An Evaluation of the Polymerase Chain Reaction in HIV-1 Seronegative Men


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Summary: The apparent detection of human immunodeficiency virus (HIV-1) DNA by the polymerase chain reaction (PCR) in seronegative individuals has been the subject of great concern. In this study, 324 seronegative participants in the San Francisco Men's Health Study were evaluated for evidence of infection using a PCR testing algorithm with multiple amplifications targeting different regions of the HIV-1 genome. While most PCR reactions were negative, 8.6% of the specimens showed weak reactivity with one or more primer sets. However, all were negative with at least one primer set and no definitively positive specimens were identified. This study addressed the possibility that some of these PCR reactions might represent latent infection or abortive exposure, leaving residual integrated DNA, rather than false-positive reactions. The frequency of such reactions was determined in homosexual men who have been at risk for HIV-1 infection and in exclusively heterosexual men who have little or no past exposure. The results demonstrate an identical frequency and distribution of equivocal PCR reactions in both populations. Assuming that there is minimal HIV-1 infection among seronegative heterosexual men in San Francisco, we conclude that PCR testing does not provide evidence for a reservoir of occult HIV-1 infection in seronegative homosexual men. Key Words: Human immunodeficiency virus—Occult infection—Polymerase chain reaction.

The polymerase chain reaction (PCR) for the in vitro amplification of DNA is an extremely sensitive method for detecting genomic sequences of infectious agents (1), including the human immunodeficiency virus, type 1 (HIV-1) (2). The reported detection by PCR of HIV-1 DNA in individuals at risk of infection who have negative results by serological testing has led to concern that there may be a substantial reservoir of occult infection (3–6). Given the potential ramifications of these reports and the failure of several studies to replicate these findings (7–11), the epidemiologic significance of positive PCRs in seronegative individuals remains controversial. A major weakness in many studies of PCR has been the failure to report the rate of positive PCRs in concurrently tested seronegative individuals without HIV-1 risk factors.

Since PCR is presumed to be more sensitive than serologic testing, there is no "gold standard" laboratory test that defines the true infection status, and a true positive PCR test cannot be distinguished from a false positive. One solution to this problem is to perform the assay in matched populations with
and without risk factors for the infection and compare the frequency of positive reactions.

We have performed such an analysis in the San Francisco Men's Health Study (SFMHS), a population-based sample of men who, in 1984, were 24–55 years of age and living in 19 contiguous census tracts of San Francisco (12). This cohort was originally composed of 396 seropositive and 410 seronegative self-reported homosexual or bisexual men and 204 exclusively heterosexual controls.

Among the 410 homosexual men who had seronegative results at entry into the cohort, 42 (10%) have seroconverted in the succeeding 5 years and there has been a stable seroconversion rate of about 1% per year for the past 3 years. Among the 204 seronegative heterosexual men, only one (0.5%) seroconverted in the same period. For the purpose of this study, we have made the assumption that the cumulative risk for exposure to HIV-1 has been negligible in the heterosexual controls compared with the risk in the homosexual and bisexual men.

METHODS

Samples

Fresh whole blood samples were collected prospectively from participants in the SFMHS from April 1989 through April 1990. A total of 375 seronegative participants visited the clinic during this period and 364 provided one or more whole blood specimens for PCR testing. Men who either declined to state a sexual preference or whose self-reported orientation changed during the 4 years of follow-up observation were also excluded from this analysis (n = 40). The resulting study population consisted of 324 individuals, 105 exclusively heterosexual men and 219 homosexual or bisexual men, all of whom had negative results for HIV-1 antibody. Peripheral blood mononuclear cells (PBMCs) were separated by centrifugation through Ficoll/diatrizoate (LSM, Organon Teknika, Durham, NC, U.S.A.) and frozen in aliquots of 1 × 10⁶ PBMCs per vial in a solution of 90% fetal bovine serum and 10% dimethyl sulfoxide. Subsequently, the frozen PBMCs were thawed and subjected to PCR testing as described below.

