Comparison of Three Nonradioisotopic Polymerase Chain Reaction-Based Methods for Detection of Human Immunodeficiency Virus Type 1

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Three nonradioisotopic polymerase chain reaction (PCR)-based detection techniques were evaluated for sensitivity and specificity in detecting human immunodeficiency virus type 1 (HIV-1) proviral DNA in peripheral blood mononuclear cells. The Roche prototype HIV-1 PCR assay, the Du Pont enzyme-linked oligonucleotide sandwich assay (ELOSAs), and the Gen-Probe hybridization protection assay (HPA) were compared with a standard radioisotopic oligonucleotide solution hybridization (OSH) technique. A panel of 111 well-characterized clinical samples that included peripheral blood mononuclear cells from 48 healthy, low-risk, HIV-1 antibody-negative subjects, 24 antibody-positive subjects with stable CD4 counts of less than 200/mm³, and 39 antibody-positive subjects with stable CD4 counts of greater than 800/mm³ were studied. Each method demonstrated good specificity, ranging between 96 and 100%; those of the OSH and ELOSAs (Du Pont) were 100%, those of the HPA (Gen-Probe) were 100% with one probe and 96% with the other probe, and that of the HIV-1 PCR assay (Roche) was 96%. Sensitivities ranged from 96 to 100% for the low-CD4-count group, with the OSH, the HIV-1 PCR assay (Roche), and the HPA (Gen-Probe) all attaining a sensitivity of 100%. For the high-CD4-count group, sensitivities ranged from 69 to 97%, with the OSH attaining a sensitivity of 97% and the HPA attaining sensitivities of 97% with one probe and 95% with the other probe. These data indicate that the nonradioisotopic techniques are sensitive and specific for the detection of HIV-1 proviral DNA in clinical samples.

The polymerase chain reaction (PCR) has proven to be an invaluable tool in molecular biology and more recently as a diagnostic technique (3). The use of PCR to identify specific nucleic acid sequences has facilitated the detection of many infectious agents, such as human immunodeficiency virus type 1 (HIV-1). Currently, HIV-1 infection is diagnosed by the presence of specific antibodies by use of an enzyme-linked immunosorbent assay (EIA) with a confirmatory Western blot (immunoblot) or by virus isolation with cocultivation techniques (1, 10). In certain clinical situations, unequivocal diagnosis has been hampered by the relative insensitivities of these more commonly used antibody tests (2, 4, 8, 9, 13, 16, 19–23). The ability of PCR to screen directly for the presence of proviral DNA or viral RNA (3, 6) has enabled the virus to be detected before the development of antibodies (7, 8). In addition, PCR is one of the few tests that can detect HIV-1 infection in infants born to HIV-1-infected mothers; in this situation the presence of maternal antibodies during the neonatal period confounds diagnosis by EIA and Western blotting (5, 11, 18, 19).

The most commonly used method for the detection of PCR-amplified HIV-1 DNA is radioisotopic oligonucleotide solution hybridization (OSH). This method demonstrates high levels of sensitivity and specificity (21) but lacks the convenience of a nonradioisotopic test. Although initial studies have demonstrated the utility of PCR for the diagno-

sis of HIV-1, wider application of the technology awaits the development of simple and reliable “kits” that can be used routinely in clinical laboratories. Recently, several PCR-based HIV-1 detection techniques which eliminate the requirement for radioisotopes and permit numerous samples to be analyzed in a relatively short period of time were developed by Roche Diagnostics Systems (Fair Lawn, N.J.), Du Pont (Billerica, Mass.), and Gen-Probe (San Diego, Calif.).

To determine the sensitivity and specificity of these prototype assays for detecting HIV-1 proviral DNA, we used a blinded, randomized panel of 111 well-characterized peripheral blood mononuclear cell (PBMC) samples from HIV-1-infected and uninfected individuals. The results of these assays were compared with those of the standardized radioisotopic method used in our laboratory. This study shows that each of these assays is specific and sensitive in detecting HIV-1 proviral DNA in PBMC samples.

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MATERIALS AND METHODS

Study participant selection. Clinical samples were obtained from individuals enrolled in the Multicenter AIDS Cohort Study (MACS), a prospective study of the natural history of HIV-1 infection in homosexual and bisexual men. These individuals were confirmed as positive or negative for antibodies to HIV-1 by both the EIA and the Western blot for at

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least 2 years prior to and 1 year after the specimen collection date. A coded panel consisting of three groups of patient samples was selected to evaluate the prototype assays. Group I consisted of 48 MACS participants who were seronegative before and have remained seronegative for 1 year after a sample was obtained for PCR. Group II consisted of 24 HIV-1-seropositive individuals with stable CD4 counts of less than 200/mm³ at the time that the sample was obtained. Group III consisted of 39 HIV-1-seropositive individuals with stable CD4 counts of greater than 800/mm³. This project was approved by the human subjects institutional review board at each of the three participating centers. Informed consent was obtained from the study participants for the purpose of this investigation.

