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Low Human Immunodeficiency Virus Type 1 (HIV-1) DNA Burden as a Major Cause for Failure To Detect HIV-1 DNA in Clinical Specimens by PCR

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To determine the sensitivity of a nested PCR procedure for detecting human immunodeficiency virus type 1 DNA in clinical specimens, 553 peripheral blood mononuclear cell samples obtained from 268 human immunodeficiency virus type 1-seropositive subjects were assayed by use of two independent primer sets for each sample. Overall, 1,088 of 1,106 (98.37%) reactions were positive. Investigation of the negative reactions showed that a low viral burden in some infected subjects, rather than primer-template mismatches, was the primary cause for the false-negative PCR results.

PCR-based procedures are now widely used to diagnose human immunodeficiency virus type 1 (HIV-1) infection. Although direct PCR amplification of HIV-1 DNA from peripheral blood mononuclear cells (PBMC) has been shown consistently to be more sensitive and convenient than virus isolation (1, 32, 38), it must be noted that a minor proportion of HIV-1-seropositive subjects have been reported to be PCR negative in a number of studies (3, 6, 10–12, 14, 16, 19–24, 30, 32, 33, 35, 36, 38, 39, 41). Data available from more than 30 major reports describing PCR detection of HIV-1 DNA in a total of >2,000 HIV-1-seropositive subjects (1–4, 6, 10–14, 16, 17, 19–26, 28–33, 35, 36, 38, 39, 41–43) indicate that 3.03% of the patients tested have been found PCR negative. In addition, there are anecdotal reports of PCR-positive samples after repeated testing (28, 33, 41). Failures to detect HIV-1 DNA in PBMC by PCR have been attributed usually to occurrence of significant primer-template mismatches, inherent lack of reproducibility of PCR, sequestration of virus in other sites, and/or low viral burden in the infected subject. However, none of these possibilities has reportedly been proved. We have previously documented a case indicating that an exceptionally low HIV-1 DNA titer in PBMC can account for a false-negative PCR result (44). We can now provide further evidence for this possibility, estimating its prevalence in a clinical diagnostic setting.

A total of 553 PBMC samples were prepared by standard Ficoll centrifugation of equal numbers of citrated blood specimens obtained from 268 HIV-1-seropositive subjects. According to the 1993 Centers for Disease Control classification system (7), 51, 81, 10, 41, 45, 8, and 32 subjects were in stages A1, A2, A3, B2, B3, C2, and C3, respectively. DNA was prepared by sodium dodecyl sulfate-proteinase K lysis of PBMC, phenol-chloroform extraction, and ethanol precipitation, quantitated by spectrophotometer, and checked for integrity by agarose gel electrophoresis. The PCR quality of the DNA samples was confirmed by a 24-cycle amplification of the human beta-globin gene GH20/PC04 region (34), by using 1.5 μg of template DNA. The presence of HIV-1 DNA was assayed by means of a nested PCR procedure previously shown to be capable of detecting a single target DNA copy by ethidium bromide staining of the agarose gel electrophoresed PCR products (45). Each DNA sample was tested with two different primer sets (primer "set" here indicates the outer pair plus the inner pair) among those four listed in Table 1, and 1.5 μg of template DNA per reaction was used. Primer sets were rotated periodically to reduce the risk of PCR product carryover possibly resulting from overextended use of the same set. Previously reported guidelines for avoiding PCR contamination (45) were strictly followed. Coordinates of the primers used are as follows, with respect to the ARV2/SF2 HIV-1 isolate (GenBank accession number KO2007): MZ8, 1316 to 1335; MZ9, 1505 to 1524; MZ13, 1224 to 1243; MZ14, 1677 to 1696; MZ17, 1833 to 1853; MZ18, 2149 to 2169; MZ19, 1878 to 1904; MZ22, 2323 to 2342; LR33, 6972 to 6993; LR34, 7367 to 7387; LR35, 7011 to 7035; LR36, 7328 to 7351; LR49, 2623 to 2642; LR50, 2716 to 2741; LR53, 3227 to 3250; LR54, 3266 to 3286. Specificity of each primer set was confirmed by failure to generate any ethidium bromide-visible PCR product when PBMC DNA samples obtained from over 100 HIV-1-seronegative controls were used.

Table 1 shows the results obtained with each of the four primer sets used. None of the primer sets detected HIV-1 DNA in 100% of the samples tested, yielding a cumulative observed sensitivity of 98.28% (19 negative reactions out of 1,106). This translated into a double-negative result for 4 (0.72%) samples and a discrepant (indeterminate) result (positive with one primer set only) for 11 (1.99%) samples (Table 2). All of these samples had been shown to be suitable for PCR analysis by control beta-globin amplification. However, the beta-globin target is expected to be present in a far larger amount (and thus more readily amplified) than HIV-1 DNA (9). To rule out any residual possibility of suboptimal PCR quality of DNA, nested amplification was repeated on the PCR-negative samples after the addition of five HIV-1 DNA copies from a titrated commercial preparation (Perkin-Elmer, Emeryville, Calif.) (18). The same primer set which gave the negative result was used for each sample. All of the reactions were positive, indicating that low-cycle-number amplification of a single-copy human gene correctly checked the amplifiabil-