PCR Procedures

The PCR testing methods used in these studies were similar to published methods for the amplification and detection of HIV-1 DNA (13). Briefly, 10⁶ frozen PBMCs were thawed, washed once, and resuspended in 0.5 ml of lysis buffer consisting of 50 mM KCl, 1 mM MgCl₂, 10 mM Tris-Cl pH 8.3, 0.5% NP-40, 0.5% Tween-20, and 60 µg/ml of proteinase K. These samples were incubated at 60°C for 60 min to release target DNA and then inactivated by incubation at 95–100°C for 10 min. A portion of each lysate equivalent to 10² PBMCs was mixed with synthetic DNA primers, heat-stable DNA polymerase, and deoxynucleotides. The target sequences were then amplified by temperature cycling. A portion of the amplified product and a labeled synthetic oligonucleotide probe, complementary to the amplified sequence, were mixed in an appropriate hybridization solution and incubated at 55°C for 10–30 min. The hybridized material was separated by electrophoresis through a 10% polyacrylamide gel and the HIV-1-specific hybrid was identified by autoradiography. A separate amplification of HLA-DQ-α was also performed on each specimen to verify the presence of adequate amplifiable DNA and was evaluated by electrophoresis in an agarose gel and visualization by ethidium bromide staining. All aliquots for which HIV-1 results were tabulated were positive for HLA-DQ-α.

Individual PCR reactions were assigned a visual intensity score on a scale of 0–4 that is illustrated in Fig. 1. For this analysis, samples with an intensity score of 1 were considered to be "weak positives." Control experiments using mixtures of infected and uninfected H9 cells indicate that this corresponds to about one infected cell in a background of 10⁶ uninfected cells. Scores of 2 and 3 were grouped as "moderate," and scores of 4 were classified as "strong positives." In our experience, seropositive SFMHS participants generally have intensity

![Visual Intensity Score](image)

FIG. 1. Visual intensity scoring for five representative PCR reactions. Detection HIV-1 was performed using DNA lysates equivalent to 1 × 10⁶ patient PBMCs that were; amplified with a gag primer pair (SK38/39), hybridized with a ³²P-labeled oligonucleotide probe (SK19), separated on a 10% polyacrylamide gel, and visualized by autoradiography.
scores of 2–4 and most of the reactions with intensity scores of 1 are observed in specimens from seronegative individuals and are negative on repeat testing with alternate primer sets (data not shown).

Testing Algorithm

Samples were subjected to a testing algorithm that involved the use of multiple amplification reactions as necessary, targeting up to three different regions of the HIV-1 genome for each specimen. Primer pairs used in these studies included two from the HIV-1 gag gene (SK38/39 and SK101/145) and one env gene (SK68/69). The sequences of these primer sets and their sensitivity for North American isolates of HIV-1 have been described elsewhere (2,9,13,14). A similar test algorithm has been previously validated in the context of a blinded multicenter proficiency trial of PCR in which this laboratory achieved an overall sensitivity and specificity of 98.0 and 97.1%, respectively. Of the 99 specimens from seropositive individuals, one had “weak positive” signals with both gag primer sets, a negative score with env primers, and was classified as indeterminate. One specimen was a strong positive but had been inadvertently switched with an adjacent negative specimen, resulting in one false negative and one false positive. The remaining 97 samples had moderate or strong signal intensities.

The complete diagnostic testing algorithm consists of amplification with two different primer pairs (one gag and one env pair or two gag pairs). Specimens were reported as positive if both reactions have intensity scores of 2–4 and as negative if both reactions have a score of 0. Reactions with intensity scores of 1 are considered discordant with both negative and positive designations. If the two results are discordant, an additional amplification is performed using the third primer pair. Where two of three of the reactions agree, a final result is reported. Specimens that remain discordant after three reactions are reported as indeterminate.

In previous studies, it was determined that the SK38/39 primer pair had extremely high sensitivity in this patient population (about 99%). For the purpose of this study, the algorithm was modified so that specimens that were negative after amplification with SK38/39 alone were designated negative and included in the analysis. Approximately half of the specimens were subjected to the full test algorithm irrespective of their SK38/39 result.

RESULTS

Testing for the presence of HIV-1 genomic sequences was performed on PBMC specimens from 324 seronegative SFMHS participants, 219 homosexual/bisexual men and 105 exclusively heterosexual men. Each specimen was subjected to the PCR testing as described earlier. The number of specimens that showed discrepancies between the first two primer pairs and the final interpretation of HIV-1 infection status after subsequent repeat testing are shown in Table 1. The frequency of specimens requiring third primer pair testing was identical in both risk groups (8.6%). No positive specimens were identified and only two specimens remained indeterminate after completion of the testing algorithm, one from a homosexual and one from a heterosexual.

The number and frequency of all nonnegative PCRs in the two risk groups as well as the distribution of intensity scores are shown in Table 2. Of 551 PCRs performed, a total of 43 (7.8%) nonnegative reactions were observed in 28 of 324 (8.6%) of the specimens. Nonnegative reactions were observed in 29 of 380 (7.6%) of the PCR reactions performed on samples from homosexual/bisexual men, and in 14 of 171 (8.2%) of the reactions performed on samples from exclusively heterosexual men.