Specimens. PBMCs were separated from heparinized whole blood by centrifugation on a Lymphocyte Separation Medium (Organon Teknika Corp.) gradient. The PBMC layer was removed and washed three times with phosphate-buffered saline. The separated PBMCs were stored either as a cell pellet of 10⁶ cells at -80°C or in RPMI medium supplemented with 50% fetal bovine serum and 10% dimethyl sulfoxide at -135°C. Before cell lysis, the dimethyl sulfoxide freezing medium was removed by two washes with phosphate-buffered saline.

Cell pellets containing 10⁶ cells were resuspended in 400 µl of lysis buffer (10 mM Tris [pH 8.3], 50 mM KCl, 2.5 mM MgCl₂, 0.5% Tween 20, 0.5% NP-40, proteinase K [100 µg/ml]), and the suspension was incubated at 60°C for 1 h. Following digestion of the proteins, the cellular DNA was incubated at 95°C for 10 min to heat inactivate the proteinase K. A 50-µl aliquot of cell lysate, equivalent to 1 µg of genomic DNA or 1.25 × 10⁵ cells, was used for each amplification reaction; the Du Pont method was done with a 25-µl aliquot, equivalent to 0.5 µg of DNA. A standard curve was prepared by spiking PBMCs from seronegative donors with an appropriate number of 8E5 cells (a cell line with one copy of proviral HIV-1 LAV DNA per cell; AIDS Research and Reference Reagent Program catalog no. 95) to obtain 0, 5, 20, and 50 copies of proviral DNA per 50 µl, equivalent to one PCR. Since the Du Pont assay was done with a 25-µl sample aliquot, these copy number standards corresponded to 0, 2.5, 10, and 25 copies for this assay. The processed lysates were stored at -20°C.

Each prototype assay was evaluated with an aliquot of the same sample lysate prepared from PBMCs isolated from each individual. The lysate samples were coded and placed in random order so that the technicians (A.J.W. and J.H.) and representatives from Du Pont (K.A.) and Roche (E.A.D.) were blinded to the infection and CD4 statuses of the samples. Sample processing, preamplification setup, and postamplification product DNA analysis were each performed in separate facilities.

Oligonucleotides. The oligonucleotides used for the OSH and Gen-Probe hybridization protection assay (HPA) methods were synthesized with an Applied Biosystems 380B DNA synthesizer by use of phosphoramidite chemistry. Primers and probes used for the Du Pont and Roche assays were supplied by their respective manufacturers. Gen-Probe provided the appropriate acridinium-labeled probes. Table 1 lists the sequences for the primers and probes and nucleotide positions relative to those of the HIV-1 BRU prototypic isolate. The sequences of the capture and reporter probes used for the Du Pont assay were not provided by the manufacturer.

Statistical analysis. The chi-square test was used to determine the statistically significant differences among the compared tests; *P* < 0.05 was considered significant.

MACS OSH method. One microgram of PBMC DNA was amplified in 100 µl of a reaction mixture containing 2.5 mM MgCl₂; 50 mM KCl; 10 mM Tris-HCl (pH 8.3); 0.01% gelatin; 0.2 mM each dATP, dGTP, dTTP, and dCTP; 0.5 µM each 5' primer and 3' primer; and 2.5 U of *Thermus aquaticus* (*Taq*) DNA polymerase (Perkin Elmer Cetus). The reaction mixture was overlaid with light white mineral oil (Sigma) to prevent evaporation. The reactions were performed with a Perkin Elmer Cetus automated 48-well thermal cycler programmed for 30 cycles of denaturation at 94°C for 50 s, annealing at 55°C for 50 s, and extension at 72°C for 1 min. After 30 cycles, primer extension was continued for 10 min at 72°C. Prior to being tested in any of the assays, primer pair GH26/27, which flanks the human leukocyte antigen-DQα locus, was used for each sample to verify that the DNA could be amplified by PCR. Product DNA was then resolved by electrophoresis on a 1% agarose gel to screen for the presence of the appropriately sized band. Each sample was then amplified with primer pairs SK68-SK69 (*env*) and SK101-SK145 (*gag*) and, in cases of discrepancies, SK38-SK39 (*gag*). Positive and negative controls amplified with each run included 0-, 5-, 20-, and 50-copy-number standards as well as a reagent control.

Product DNA was detected by hybridization in solution with specific internally conserved ³²P-end-labeled probes. Oligonucleotide probes were 5' end labeled with [γ-³²P]ATP by use of polynucleotide kinase (Boehringer Mannheim Biochemicals). Unincorporated [γ-³²P]ATP was removed by elution through a Sephadex G-25 spun column. A 30-µl aliquot of the amplified product was added to 10 µl of a hybridization mixture containing 750,000 cpm of labeled probe, 60 mM NaCl, and 10 mM EDTA. A 50-µl aliquot of light white mineral oil (Sigma) was added before sample addition to reduce aerosolization. The amplified product was heat denatured at 95°C for 5 min and then hybridized to the probe at 56°C for 15 min. An 8-µl aliquot of 6× loading dye (1× loading dye is 2.5% Ficoll 400, 0.042% bromophenol blue, and 0.042% xylene cyanol) was added to each sample, and 20 µl of this mixture was resolved by electrophoresis on a 10% polyacrylamide gel with 1× TBE (0.89 M Tris-borate, 2 mM EDTA). Unhybridized probe was removed by cutting the gel immediately below the xylene cyanol dye front. The gel was then transferred to Whatman 3MM paper, wrapped in plastic wrap, and exposed to Kodak XAR-5 film at -80°C with an intensifying screen for 12 to 16 h.

