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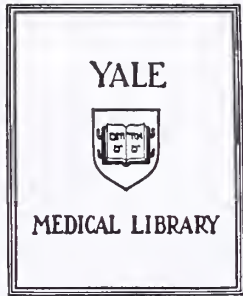



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A COMPARISON OF POLYMERASE CHAIN REACTION,
ELISA, AND WESTERN BLOT IN DETECTING
EVIDENCE OF HIV-1 INFECTION IN THE NEEDLES
OF INJECTION DRUG USERS

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A Comparison of Polymerase Chain Reaction, ELISA, and Western
Blot in Detecting Evidence of HIV-1 Infection in the Needles of
Injecting Drug Users

A Thesis Submitted to the Yale University
School of Medicine in Partial Fulfillment
of the Requirements for the Degree of
Doctor of Medicine

by

Samuel Skipworth Myers

1992

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In June of 1981 an astute worker at the Centers for Disease Control (CDC) observed a new pattern of disease. In the previous eight months, physicians had suddenly made five requests for an experimental drug which had only been requested twice before between 1967 and 1979 for use in adults. Dispensed only by the CDC and used to treat an exceedingly rare infection, the drug was pentamidine isethionate. The infectious agent was *Pneumocystis carinii*.

About the same time, reports began to flow into the CDC detailing the rise in incidence of another rare disease, a form of cancer called Kaposi's sarcoma. Simultaneously, and sometimes in association with the pneumocystis or kaposi's cases, the CDC noted a rise in chronic lymphadenopathy and diffuse, undifferentiated, non-Hodgkin's lymphoma. It was remarkable that all of these conditions had previously been found primarily in immunocompromised hosts. It was also notable that the vast majority of early cases were diagnosed in young homosexual men clustered around New York City, Los Angeles and San Francisco. By 1982, based on these epidemiological findings, the syndrome had been given a name, acquired immunodeficiency syndrome, or AIDS.

Initially described in homosexuals, AIDS was soon found to affect other segments of the population. In 1982 AIDS was described in hemophiliacs, other blood-transfusion recipients, and intravenous drug users (IVDU's) who had shared needles with other users. By January of 1983 there were two well-documented cases of AIDS in heterosexual partners of IVDU's, making it clear that AIDS could be transmitted by heterosexual as well as homosexual contact. In late 1983, the HIV-1 virus was isolated and soon afterwards serological testing for HIV-1 became available.¹

While the majority of early AIDS cases occurred in homosexual men, there has been an alarming rise in the percentage of cases attributable to intravenous drug use. In the U.S, 19% of AIDS cases from 1985 to 1987 were associated with intravenous drug use; however, this figure had climbed to 26% by 1988.² In Europe, the trends have been even more dramatic. Associated with only 2% of AIDS cases in 1983, intravenous drug use was responsible for an estimated 15% in 1985 and 34% in 1988. By the end of 1989, the year's newly diagnosed AIDS cases in IVDU's would exceed those in homosexuals.² These increasing percentages are reflected in the significantly shorter doubling times of AIDS cases in IVDU's compared to homosexual AIDS cases. For example, in Europe, the time between the doubling of the number of AIDS patients from 1983 to 1985 was 4.4 months for IVDU's and 8.8 months for homosexuals. In 1986 to 1988 it was 10.8 months and 20.4 months respectively.

AIDS among IVDU's is not limited to the developed world. There have been a rise in reported cases from South America (Brazil), Africa, Asia (Thailand), and Eastern Europe.² It has been shown that the spread of AIDS among IVDU's can be quite rapid. In Manhattan, historically collected sera from IVDU's showed seroprevalence rising to >40% within three years after the first seropositive sample was collected.³ Edinburgh had an even more dramatic rise to 50% only two years after the first seropositive sample.⁴ In Bangkok, seroprevalence was measured at 0% in 1987 and 30% in 1988.² AIDS in IVDU's is also associated with spread to the heterosexual and pediatric populations. In 1988, 55% of AIDS cases associated with heterosexual transmission in the U.S. were partners of IVDU's. Seventy percent of pediatric AIDS cases were born to parents who were IVDU's or partners of IVDU's.² There are an estimated 1.1 to 1.3 million IVDU's in the

U.S.⁵ Thus by the late 1980's it was clear that AIDS among IVDU's was a major public health problem. It was international in scope and increasing at an alarming rate both within the intravenous drug using community and in heterosexual and pediatric populations as well.

