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Microplate-Based DNA Hybridization Assays for Detection of Human Retroviral Gene Sequences

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Nonisotopic, microwell-based DNA hybridization assays for the specific detection of human immunodeficiency virus type 1 (HIV-1) *gag*, human T-cell lymphotropic virus type I (HTLV-I) *pol*, and HTLV-II *pol* DNA sequences were evaluated. The performances of these detection kits (Gene Detective enzyme oligonucleotide assays; Cellular Products, Inc., Buffalo, N.Y.) were assessed by using clinical samples whose infection status were established by amplification by PCR and then liquid hybridization detection by using virus-specific probes. Peripheral blood mononuclear cell lysates from 59 HIV-1-, 35 HTLV-I-, and 19 HTLV-II-infected individuals and from 15 healthy blood donors were used as substrates for PCR amplification. The results of the study demonstrated a clinical sensitivity of 100%. In addition, the enzyme oligonucleotide assays were able to detect 1 to 10 proviral copies subsequent to PCR amplification, indicating an analytical sensitivity comparable to that of liquid hybridization.

Gene amplification technology (3, 17, 20), such as PCR, has provided molecular biologists with the means to detect gene sequences refractory to detection by conventional nucleic acid hybridization techniques. An important application of this approach is the detection of genomes of infectious organisms, such as retroviruses, which may be present in only a small percentage of cells or at very low copy numbers (2, 12, 15, 18).

In the present study, we evaluated the performances of a new group of rapid, nonisotopic, microplate-based DNA hybridization assays. These kits, designated enzyme oligonucleotide assays (EOAs), specifically detect human immunodeficiency virus type 1 (HIV-1) or human T-cell lymphotropic virus type I (HTLV-I) or II (HTLV-II) DNA. Microplate-based detection is extremely desirable for research or clinical laboratories because it allows for high sample throughput and eliminates the hazards associated with the handling and disposal of radioisotopes. In contrast to the use of ^{32}P-labeled DNA probes in liquid hybridization (LH) or Southern hybridization, which may take 1 to several days, infection status can be determined within hours.

In the present study, samples analyzed with these kits included DNA that was amplified by PCR from infected peripheral blood mononuclear cells (PBMCs) and from cell lines containing defined numbers of viral genomes. These results demonstrated that EOA and LH yield comparable clinical and analytical sensitivities. Since the EOA approach is nonisotopic and more rapid than LH, it is a preferred alternative to conventional hybridization technologies.

Detection of HIV-1, HTLV-I, and HTLV-II DNAs in cell lines. 8E5/LAV cells containing one proviral copy per cell (8) were subjected to 35 cycles of PCR amplification by using the HIV-1 *gag* gene-specific primer pair SK38-SK39 (18). Optimized reaction parameters were used (1, 6, 16, 19). LH detection involved incubation of 30 μl of the amplified material to a 5′ ^{32}P-end-labeled probe (19). Virus-specific radiolabeled probes included SK19 (HIV-1) (18), SK112 (HTLV-I), and SK188 (HTLV-II) (14) (Table 1). Samples were scored as positive by LH if a band was observed on the autoradiogram in the experimental lanes and not in the negative control lanes. Negative controls for LH were PCR amplification products prepared in the absence of specific target DNA. Quantitative results were obtained by densitometry.

EOA detection of PCR-amplified material was carried out by using Gene Detective EOA kits (Cellular Products, Inc., Buffalo, N.Y.). Briefly, 30 μl of the DNA sample was heat denatured and then simultaneously incubated with a solid-phase capture probe and a solution-phase reporter probe in a microwell at 37°C for 75 to 90 min. The microwells were washed, substrate (*para*-nitrophenyl phosphate) was added to each well (2 h at 37°C), and the A_{405} was determined for each microwell. The reactive threshold value for each experiment was equal to 0.075 units at an optical density at 405 nm (OD_{405}) plus the mean OD of the negative control wells (0.075 OD_{405} units are 5 standard deviations above the mean OD for material from the healthy population). A sample whose OD_{405} was equal to or greater than the reactive threshold value was considered reactive.