Roche method (prototype HIV-1 PCR assay) (Fig. 1). Samples were amplified in duplicate in 100-µl reaction mixtures containing 50 µl of cell lysate and 50 µl of Master mix consisting of dATP, dCTP, dGTP, dUTP, Bio-SK462, Bio-SK431, *Taq* polymerase, uracil *N*-glycosylase, and salts. Bio-SK462 and Bio-SK431 are biotinylated primers which create a biotinylated product when used for PCR amplification. Following the addition of cell lysate and Master mix, samples were initially incubated for 10 min at room temperature to allow sufficient time for uracil *N*-glycosylase to destroy any dUTP-containing amplified product that was inadvertently carried over from previous amplifications. This incubation was followed by 30 cycles at 95°C for 10 s and 55°C for 30 s in a 96-well thermal cycler (Perkin Elmer Cetus). After the initial results had been tabulated and the samples had been decoded, the Roche cycling parameters were changed to 50°C for 2 min, 5 cycles at 95°C for 10 s, 55°C for 10 s, and 72°C for 10 s, and 30 cycles at 90°C for 10

TABLE 1. Oligonucleotide primers and probes

Test and primer or probe	Region ^a	Sequence (5' to 3')	Nucleotide positions
OSH			
GH26 (primer)	HLA	GTGCTGCAGGTGTAAACTTGTACCA	
GH27 (primer)	HLA	CACGGATCCGGTAGCAGCGGAGAGTTG	
SK38 (primer)	<i>gag</i>	ATAATCCACCTATCCCAGTAGGAGAAAT	1551-1578
SK39 (primer)	<i>gag</i>	TTTGGTCCTTGTCTTATGTCCAGAATGC	1638-1665
SK19 (probe)	<i>gag</i>	ATCCTGGGATTAAATAAAATAGTAAGAATGTATAGCCCTAC	1595-1635
SK101 (primer)	<i>gag</i>	GCTATGTCAGTTCCCTTGGTTCTC	1482-1506
SK145 (primer)	<i>gag</i>	AGTGGGGGACATCAAGCAGCCATGCAAAAT	1366-1395
SK102 (probe)	<i>gag</i>	GAGACCATCAATGAGGAAGCTGCAGAATGGGAT	1403-1435
SK68 (primer)	<i>env</i>	AGCAGCAGGAAGCACTATGG	7801-7820
SK69 (primer)	<i>env</i>	CCAGACTGTGAGTTGCAACAG	7922-7942
SK70 (probe)	<i>env</i>	ACGGTACAGGCCAGACAATTATTGTCTGGTATAGT	7841-7875
Roche			
Bio-SK431 (primer)	<i>gag</i>	TGCTATGTCAGTTCCCTTGGTTCTCT	1481-1507
Bio-SK462 (primer)	<i>gag</i>	AGTTGGAGGACATCAAGCAGCCATGCAAAAT	1366-1395
SK102 (probe)	<i>gag</i>	GAGACCATCAATGAGGAAGCTGCAGAATGGGAT	1403-1435
Gen-Probe			
SK38 (primer)	<i>gag</i>	ATAATCCACCTATCCCAGTAGGAGAAAT	1551-1578
SK39 (primer)	<i>gag</i>	TTTGGTCCTTGTCTTATGTCCAGAATGC	1638-1665
gag 1 (probe)	<i>gag</i>	GATGGATAATCCTGGGATTAAATAAAATAGTAAGAATGTATAGC	1587-1630
gag 2 (probe)	<i>gag</i>	-----ATCCTGGGATTAAATAAAATAGTAAGAATGTATAGCCCTACCA	1595-1637
SK101 (primer)	<i>gag</i>	GCTATGTCAGTTCCCTTGGTTCTC	1482-1506
SK145 (primer)	<i>gag</i>	AGTGGGGGACATCAAGCAGCCATGCAAAAT	1366-1395
gag 3 (probe)	<i>gag</i>	AGAGACCATCAATGAGGAAGCTGCAGAATG	1392-1421
gag 4 (probe)	<i>gag</i>	-----CCATCAATGAGGAAGCTGCAGAATGGGATAG	1397-1427
Du Pont			
5'Pol (primer)	<i>pol</i>	CTAAAGGAAGCTCTATTAGA	1889-1908
3'Pol (primer)	<i>pol</i>	GTCAATGGCCATTGTTTAAC	2192-2211

^a HLA, human leukocyte antigen locus.

s, 60°C for 10 s, and 72°C for 10 s. (These modifications were introduced to improve the sensitivity of the assay in detecting a low copy number of HIV-1 DNA.) Samples were then cooled to 4°C, and an equal volume (100 µl) of denaturation solution containing NaOH (Roche) was added immediately.