Nowhere is AIDS among IVDU's more of a problem than in the North Eastern region of the United States. In Connecticut the demographics of AIDS are skewed significantly from those of the country at large. While 59% of reported AIDS cases nationally are gay/bisexual men, in Connecticut only 31% of AIDS cases occur in this population. Conversely IVDU's constitute 47% of Connecticut cases as compared to 26% nationally. These statistics are even more skewed in the city of New Haven. The spread of AIDS to women and children in New Haven reflects these demographics. In New Haven, 28% of adult cases are women compared to 10% nationally, and one third of the state's pediatric AIDS cases are from New Haven.⁶

As these alarming figures and trends became evident, community leaders, public health workers, and legislators in Connecticut sought ways to address the epidemic of AIDS in IVDU's. In 1987 the New Haven Mayor's Task Force on AIDS was formed in order to develop a comprehensive strategy to break the connection between AIDS and intravenous drug use. In October, 1987, the Task Force went to work. Multicultural indigenous workers delivered AIDS information to addicts in areas of concentrated drug activity, helped users enter treatment programs and distributed condoms and "safety kits" filled with bleach and water to clean injection equipment. At the same time, the Task Force began to raise consciousness about the need for access to clean needles and to build support to legalize needle exchange programs (NEP's). A first step was to interview drug users about their injection behavior. IVDU's reported that they did not share needles as a

ritual of the drug culture as some had believed. Rather, they feared arrest for the possession of needles/syringes.⁶ Furthermore, access to clean needles was limited due to scarcity and cost. On the basis of these findings and the alarming scope of the AIDS problem among IVDU's the Task Force in 1989 proposed a NEP before the Public health Committee of the Connecticut General Assembly.

While there have been no controlled trials to show that NEP's are effective in curbing the transmission of HIV-1 among IVDU's,⁷ there is good theoretical and circumstantial evidence to support this assertion. The theoretical evidence comes from epidemiological studies of intravenous drug using populations when stratified by injection behavior. A number of studies have shown that HIV-1 antibody seroprevalence rises with self-reported sharing of needles.^{4, 8-11} A more detailed study of IVDU's in New York City shows that the seroprevalence rises with the percent of total injections in shooting galleries, the percent of total injections with used needles, and the percent of total injections with needles shared with strangers or acquaintances.¹² Finally, a study of IVDU's in Baltimore showed an HIV-1 seroprevalence of 9.8% in diabetic IVDU's compared to 24.3% in non-diabetics. The investigators conclude that the ready access to sterile needle/syringes among the diabetics has a protective effect obviating the need for sharing among drug users.⁷ Thus, there is ample theoretical evidence to suggest that a NEP, by providing sterile injection equipment and reducing the need for needle sharing, would decrease the transmission of HIV-1 virus among IVDU's.

Other evidence comes directly from experience with NEP's. There are a number of reports documenting diminished high-risk behavior in populations served by NEP's. Researchers affiliated with NEP's in the UK,

Australia, Amsterdam, Rotterdam and Sweden have all documented reduction in self-reported needle-sharing within their programs' populations.¹³ Given the clear evidence that needle sharing is at the heart of HIV-1 transmission in these populations, it is reasonable to assume that such behavior modifications should reduce transmission of this virus. The few seroprevalence studies of NEP populations also support the effectiveness of such programs. In Amsterdam a NEP was instituted in 1985. In 1986, the seroprevalence of NEP participants was 33%. In 1987 and 1988 this figure remained stable at 32% while similar populations without NEP's rose to approximately 60%.² Three years after the early institution of a NEP in Lund, in southern Sweden, the seroprevalence for HIV-1 remained 1% while similar Scandanavian populations without such programs approached 60%.¹⁴ Thus there is good circumstantial as well as theoretical support for the assumption that NEP's reduce transmission of HIV-1.

Nonetheless, the research to date is not conclusive. No study has shown a decrease in HIV-1 rates among populations served by a NEP. Further, most studies have been limited to testing seroprevalence in populations served by a NEP without testing the used needles directly. Without showing a decrease in the infectedness of needles used by program participants, it is impossible to make definite conclusions about the efficacy of such a program.

Because of this lack of good evidence and concern about encouraging drug use, the Public Health Committee proved reluctant to establish a legal exchange. However, the following year, with mounting public and medical support for such a program, representatives Bill Dyson (New Haven) and Joe Grabarz (Bridgeport) introduced a bill calling for decriminalization of sale and possession of needle/syringes statewide and the institution and funding of

three NEP's in Connecticut cities with a high concentration of intravenous drug use and AIDS. In March of 1990 the Public Health Committee voted in favor of a compromise bill authorizing the funding of one demonstration NEP by a vote of 15-5. The bill was then passed by a majority of Connecticut legislators with a vote of 99-36 and was signed into law by Governor O'Neill effective July 1, 1990.