Figure 1A depicts the results obtained when DNA prepared from 8E5/LAV cells was amplified by PCR and subjected to detection by using the HIV-1 *gag* EOA and LH. PCR amplification of 8E5/LAV cells followed by EOA analysis demonstrated that one HIV-1 copy was detectable. Similarly, HTLV-I-infected cells (HSC-CTCL-11B) (7) and HTLV-II-infected cells (MoT) (5) were lysed and amplified by PCR for 45 cycles with 10 pmol of primers SK110-SK111 (14). Following amplification, 30 μl of reaction product was subjected to detection by LH and the HTLV-I *pol* EOA or HTLV-II *pol* EOA. The results of these experiments demonstrated that both EOA and LH detected 10 HTLV-I copies (Fig. 1B); one proviral copy of HTLV-II (Fig. 1C) was detectable by each technique. The slopes of the EOA curves in Fig. 1A to C were greater than the slopes of the LH

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TABLE 1. Genomic locations of EOA and LH probes

Virus	Solid-phase EOA capture probe (bp)	Enzyme-labeled EOA reporter probe (bp)	5'-Radiolabeled LH probe (bp)	Virus gene
HIV-1[a]	1595–1614 (5' biotin)	1616–1635 (3' alkaline phos[b])	SK19 (1595–1635)	*gag*
HTLV-I[c]	SK115 (4870–4902; 3' biotin)	SK112 (4825–4850; 5' Alk.Phos.)	SK112 (4825–4850)	*pol*
HTLV-II[d]	SK115 (4848–4880; 5' biotin)	SK188 (4880–4898; 3' Alk.Phos.)	SK188 (4880–4898)	*pol*

[a] GenBank accession number, K02007.
[b] Alk.Phos., alkaline phosphatase.
[c] GenBank accession number, J02029.
[d] GenBank accession number, M10060.

curves; however, both detection methods exhibited similar analytical sensitivities.

Characterized field isolates. Patient samples were obtained from North Americans who were either healthy volunteer blood donors or patients referred to Health Sciences Center, State University of New York, Syracuse, for the diagnosis of retroviral infection. All patient sera were screened with commercially available HIV-1 and HTLV-I enzyme-linked immunosorbent assays (Cellular Products, Inc.). Repeatably reactive samples were confirmed as antibody positive by using Western blots (immunoblots) (Dupont for HIV-1 and Diagnostic Biotechnology for HTLV-I and HTLV-II). In the case of the patients infected with HIV-1, all had been shown to be positive for HIV-1 in a 28-day cell coculture with a p24 antigen capture assay as the monitor (Cellular Products, Inc.).

PBMCs isolated from infected patients or healthy blood donors were lysed and amplified by PCR by using virus-specific primers and were detected by LH by using virus-specific probes (Table 1). Only specimens that were PCR and LH positive were used to evaluate the clinical sensitivities of the EOA kits.

Detection of HIV-1 DNA in PBMCs from infected patients and controls. PBMCs were isolated from whole blood by a Ficoll-Hypaque step gradient, and 10^6 cells were lysed for 1 h at 60°C in 50 μl of a solution containing 100 mM KCl, 10 mM Tris (pH 8.3), 2.5 mM $MgCl_2$, 1% Tween 20, 1% Nonidet P-40, and 60 μg of proteinase K per ml. Proteinase K was then inactivated by boiling for 60 min. Insoluble material was removed by centrifugation, and 50 μl of the supernatant was used as substrate for PCR amplification by using primers SK38-SK39. EOA analysis of PCR products from 59 HIV-1-infected patients yielded sample/cutoff values over a wide range (14.9 to 27.9), with a mean and standard deviation of 21.4 ± 6.5 (Fig. 2). The specificities of EOAs were studied by using PCR products from HTLV-I- or HTLV-II-infected patients and healthy blood donors that were amplified by PCR with primers SK38-SK39. None of these samples was reactive in the HIV-1 *gag* EOA, with means and standard deviations of 0.15 ± 0.19 (n = 28), 0.25 ± 0.21 (n = 16), and 0.24 ± 0.20 (n = 11), respectively (Fig. 2).