Microtiter plates containing wells coated with a specific oligonucleotide probe (SK102) were removed from 4°C and allowed to reach an ambient temperature. Each sample was detected in triplicate by adding 100 µl of hybridization solution (Roche) and 25 µl of denatured amplified product to each well, gently mixing, and then incubating the mixture at 37°C for 60 min to allow hybridization of the biotinylated product to the SK102-coated wells. To remove any unhybridized product, we washed the plate four times with Wash Buffer I (Roche) by using an automated plate washer. The plate was then incubated at 37°C for 15 min with an avidin-horseradish peroxidase conjugate (Roche). To remove unbound conjugate, we again washed the plate four times (automated) with Wash Buffer I and once (manual) with Wash Buffer II (Roche). Tetramethylbenzidine (Roche), a chromogenic substrate for horseradish peroxidase, was added to each well, and the contents of the plate were allowed to develop at room temperature for 10 min. The color reaction was stopped with 100 µl of stop solution (Roche), and the plate was read at 450 nm on a microtiter plate reader (Bio-Tek Instruments). The optical densities (OD) can range from 0.0 to 3.0 or more. Readings greater than 3.0 were off-scale and were therefore not assigned a value.

Du Pont method (ELOS) (Fig. 1). PCR was performed in duplicate with 25 µl of each lysed cell sample. The reaction

mixture (total volume, 50 µl) contained 50 mM Tris (pH 9.0), 20 mM (NH₄)₂SO₄, 1.5 mM MgCl₂, 200 µM each deoxynucleoside triphosphate (dNTP), 1 µM 5'Pol primer, 1 µM 3'Pol primer, and 1 U of *Thermus flavus* DNA polymerase. Reactions were cycled 35 times in a programmable 48-well thermal cycler (Perkin Elmer Cetus); the first cycle was done at 94°C for 2 min, 45°C for 1 min, and 72°C for 1 min, and all additional cycles were done at 94°C for 1 min, 45°C for 1 min, and 72°C for 1 min.

The HIV-1 DNA products were detected by the HIV-1 enzyme-linked oligonucleotide sandwich assay (ELOS). The assay was performed as follows. A 20-µl aliquot of the heat-denatured PCR sample was added to a streptavidin-coated microplate well in duplicate. A 100-µl aliquot of hybridization buffer containing a biotin-labeled capture oligonucleotide and a horseradish peroxidase-labeled reporter oligonucleotide complementary to the same strand of the PCR product (323 bases, from 1889 to 2211, of HIV-1 BRU) was added to each well, and the mixture was hybridized for 60 min at 37°C. When a sample contained the specific HIV-1 PCR product, a hybridization complex was formed among the PCR product, the capture probe, and the reporter probe (collectively termed "detector" in Tables 2 and 3), and the complex was bound to the microplate well by biotin-streptavidin affinity. Uncomplexed reporter probe was removed by washing of the microplate with an automated plate washer (Bio-Tek Instruments).

A 100-µl aliquot of ortho-phenylene diamine (OPD) substrate and buffer was added, and the colorimetric reaction was allowed to develop at room temperature for 60 min. Enzyme development was stopped by the addition of 4 N

FIG. 1. Comparison of nonradioisotopic HIV-1 detection assays. (a) Roche prototype HIV-1 PCR assay. (b) Du Pont ELOSA. (c) The Gen-Probe HPA. ●, horseradish peroxidase; AV, avidin; B, biotin; TMB, tetramethylbenzidine; ODP, ortho-phenylene diamine; Strep, streptavidin; RLU, relative light units. $\text{O}^\ominus$, acridinium ester (not correct chemical structure).

H_2SO_4 , and the OD of each sample well was read at 490 nm on a microtiter plate reader. The OD can range from 0.0 to 3.0 or more.

Gen-Probe method (HPA) (Fig. 1). Samples were amplified under the same conditions as those used in the MACS OSH method. Each sample was amplified one time each with the SK101-SK145 and SK38-SK39 primer pairs, except in the case of primer pair discrepancy, in which the sample was reamplified with the primer pair that produced the negative result.

For the HPA, 25 μl each of amplified DNA and TE buffer (10 mM Tris-HCl, 1 mM EDTA) were added in duplicate to polypropylene tubes (12 by 75 mm). The mixture was heat denatured at 95°C for 5 min and then quickly chilled on ice. A probe solution consisting of 2 μl (0.02 pmol) of either gag 3-gag 4 (acridinium ester-labeled oligonucleotides specific for SK101-SK145 products) or gag 1-gag 2 (acridinium ester-labeled oligonucleotides specific for SK38-SK39 products) and 48 μl of hybridization buffer (0.1 M lithium succinate buffer [pH 4.7], 2% [wt/vol] lithium lauryl sulfate, 1.2 M lithium chloride, 20 mM EDTA, 20 mM ethylene glycol-bis(β -aminoethyl ether)- N,N,N',N' -tetraacetic acid) was added to each sample. Hybridization was done for 10 min in a 60°C water bath. Unhybridized probe was hydrolyzed and therefore rendered nonchemiluminescent by the addition of 300 μl of hydrolysis buffer (0.6 M sodium borate [pH 8.5], 1% Triton X-100), and then the mixture was vortexed and

incubated for 6 min at 60°C. The tubes were placed in an ice bath for 1 to 5 min and subsequently allowed to equilibrate to room temperature for 1 to 5 min.