The city chosen to institute Connecticut's first NEP was New Haven. The NEP law mandated that 1) the program should provide for anonymous free exchanges of needle/syringes with an equal exchange of new for used needles up to a cap of five per exchange, 2) all needle/syringes distributed be marked and checked for return rates, 3) the program was to simultaneously offer education about HIV-1 transmission and prevention and assist participants in obtaining drug treatment services.

The program must also monitor 1) return rate of needle/syringes distributed, 2) behavioral change of program participants such as needle sharing and condom use, 3) program participation rates and success rates in initiating and completing drug treatment services, 4) the incidence of intravenous drug use to determine whether any change occurred as a result of the program.

A final aspect of the program set it apart from other similar programs around the world. A syringe tracking and testing system was designed to directly evaluate the program's effect in reducing the spread of HIV-1 among IVDU's. To do this all syringes distributed are marked with a unique number. When conducting an exchange, NEP workers record the sequence numbers of the syringes, the code name of the participant receiving the syringes (every NEP participant must carry a card with his or her code name on it) and the date and location of the exchange. When syringes are returned

they are placed in a cannister with the code name, date and location. The cannisters are then taken to the laboratory where this information is recorded as well as the sequence numbers in each cannister. Finally, a sample of these needles must be tested for evidence of the HIV-1 virus.

The results of these tests are then analyzed using statistical models developed by Edward Kaplan at the Yale School of Management. Essentially, these models use the initial prevalence of HIV-1 positive needles and a number of fixed variables to project what the expected seroprevalence of HIV-1 in needles would be over time were the program to have no effect. These projections can then be compared to actual observations to provide a quantitative assessment of the NEP's effect on HIV-1 transmission.

Central to this process of evaluation, then, was the need for a dependable technique for detecting evidence of HIV-1 in needle/syringe combinations returned by IVDU's. This application represented a new frontier in HIV-1 testing and researchers at the Yale School of Medicine were asked for assistance. The special circumstances of this application required an extremely sensitive assay given the very small sample sizes (often less than 1 microliter of dried blood). Ideally, the technique would also be specific, inexpensive, and easy to perform in large batches. In choosing a detection technique these investigators considered the five general classes of HIV-1 assay. These include testing for the virus itself, viral antigens, viral RNA, human antibodies to the virus or human white cell HIV-1 proviral DNA.

Culturing for virus directly was excluded on the grounds that it would be extremely labor intensive for such a large number of samples (tens of thousands). Furthermore, free virus tends to be present in relatively small copy numbers until late in infection.¹⁵ Testing for viral antigen was also excluded for this second reason. A third possibility was a polymerase chain

reaction (PCR) assay using reverse transcriptase to amplify viral RNA. While in theory this test is the most sensitive available, it is both labor intensive and technically difficult and, therefore, inappropriate for such a large number of samples.

Using an enzyme-linked immunosorbent assay (ELISA) was a fourth possibility. Commonly performed as a screening technique for blood donors or as the first diagnostic test in a clinic or hospital setting, the ELISA is a serological test to detect the presence of antibody to HIV-1 virus. There was little documentation of its use in such small samples (which were whole blood not sera) with the exception of one study done by investigators in Florida. These investigators collected used needle/syringe combinations from three shooting galleries in South Florida and tested for antibody using the Abbott ELISA. They found 15 of 148 needles (10.1%) tested positive. Of the needles with visible blood 20.0% were positive while only 5.1% without visible blood showed presence of antibody¹⁶. While these results were interesting, the Yale group noted that the seroprevalence in the IVDU's using these shooting galleries was reported at approximately 36%. They were concerned that the ELISA results must therefore be an unacceptably low estimate of the true infectedness of the gallery needles. This concern was bolstered by the fact that the ELISA was positive in only 5.1% of needles without visible blood. It appeared, at least from this study, that the ELISA might lack the sensitivity for such small samples.

The final alternative was to use a PCR assay to amplify and detect HIV-1 proviral DNA in human white cells. By this time the ability of PCR to detect sequences from the HIV-1 genome in human white cells had been well described,¹⁷ although no one had attempted its application in this type of sample. The great strength of PCR is its ability to amplify a specific DNA

sequence in very small copy numbers (down to one molecule) into the millions and thereby allow its detection. The technique seemed ideally suited for this application and was therefore, chosen as the best alternative.