Detection of HTLV-I DNA from PBMCs of infected patients and controls. PBMCs from healthy blood donors and individuals infected with either HTLV-I, HTLV-II, or HIV-1 were used as substrates for PCR amplification with primers SK110-SK111. Following amplification, reaction products were subjected to detection by LH and by the HTLV-I *pol* EOA.

Of the 35 specimens from HTLV-I-infected individuals that were examined, EOA was able to detect HTLV-I-specific sequences in all of these specimens. HTLV-I sequences were undetectable in the specimens from healthy blood donors and in the specimens from HIV-1- and HTLV-II-infected individuals following amplification by PCR with primers SK110-SK111 (Fig. 3). Each of the HTLV-I samples yielded sample/cutoff values over a wide range (6.4 to 18.2), with a mean and standard deviation of 12.3 ± 5.9. Analysis of specimens from healthy blood donors and HTLV-II- and

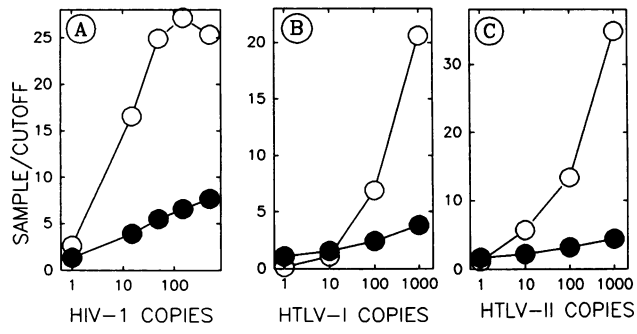

FIG. 1. Specific detection of HIV-1, HTLV-I, and HTLV-II proviral DNAs in cultured cells. Cellular DNA was subjected to PCR amplification with primer pairs SK38-SK39 (A) or SK110-SK111 (B and C). Reaction products (30 μl) were detected by EOA (○) and LH (●) analyses. A specimen whose sample/cutoff value was ≥1 was considered reactive. Each EOA datum point represents quadruplicate measurements; LH datum points represent duplicate measurements (standard deviations, less than 10%).

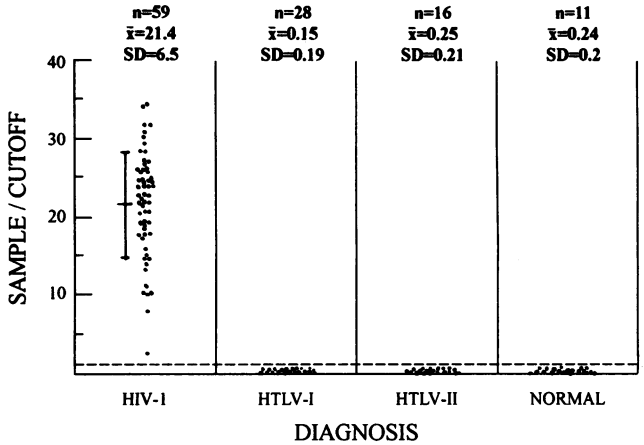

FIG. 2. Detection of HIV-1 DNA in PBMCs from infected patients and controls. A total of 10^6 PBMCs from infected individuals and healthy blood donors were lysed, amplified by PCR with primers SK38-SK39, and detected by the HIV-1 *gag* EOA. The dotted line denotes a sample/cutoff value of 1. A specimen whose sample/cutoff value was ≥1 was considered reactive.