Samples were analyzed with a Leader I luminometer (Gen-Probe), which automatically injects two detection reagents, one containing 0.1% hydrogen peroxide and 1 mM nitric acid and the other containing 1 N sodium hydroxide and a surfactant. Units of detection are given in relative light units, which remain linear from 0 to 1,000,000 (17).

Data analysis. The results for the OSH method were determined in accordance with a preestablished algorithm. Each sample was amplified with two primer pairs, SK68-SK69 and SK101-SK145. When the results obtained with these two primer pairs were in agreement, no further amplification was performed on the sample. However, when one primer pair produced a negative result and the other produced a positive result, the sample was amplified with a third primer pair, SK38-SK39. When the SK38-SK39 amplification produced a positive result, the sample was considered positive, but when it produced a negative result, the sample was retested with the primer pair producing the positive result. When the retesting yielded a positive result, the sample was considered indeterminate. The sample was considered negative when the retesting yielded a negative result. Positive samples are indicated in Table 2 as one, two, or three plus signs. One plus sign indicates that the band on the autoradiograph corresponded in intensity to the 5-copy-

TABLE 2. Results for high-CD4-count seropositive samples^a

Sample	CD4 count (no. of cells/ mm ³)	Result obtained with:						
		OSH		Algorithm	Roche		Gen-Probe	
		SK70 and SK68/SK69	SK102 and SK101/SK145		SK102 and SK431/SK462	Retrial	gag 1-gag 2 and SK38/SK39	gag 3-gag 4 and SK101/SK145
112	804	+++	+++	+++	+++		+++	++
18	805	++	+++	+++	++		++	+++
90	806	+++	+++	+++	++		+	+
51	819	+++	+++	+++	++		+++	+++
24	840	++	+++	+++	+++		++	+++
39	848	++	++	++	-	++	+	+
75	869	+++	+++	+++	++		+++	+++
86	876	+++	+++	+++	+		++	+
7	880	++	+++	+++	+++		++	+
77	890	+++	+++	+++	-	+++	+	+
28	898	++	+	++	+		++	+
29	901	-	+	+	+		+	+
13	902	++	+++	+++	++		++	+
89	924	++	++	++	-	++	+	+
91	925	+	+	+	-	++	+	±
55	945	+	++	++	-	+++	+	+
36	956	++	+++	+++	+++		++	+
71	968	++	++	++	+		++	+++
52	1,014	-	++	++	-	+	+	+
109	1,036	+++	+++	+	+		++	+
3	1,041	+	++	++	+		+	++
105	1,044	++	++	++	-	+++	+	+
78	1,081	+++	+++	+++	-	+++	++	+
67	1,120	++	+++	+++	++		+++	+++
64	1,128	++	+++	+++	+		++	+++
110	1,139	-	++	+++	+++		+	+
8	1,161	+	++	++	+		+	+++
114	1,215	++	++	++	+++		++	+
53	1,243	-	++	+++	+		+	+
84	1,304	+	+	+	-	-	+	+
69	1,310	+++	+++	+++	+++		+++	+++
17	1,320	-	-	-	-	+	-	-
88	1,320	++	+++	+++	+		+	+
73	1,417	-	++	+++	+++		+++	+++
72	1,446	+	+	+	-	+++	+	+
68	1,533	++	+	++	-	++	+	+
113	1,552	+	++	++	+		+	++
111	1,619	+	++	++	++		+	+
60	1,681	++	++	++	+		+	+

^a -, negative; ±, indeterminate.

number standard, two plus signs indicate correspondence to the 20-copy-number standard, and three plus signs indicate correspondence to the 50-copy-number standard (or more). The radioisotopic method consistently detected the five-copy-number standard.

For the Roche detection method, each sample lysate was amplified with one primer pair, SK431-SK462, in duplicate, and each amplified sample was detected in triplicate, resulting in six datum points for each sample. Background values corresponding to reagent lysate buffer blanks for each run were averaged and subtracted from each datum point. Any deviants (greater than 20% coefficient of variation) in the triplicate values were discarded, and the remaining two values were averaged. An OD of 0.2 was used to differentiate positives from negatives. Thus, all values 0.2 and above were positive, and all those below were negative. This value of 0.2 was subtracted from all the OD means; one plus sign was assigned to values ranging from 0.000 to 0.999, two plus signs were assigned to values ranging from 1.000 to 1.999, and three plus signs were assigned to values ranging from 2.000 to 2.800. For the control standards, one plus sign

corresponded to 20 copies, and two plus signs corresponded to 50 copies. The average value obtained for the five-copy-number standard was 0.182, below the 0.200 cutoff value, and therefore was not assigned one plus sign.