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TABLE 1. Nested PCR detection of HIV-1 DNA in 553 clinical samples[a] obtained from 268 infected subjects: distribution of results with respect to primer sets

Primer set	No. of positive reactions/ total no. of reactions	No. of negative reactions due to:		
		Primer-template mismatches	Low HIV-1 DNA load	Unknown causes
MZ13/14 + MZ8/9 (*gag1*)	238/242 (98.35)[b]	0	4	0
MZ17/22 + MZ19/18 (*gag2*)	135/137 (98.54)	1	1	0
LR49/54 + LR50/53 (*pol*)	326/332 (98.19)	0	5	1
LR33/34 + LR35/36 (*env*)	388/395 (98.23)	2	4	1
All sets	1,087/1,106 (98.28)	3 (0.27)	14 (1.27)	2 (0.18)

[a] Each sample was assayed by two different primer sets.
[b] Values in parentheses are percentages.

ity of PBMC DNA samples. To investigate potential sources for PCR failures, all of the 19 negative reactions obtained initially were repeated in quintuplicate with 1 μg of template DNA per tube. Only three cases were confirmed to be repeatedly negative, one with the *gag2* primer set and two with the *env* primer set. Results obtained with separate substitutions of primers were compatible with failure of primer MZ22 to anneal to its target sequence in the first case (data not shown). The two *env*-negative reactions could not be investigated similarly but, notably, were obtained on two samples from the same individual, suggesting the presence of a primer-template mismatch(es) as a source for the false-negative results.

Two of the initially negative samples (one with the *pol* set and one with the *env* set) proved to be positive in all of the subsequent replicate tests. Since neither primer-template mismatching nor sampling error due to low viral load (see below) could be demonstrated, no definite cause could be found for these two occasionally negative reactions (obtained on samples from two different individuals).

For all of the 14 remaining PCR-negative reactions (obtained on 10 samples from six subjects), quintuplicate testing yielded a combination of positive and negative results. This ruled out the possibility of primer-template mismatches since, in the absence of any modification of the experimental conditions (including reaction components, cycling profile, and thermal reactor), primer-template mismatches are expected to affect PCR in a reproducible manner, both interassay and intra-assay. In addition, sequence data obtained for the *pol* gene in three samples which were initially but not repeatedly recorded as *pol* negative failed to identify any primer-template mismatch (not shown). A more extensive limiting dilution analysis performed with the *gag1* set showed that decreasing or increasing the amount of template DNA for replicate testing resulted in a lower or higher frequency of positive reactions, respectively (Table 3). Results obtained with the *gag1* set were confirmed with a second set (*pol* or *env*) in four samples for which sufficient volume was available (i.e., samples 1a, 3a, 3b, and 6a). This pattern is compatible with a low HIV-1 DNA load in the original samples, raising the possibility that no target molecule is included in the amount being tested (sampling error) (37, 40). Poisson analysis of the results obtained by replicate testing of these 10 samples (40) indicated a mean HIV-1 DNA content of 0.58 copy per μg of PBMC DNA (range, 0.2 to 1.6 copies). Thus, when results of the investigation on the sources for false-negative reactions (Table 1) were analyzed on a sample basis (Table 2), all of the four double-negative samples and 6 of the 11 indeterminate samples could be explained as the result of sampling error due to an extremely low viral burden. Such low HIV-1 DNA burdens in the PBMC of infected subjects have been reported consistently in quantitative PCR studies (3, 5, 8, 15, 37). On the other hand, primer-template mismatches and other presently undefined causes were responsible for three and two of the indeterminate samples, respectively.

We next analyzed available data for the six individuals from whom the 10 low-titer HIV-1-positive samples had been obtained (Table 4). None of the subjects had ever shown any clinical sign of HIV-1 infection, including the acute syndrome often associated with primary infection. Interestingly, four subjects (subjects 3, 4, 5, and 6; all in CDC stage A1) were tested for HIV-1 DNA shortly (0 to 4 months) after the first serological evidence of HIV-1 infection. Since these subjects were regularly attending a follow-up study at 3-month inter-

TABLE 2. Nested PCR detection of HIV-1 DNA in 553 clinical samples[a] obtained from 268 infected subjects: distribution of results with respect to samples

No. (%) of samples	Result with two primer sets	No. of misdiagnosed samples due to:		
		Primer-template mismatches	Low HIV-1 DNA load	Unknown causes
538 (97.29)	Positive, positive	0	0	0
11 (1.99)	Positive, negative	3	6	2
4 (0.72)	Negative, negative	0	4	0

[a] Each sample was assayed by two different primer sets.