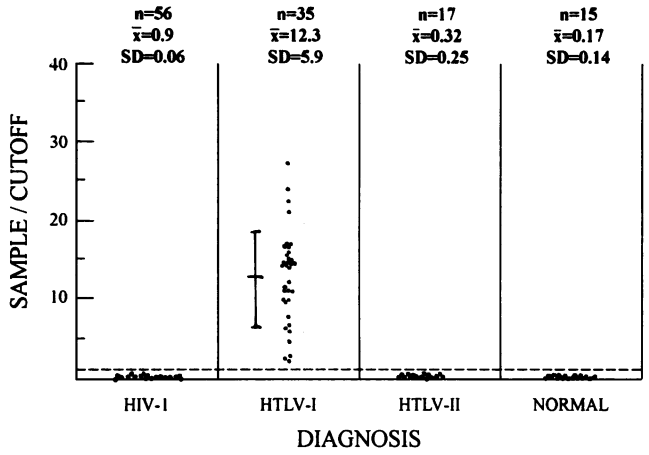

FIG. 3. Detection of HTLV-I DNA in PBMCs from infected patients and controls. A total of 10^6 PBMCs from infected individuals and healthy blood donors were lysed, amplified by PCR with primers SK110-SK111, and detected by the HTLV-I *pol* EOA. The dotted line denotes a sample/cutoff value of 1. A specimen whose sample/cutoff value was ≥1 was considered reactive.

HIV-1-infected individuals demonstrated the specificity of the HTLV-I *pol* EOA. These sample/cutoff values were similar to the negative control values, exhibiting means and standard deviations of 0.17 ± 0.14 (n = 15), 0.32 ± 0.25 (n = 17), and 0.09 ± 0.06 (n = 56), respectively.

Detection of HTLV-II DNA from PBMCs of infected patients and controls. PBMCs from healthy blood donors and from individuals infected with HTLV-I, HTLV-II, or HIV-1 were used as substrates for PCR amplification with primers SK110-SK111. Following amplification, the reaction products were subjected to detection by HTLV-II *pol* EOA. Of the 19 specimens from HTLV-II-infected individuals examined, EOA was able to detect HTLV-II sequences in all of these specimens (Fig. 4). The HTLV-II samples yielded sample/cutoff values over a wide range (14.1 to 36.3), with a mean and standard deviation of 25.2 ± 11.1. HTLV-II was undetectable in the specimens from HIV-1- and HTLV-I-infected individuals and in the specimens from healthy blood donors, with means and standard deviations of 0.13 ± 0.12 (n = 59), 0.20 ± 0.14 (n = 35), and 0.21 ± 0.14 (n = 15), respectively.

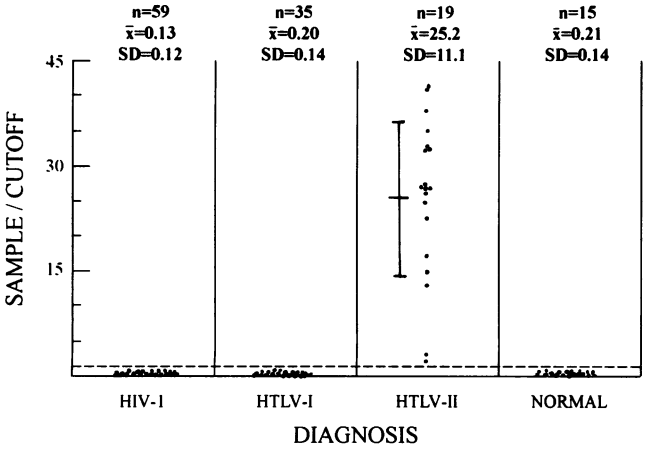

FIG. 4. Detection of HTLV-II DNA in PBMCs from infected patients and controls. A total of 10^6 PBMCs from infected individuals and healthy blood donors were lysed, amplified by PCR with primers SK110-SK111, and detected by the HTLV-II *pol* EOA. The dotted line denotes a sample/cutoff value of 1. A specimen whose sample/cutoff value was ≥1 was considered reactive.

The combination of PCR amplification and detection of viral genes by using a nonisotopic hybridization assay offers exquisite sensitivity and simplicity. The EOA approach provides a format which allows for high sample throughput without compromising sensitivity or specificity. The results obtained in the present study demonstrated that EOA is a viable alternative to standard radioisotopic hybridization methods, such as LH, for the detection of gene amplification products.

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