With the Du Pont detection system, each sample lysate was amplified with one primer pair, 5' Pol-3' Pol, in duplicate, and each amplified sample was detected in duplicate, producing four datum points for each sample. Background values, determined from the substrate blank, were subtracted, and duplicates from each amplification were averaged. When the two averages for a sample produced different results, that particular sample was reamplified. Samples with an OD below 0.025 were considered negative. Samples with OD ranging from 0.025 to 0.075 were considered indeterminate. Samples with an OD above 0.075 were considered positive. For the assignment of plus signs, 0.024 was subtracted from each OD mean; one plus sign was assigned to values between 0.051 and 0.999, two plus signs were assigned to values between 1.000 and 1.999, and three plus signs were assigned to values between 1.999 and 2.976. The ELOSA produced a linear standard curve at between 5 and

TABLE 3. Specificities and sensitivities^a

Test	Seronegatives (n = 48)				Seropositives with CD4 count/mm ³ of:							
	Nega- tive	Indeter- minate	False- positive	Speci- ficity	<200 (n = 24)				>800 (n = 39)			
					Posi- tive	Indeter- minate	False- negative	Sensi- tivity	Posi- tive	Indeter- minate	False- negative	Sensi- tivity
OSH												
SK70 and SK68/ SK69	47	0	1	98	23	0	1	96	33	0	6	85
SK102 and SK101/ SK145	48	0	0	100	24	0	0	100	38	0	1	97
Algorithm	48	0	0	100	24	0	0	100	38	0	1	97
Roche												
SK102 and SK431/ SK462	46	0	2	96	24	0	0	100	27	0	12	69
Retrial	47	0	1	98					38	0	1	97
Gen-Probe												
gag 1-gag 2 and SK38/SK39	46	2	0	96	24	0	0	100	38	0	1	97
gag 3-gag 4 and SK101/SK145	48	0	0	100	24	0	0	100	37	1	1	95
Du Pont detector and 3'Pol-5'Pol	48	0	0	100	23	0	1	96	33	1	5	85

^a Data are reported as numbers of samples, except for specificities and sensitivities, which are reported as percentages.

50 copies. Therefore, one plus sign corresponded to 5 copies, two plus signs corresponded to 20 copies, and three plus signs corresponded to 50 copies or more.

For detection with the Gen-Probe system, each sample lysate was amplified with two sets of primer pairs, SK101-SK145 and SK38-SK39. Each amplified product was detected in duplicate, resulting in four values for each sample. The duplicate values were averaged for each primer pair, and sample values were compared with assay background values, which were determined with zero-copy-number standards for each run. Samples with values greater than two times the background were considered positive, and those with values equal to or less than the background were considered negative. Samples with values ranging between the background and two times the background were considered indeterminate. The samples were reanalyzed when the results from both primer pairs were discordant or when values were within the indeterminate range. For the assignment of plus signs, two times the background (zero-copy-number standard) was subtracted from the mean of each sample value. One plus sign corresponded to values from 500 to 50,000 relative light units. Two plus signs were assigned to values from 50,001 to 150,000 relative light units. Three plus signs were assigned to values of 150,001 relative light units and above. Because of the broad linear range of the HPA, plus values could not be correlated with the copy number standards.

RESULTS

For analysis purposes, the 111 coded clinical samples were divided into three groups according to HIV-1 antibody status and CD4 cell number. These included 48 samples from low-risk, HIV-1 antibody-negative subjects, 24 samples from low-CD4-count (<200 cells/mm³) seropositive subjects, and 39 samples from high-CD4-count (>800 cells/mm³) seropositive subjects. Copy number standards and appropriate positive and negative controls were run concurrently.

The results for the 48 seronegative samples revealed differences among the four assays (data not shown). Both the OSH and the Du Pont assays achieved an overall specificity of 100%. Only in one case was there a false-positive PCR assay result (sample 19, primer pair SK68-SK69) but, as dictated by the PCR algorithm, this sample retested negative with SK68-SK69 and therefore was judged negative. The Roche assay correctly identified 46 of the 48 seronegative samples and therefore achieved 96% specificity. To examine the possibility that the two false-positives encountered with the Roche assay could have been attributed to sample contamination, we analyzed the same amplified products by the OSH method; no bands were apparent on the autoradiograph. Similarly, for the Gen-Probe assay, 46 samples were found negative and 2 were found indeterminate with the SK38-SK39-gag 1-gag 2 primer-probe set, and all 48 were found negative with the SK101-SK145-gag 3-gag 4 primer-probe set. Therefore, the SK38-SK39-gag 1-gag 2 primer-probe set demonstrated 96% specificity, and the SK101-SK145-gag 3-gag 4 primer-probe set demonstrated 100% specificity. The differences in specificities among the four tests were not found to be statistically significant.