TABLE 3. Results of limiting dilution PCR analysis of the 10 samples initially misdiagnosed because of low viral load

Sample[a]	Initial result with two primer sets with 1,500 ng of DNA per reaction	No. of positive reactions/total no. of reactions[b] with template DNA in amt of:			
		2,000 ng	1,000 ng	500 ng	250 ng
1a	*env* positive, *pol* negative	6/8	4/8	2/8	2/8
1b	*pol* negative, *gag2* negative	1/4	4/10	1/8	NT[c]
1c	*pol* positive, *env* negative	4/4	3/6	2/4	1/4
2a	*gag1* positive, *pol* negative	3/4	3/6	2/6	NT
2b	*gag2* positive, *gag1* negative	NT	6/8	4/6	2/6
3a	*env* negative, *gag1* negative	2/8	4/10	0/8	NT
3b	*pol* negative, *env* negative	2/8	3/10	1/8	NT
4a	*pol* positive, *gag1* negative	7/8	5/8	1/8	NT
5a	*gag2* positive, *env* negative	6/8	4/8	1/8	NT
6a	*gag1* negative, *pol* negative	4/8	3/10	1/8	1/8

[a] Samples with the same number refer to the same subject.
[b] Availability of small volumes of samples limited the extent of replicate testing. Tests were performed with the *gag1* primer set, except for 5 (for initially indeterminate samples) or 10 (for initially double-negative samples) of the 1,000-ng reactions for which the same primer sets were used as in the initial negative reactions.
[c] NT, not tested.

TABLE 4. Available data for the six HIV-1-seropositive subjects whose samples had been initially misdiagnosed because of low viral load

Sample[a]	HIV-1 DNA copies/μg[b]	CD4 cells/mm^3	CDC clinical stage[c]	Month	
				Infection[d]	ZDV[e]
1a	0.7 ± 0.2	230	A2	28	3
1b	0.2 ± 0.1	216	A2	32	7
1c	0.8 ± 0.4	257	A2	38	13
2a	0.7 ± 0.3	197	A3	19	12
2b	1.6 ± 0.5	234	A3	21	14
3a	0.2 ± 0.1	872	A1	2	
3b	0.2 ± 0.1	1,174	A1	4	
4a	0.6 ± 0.2	604	A1	1	
5a	0.5 ± 0.2	528	A1	0	
6a	0.3 ± 0.1	872	A1	1	

[a] Samples with the same number refer to the same subject.
[b] Calculated as reported previously (40) from data shown in Table 3.
[c] Clinical stage based on the 1993 Center for Disease Control classification system (7).
[d] Months elapsed since the first serological evidence of HIV-1 infection.
[e] Months elapsed since initiation of zidovudine (ZDV) therapy.

vals, it is reasonable to consider that HIV-1 infection had been acquired quite recently with respect to sampling for PCR. A low viral burden shortly after seroconversion, possibly resulting from immune clearance of the large amount of initially virus-infected cells, is in agreement with the current model of the natural history of HIV-1 infection (reviewed in reference 27). Consistent with the model, we have to date been unable to find any PCR-low-positive seronegative subjects after replicate testing PBMC DNA samples (up to a total of 10 μg) obtained from 40 high-risk seronegative regular sexual partners of HIV-1-infected individuals. However, only quantitative serial testing of a large number of subjects before and after seroconversion will clarify whether and what alternative patterns with regard to HIV-1 DNA load can be found immediately following HIV-1 infection (PBMC samples obtained from patients 3, 4, 5, and 6 before seroconversion were, unfortunately, not available). Our ongoing longitudinal quantitative analysis of HIV-1 DNA in infected subjects indicates that the 6-month period after seroconversion is not necessarily associated with such a low viral burden since other seroconverters exhibit copy numbers 1 to 3 orders of magnitude higher than the low-titer infected ones presented in this study (data not shown).

The other two subjects (subjects 1 and 2; CDC stage A2 and A3, respectively), accounting for half of the low-titer HIV-1-positive samples found in the present study, had been HIV-1 infected for >19 months when blood samples were obtained for PCR. Both subjects were under zidovudine therapy, and both had <300 CD4 cells per mm^3 at every sampling. Since we have found <1 to >1,000 HIV-1 DNA copies per μg in a total of >30 zidovudine-treated subjects (not shown), we deduce that, as for the small group of the low-titer infected seroconverters, the very low HIV-1 DNA load found in these two subjects may actually represent the exceptionally lower limit of the range and is to be expected at low frequency.

In summary, by using two primer sets to test 553 PBMC samples by means of a single-copy-sensitive nested PCR procedure (45), we found that a low viral burden was responsible for 14 of 19 (73.68%) false-negative reactions, with four samples (0.72%) scored as double negative. On a subject basis, such a low HIV-1 DNA load was found in 6 of the 268 (2.24%) patients tested. Primer-template mismatches or other presently undefined causes accounted for a minority of PCR failures, causing indeterminate, but never double-negative, results. Different data may be expected in other HIV-1 diagnostic PCR studies, depending on the sensitivity of the method, the primer pairs used, and the population and geographic area considered. We invite all those using PCR methods for direct detection of HIV-1 DNA in PBMC to investigate properly PCR failures in HIV-1-seropositive subjects and to confirm negative results on seronegative high-risk individuals. Extending this analysis is expected to help define the impact of sampling error on PCR diagnostics and avoid misinterpretation of primer pair efficiency, thereby providing a valuable contribution for a correct diagnostic use of the technique.

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