainson, R. Cuthbert, C. M. Steel, J. F. Peutherer, and C. A. Ludlam.** 1988. HIV antigen and antibody detection: variable responses to infection in the Edinburgh haemophilic cohort. *Br. Med. J.* **296**:593–598.
22. **Snisky, J. J., and S. Kwok.** 1990. Detection of human immunodeficiency viruses by the polymerase chain reaction. *Arch. Pathol. Lab. Med.* **114**:259–262.
23. **Verani, P., S. Buttò, B. Taddeo, M. Federico, and G. B. Rossi.** 1993. HIV variability and perspectives for a vaccine. *Vaccine* **11**:542–544.
24. **Wethsell, A. J., J. B. Drew, G. Milman, R. Hoff, E. A. Dragon, K. Adler, J. Hui, P. Otto, P. Gupta, H. Farzadegan, and S. M. Wolinsky.** 1992. Comparison of three nonradioisotopic polymerase chain reaction-based methods for detection of human immunodeficiency virus type I. *J. Clin. Microbiol.* **4**:845–853.
25. **Zazzi, M., L. Romano, A. Brasini, and P. E. Valensin.** 1992. Nested polymerase chain reaction for detection of human immunodeficiency virus type 1 DNA in clinical specimens. *J. Med. Virol.* **38**:172–174.
26. **Zazzi, M., L. Romano, A. Brasini, and P. E. Valensin.** 1993. Simultaneous amplification of multiple HIV-1 DNA sequences from clinical specimens by using nested-primer polymerase chain reaction. *AIDS Res. Hum. Retroviruses* **9**:315–320.
27. **Zimmerman, K., K. Pischinger, and J. W. Mannhalter.** 1993. Rapid nonradioactive detection of HIV-1 RNA from a single-cell equivalent by reverse transcription PCR with nested primers. *BioTechniques* **15**:806–808.