The results for the HIV-1-seropositive, low-CD4-count group were highly concordant among all the assays, with a few exceptions. The OSH method achieved an overall sensitivity of 100%. Only in the case of sample 43 was the third primer pair (SK38-SK39) required to determine the algorithm result. The Roche assay also correctly determined all 24 low-CD4-count samples as positive and therefore achieved 100% sensitivity for this group. The Du Pont assay achieved 96% sensitivity; one sample was determined as false-negative. Both primer-probe sets used in the Gen-Probe assay demonstrated 100% sensitivity for the low-CD4-count group. No statistically significant differences were calculated among the assays for this sample group.

Table 2 lists the results for samples from HIV-1-seropositive individuals with high CD4 cell levels (greater than 800

cells/mm³), ranked from lowest to highest. Positive results in Table 2 are expressed as one plus sign, two plus signs, or three plus signs, reflecting the relative intensities determined by each detection technique. Table 3 summarizes the results for all three sample groups and expresses specificities and sensitivities as percentages.

In the high-CD4-count group, with a theoretically lower HIV-1 copy number (Table 2), significant differences in sensitivities were found among the four assays. For the OSH method, primer pair SK68-SK69 demonstrated 85% sensitivity, while primer pair SK101-SK145 demonstrated 97% sensitivity. Because of discrepant results with different primer pairs, five samples (29, 52, 110, 53, and 73) required a third amplification with SK38-SK39 to determine the overall algorithm result of 97% sensitivity (Table 2; SK38-SK39 results not shown). The Roche assay successfully detected 27 of the 39 high-CD4-count seropositive samples and therefore achieved 69% sensitivity. The Du Pont assay detected 33 high-CD4-count seropositive samples as positive, 5 as negative, and 1 as indeterminate and therefore achieved 85% sensitivity. The Gen-Probe assay detected 38 samples as positive with the SK38-SK39-gag 1-gag 2 primer-probe set and 37 as positive with the SK101-SK145-gag 3-gag 4 primer-probe set and therefore achieved 97 and 95% sensitivities, respectively (Table 3). The DuPont assay was less sensitive than the other assays ($P < 0.05$).

By the time the initial comparison of the nonradioisotopic assays had been completed, the Roche assay had been optimized to improve sensitivity. Upon retesting of the missed seropositive samples with the improved amplification method, only one false-positive and one false-negative were obtained. The two false-positives from the original analysis were resolved; a different seronegative sample, however, was assigned a positive result. The modified test resulted in an overall improvement in sensitivity from 96 to 98% for the low-CD4-count group as well as an improvement in sensitivity from 69 to 97% for the high-CD4-count group. These samples were not analyzed in a blinded fashion, since the samples had already been decoded at the time of retesting.

Sample 17 from a seropositive individual with a low CD4 count was found negative by all of the assays (the Roche assay detected this sample upon reanalysis). To exclude the possibility of a specimen mix-up, we typed the sample for human leukocyte antigen by using the AmpliType HLA-DQ α forensic DNA amplification and typing kit (Perkin Elmer Cetus) and compared it with another sample collected at a different time from the same individual. The two samples proved to have the same human leukocyte antigen type, greatly reducing the possibility that the discrepant sample was actually derived from a seronegative donor. This sample was assumed to be a very weakly positive sample and, according to the Poisson distribution, would require several amplification trials to detect. After calculation of the final results, detection of this sample was achieved by the OSH method by increasing the length of time for hybridization and autoradiography. Subsequent amplification trials with the Gen-Probe and Du Pont methods were not performed.

DISCUSSION

The results of this double-blind comparative study confirm that commercially produced kits with nonradioisotopic detection techniques can be used to identify amplified HIV-1 proviral sequences in clinical samples. The sensitivity and specificity of the nonradioisotopic detection techniques were

generally comparable to those of the OSH procedure in detecting PCR-amplified HIV-1 DNA.

Any HIV-1 PCR method intended for clinical use must demonstrate the utmost degree of specificity, which is achieved by effective avoidance of false-positives. False-positives can occur as a result of sample contamination at the amplification level or, as observed in this study, as a result of high background at the detection level. Precautions such as physically isolating PCR preparations from PCR products and using proper negative controls have eliminated much of the concern involving sample lysate contamination (12). The use of the uracil *N*-glycosylase system may also prove to be an effective safeguard against sample carryover, as demonstrated by its use with the detection system developed at Roche.

In all the amplifications and detections that were performed, only three false-positive results were encountered (sample 19 in the OSH method with SK68-SK69 and samples 32 and 107 in the Roche method). Sample 19, which was discordant with SK68-SK69 and SK101-SK145, was determined to be negative following amplification with the third primer pair (SK38-SK39), as dictated by the MACS PCR algorithm.

The two false-positive results found with the Roche method were apparently not the result of sample lysate contamination, since subsequent amplification and detection by other methods did not produce false-positive results. (The Du Pont and Gen-Probe assays were performed after the Roche assay.) In addition, sample contamination was also excluded by verification of the absence of an amplified product in the Roche-amplified samples by the OSH method (see Results). Therefore, these two false-positive results were probably due to the high background levels encountered with the Roche test. The other false-positive encountered during the Roche retest was probably the result of excessive handling of the sample, as the tube had been opened several times at our facility as well as at the Roche Diagnostics Systems facility. In general, the methods demonstrating the fewest number of false-positives and/or indeterminates were those reporting the lowest background levels and the highest ratio of positive to negative samples. On the basis of these results, Roche has reformulated the wash buffer to reduce background levels, therefore improving specificity. As noted in the retest results, optimization of the Roche amplification procedure has also greatly improved sensitivity.