Nonnegative reactions occurred with all three primer sets, SK38/39, SK101/145, and SK68/69, at rates of 6.3, 14.7, and 4.4%, respectively, and the relative proportions were similar in both risk groups. While HIV-1 sequence variation can affect

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of patients</th>
<th>Initial discrepant, no. (%)</th>
<th>No. positive</th>
<th>Final interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homosexual</td>
<td>219</td>
<td>19 (8.7)</td>
<td>0</td>
<td>218</td>
</tr>
<tr>
<td>Heterosexual</td>
<td>105</td>
<td>9 (8.6)</td>
<td>0</td>
<td>104</td>
</tr>
<tr>
<td>Total</td>
<td>324</td>
<td>28 (8.6)</td>
<td>0</td>
<td>322</td>
</tr>
</tbody>
</table>

the relative performance of PCR primers, our experience indicates that large fluctuations in the specificity of a primer set also occur in proportion to the volume of PCR testing performed with that primer set. During a previous study (14), most of the nonnegative reactions in seronegative individuals were attributable to SK38/39, reflecting the heavy use of this primer set and increasing the potential for the contamination of negative samples with previously amplified product.

Finally, most of the nonnegative reactions had the lowest relative intensity, including 21 of 29 (72%) from homosexuals and nine of 14 (64%) from heterosexuals. Only one reaction had an intensity score of 4 (Table 2). This reaction occurred with one of the gag region primer sets (SK38/39) and was negative using the alternate gag primers (SK101/145) and the env region primer set (SK68/69).

DISCUSSION

Since the first reports of positive PCRs in seronegative individuals (3,4), there has been considerable concern that there might be a substantial reservoir of occult HIV-1 infection with the potential for additional transmission that would not be prevented by present testing and counseling strategies. Since that time there have been several reports describing the propensity for false-positive results inherent in PCR testing (15,16), and several attempts have been made to establish the frequency of HIV-1 infection in seronegative populations with risk factors for infection (7–11). A major deficiency in many of these studies has been the absence of sufficient concurrent “true” negative controls to establish the background false-positive rate. Another difficulty with many of these studies is the failure to use other confirmatory methods such as antigen detection or HIV isolation since the PCR is assumed to be inherently more sensitive. In one report (3), isolation of HIV was used to confirm PCRs but the culture and PCR were not performed on the same specimen.

Finally, longitudinal studies of seronegative individuals who eventually seroconverted showed only sporadic PCR reactivity with generally weak signal intensity (4). We have suggested that if this is not a laboratory artifact, it might be evidence for some kind of abortive exposure resulting in DNA integration but insufficient viral replication to induce antibody formation (17).

In this report we have taken a statistical approach in which the frequency of nonnegative PCR reactions has been compared in populations with different levels of risk for exposure to HIV-1. If such sporadic PCR reactions represent true biologic phenomena, one would expect to detect a difference between groups with substantially different histories of exposure to HIV-1. The results presented here demonstrate that there is an identical frequency and distribution of nonnegative reactions (about 8% of all reactions) in seronegative populations with extremely different historical risk for exposure to HIV-1. We conclude that stable occult or latent HIV-1 infection in seronegative homosexual men, if it exists at all, is an extremely rare phenomenon that is of little or no epidemiologic significance.

It is of interest to note that, in a previous study of seronegative regularly repeating blood donors (14), we observed a similar rate of indeterminate specimens (one of 105) and a somewhat higher rate of nonnegative PCRs (38 of 246, or 15%). These results illustrate the presence and variability of false reactivity, inherent in current PCR methods, and argue for the simultaneous performance of many negative controls to monitor changing false-positive rates.

This study does not directly address the possibility that, in some cases, weak and sporadic PCR positivity might be present during a long incubation period between infection with HIV-1 and the first appearance of specific antibody. The SFMHS co-
hort has had a stable seroconversion rate of 1% per year for the past 3 years. Therefore, an incubation period as long as 2 years would result in only about 10 individuals in this population incubating virus during the study period. Weak and sporadic PCRs in these individuals might not be detectable over a background rate of almost 9%. However, the failure to identify any definitively positive specimens is consistent with the conclusion that PCR does not detect a significant window period between infection and seroconversion. Finally, none of the 28 individuals in this study with nonnegative PCR reaction have seroconverted during the subsequent 12 months of follow-up observation. A definitive answer to the seroconversion question will require a careful retrospective analysis of seroconvertors, which is presently under way using blinded testing in two laboratories, a large number of "true-negative" controls, and established PCR testing algorithms.

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REFERENCES


8. Pan L-Z, Royce R, Winkelstein W, Levy JA. Human immuno-