We found that an important factor involved in avoiding false-positives and therefore increasing specificity was the use of alternate primer pairs for amplification. For example, if a sample does become contaminated with an amplified product, it is unlikely that the contaminating product will contain more than one amplified region. Consequently, false-positives and indeterminates can often be identified when the sample is analyzed with a second primer pair amplifying a different region. Of the commercial methods evaluated, only the Gen-Probe method currently supplies more than one primer-probe set for detection. The Roche HIV-1 assay addresses this issue by use of the uracil *N*-glycosylase carryover containment technology. Product DNA previously amplified with dUTP is specifically degraded and therefore would not be amplified.

It is also necessary that specificity be maintained with adequate sensitivity. Without a sensitive method of detection, the advantages of PCR technology are essentially lost. In this study, sensitivity was evaluated at two levels. A previous study showed that low CD4 cell numbers corre-

spond to a high proviral load and that high CD4 cell numbers correspond to a low proviral load (14). For this reason, the HIV-1-seropositive samples were divided into two groups, those with high CD4 cell numbers and those with low CD4 cell numbers. Since all detection systems were expected to confirm the presence of DNA in the low-CD4-count group and therefore reveal the least valuable information regarding sensitivity, this group contained the fewest number of samples. Indeed, all methods of detection displayed 100% sensitivity for the low-CD4-count group, with the exception of the Du Pont method (92%). The most challenging evaluation of sensitivity, however, is the detection of proviral DNA in samples from individuals with high CD4 cell numbers, since these samples contain the lowest concentration of HIV-1 proviral DNA.

Sensitivity was highest with the MACS radioisotopic method, followed by the HPA (Gen-Probe), the ELOSA (Du Pont), and the Roche method. The superior sensitivity achieved by both the OSH and the Gen-Probe methods may be attributed to the use of multiple primer pairs, which sometimes elucidated true-positives that failed to be amplified with a single primer pair. The use of more than one primer pair often improves the level of detection, because the possibility of genetic variants of HIV-1 within the sample lysate often prevent amplification of certain regions but not others (22). In addition, low copy numbers will be detected with multiple amplifications, as dictated by the Poisson distribution.

Another aspect of the evaluation of PCR detection assays is the speed and ease with which they can be performed. In this respect, the three prototype nonradioisotopic HIV-1 PCR detection methods represent a considerable advance over our more time- and labor-intensive radioisotopic method. The HPA is the simplest procedure to perform, as it requires the fewest number of manipulations and the shortest incubation times. For example, 30 samples can be analyzed within 30 min. However, both the Roche and the ELOSA methods possess advantages over the HPA in that they are microtiter plate based and can take advantage of the automated equipment that is available for microtiter plates (e.g., pipettors, plate washers, and readers). Therefore, when a large number of samples are being processed, the greater number of manipulations and longer incubation times are compensated for by the microtiter plate arrangement. In addition, laboratories already equipped for EIAs would also be equipped for the Roche or Du Pont assays.

Nonradioisotopic PCR detection methods also have the potential for producing semiquantitative output based on input copy number (15, 17), a valuable technique for monitoring disease progression and antiretroviral therapy. For quantitation, the number of amplification cycles not only must be sufficiently sensitive to produce a detectable product but also must be able to measure the product over a range of concentrations of input target DNA without reaching saturation. A previous study showed that a linear correlation between the concentration of input target DNA and the product DNA signal is limited to between 10 and 1,000 molecules for the Gen-Probe and OSH tests (17). Each nonradioisotopic assay could measure products in a range in which semiquantitation of the input DNA could be achieved relative to serial dilutions or stepped amplification (i.e., stopping amplification at successive cycling intervals) of an internal or an external calibrated standard. In this study, the relative signal strengths obtained with each assay for an undiluted sample were correlated. For example, sample 29 was weakly positive and sample 69 was strongly positive in

all assays. There were some exceptions to this pattern, such as sample 110, which was weakly positive in the Gen-Probe assay and strongly positive in all the other assays. The samples that were found false-negative by the less sensitive Du Pont and Roche assays were usually found weakly positive by the OSH and Gen-Probe assays.

In summary, the data from this study show that the nonradioisotopic assays are comparable to the OSH assay for the specific and sensitive detection of HIV-1 proviral DNA. In addition, the nonradioisotopic tests can provide rapid and potentially semiquantitative detection of HIV-1 genomic sequences. Nonradioisotopic prototype PCR assays offer promise for the routine application of PCR technology in clinical laboratories in association with suitable standards and means for quality assurance. These simplified methods would expedite the effective use of PCR for the diagnosis of HIV-1 infection and evaluation during treatment.

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