It was at this stage, in November 1990, that I joined Dr. Edwin Cadman and Robert Heimer, Ph.D. as part of the laboratory research group responsible for testing returned needle/syringes. Initially involved in trying to develop the optimal PCR technique for detecting HIV-1 proviral DNA in needles, I became increasingly interested in the theoretical limitations of this assay and began to wonder if it really was the most appropriate technique for this application.

Because HIV-1 proviral DNA is found almost exclusively in CD4+ cells,¹⁸ the sensitivity of PCR in testing a given volume of blood is proportional to the CD4 count/microliter multiplied by the concentration of proviral DNA in that patient's CD4 population. I was concerned that the sensitivity of the PCR assay would be limited by the relatively small copy number of HIV-1 proviral DNA sequences in a population of host CD4+ cells. I was further concerned that in HIV+ patients, as the CD4 count fell, detection of proviral DNA might be even more difficult. A number of studies have used the PCR to attempt to quantify the number of white cells required to detect a single copy of HIV-1 proviral DNA. Schnittman et al report approximately one copy of HIV-1 proviral DNA per 100 CD4+ cells in patients with AIDS.¹⁸ A second study performed by Simmonds et al found there was one copy of HIV-1 proviral DNA in 108-9130 CD4 cells with fewer copies in less advanced disease.¹⁹ Donovan et al, however, found one copy of HIV-1 proviral DNA per 10-200 peripheral blood mononuclear cells (PBMC's) in six patients with AIDS.²⁰ These experiments are consistent, then, with an

infection rate that covers a range from approximately 1: 1-100 CD4 cells in symptomatic patients with AIDS.

Most of the needles returned from the NEP contained less than one microliter of dried blood. In a healthy, HIV negative person one would expect approximately 500 CD4 cells in this volume. Thus, if we assume 500 CD4 cells per sample (a generous assumption in both the volume of blood and the CD4 count per microliter) we are close to the cutoff point where we would not expect to find a single molecule of HIV-1 proviral DNA. In fact, of the twelve patients studied by Simmonds, only four averaged one copy of proviral DNA in less than 600 CD4 cells.¹⁹ Furthermore, the vast majority of HIV+ NEP participants are presumably asymptomatic, or at least are not suffering full-blown AIDS. There is little literature on the proviral burden in asymptomatic patients. Simmonds cites unpublished data to give an estimate of one copy per 1,000-10,000 CD4 cells in such patients.¹⁹ Thus, while asymptomatic HIV+ patients are likely to have higher CD4 counts than AIDS patients, they are also likely to have a lower concentration of proviral DNA within their CD4 cell population. One could only speculate on the net effect of these opposed trends on the sensitivity of PCR. If higher CD4 counts outweighed lower proviral copy number, then PCR would be more sensitive in asymptomatic patients. However, the inverse is also possible. Given this arithmetic I became concerned that needles with small amounts of blood from HIV+ participants might routinely test negative by our techniques. From a practical standpoint, the PCR assay was labor intensive, time-consuming, and expensive, and switching to an ELISA would probably represent improvements in all these areas.

In an initial experiment I found that the Abbott ELISA was sensitive enough to detect anti-HIV-1 antibody in as little as .02 microliters of dried

blood left overnight in a syringe in ten of ten known positive samples and as little as .002 microliters in eight of ten. Given these encouraging results, I designed an experiment to compare PCR with ELISA directly by using serial dilutions of known positive and negative blood. I would then correlate the results with the subjects' CD4 counts to see whether the sensitivity of PCR dropped off with falling CD4 counts.

Materials and Methods

We received approval for our protocol from the Human Investigations Committee. Twenty volunteers at the Nathan Smith Clinic (a clinic for HIV+ patients) donated four tubes of blood each. Five of these patients were asymptomatic, with CD4 counts of 500 or greater and not being treated with AZT or other antiretroviral agents. Two tubes of blood were used to measure T-cell counts, and one tube was used to prepare sera. Using the final tube, we prepared ten needle/syringes by drawing up five microliters of blood and leaving to dry for 24 hours. Additional samples were obtained from three HIV- volunteers from which sera and needle/syringes were prepared. We then used the PCR and ELISA assays to test serial dilutions from the extracts of these needles. Western blots were also performed on these specimens to determine the limits of sensitivity of this technique and its potential for application in confirming ELISA results. Finally, the PCR, ELISA, and western results were compared to each other and to the T-cell studies to determine which technique was most sensitive, whether the sensitivity of PCR was effected by a patient's T-cell profile, and whether there was a difference in sensitivities between symptomatic and asymptomatic HIV+ patients.

T-cell Studies

After phlebotomy of HIV+ volunteers, one tube of blood was sent directly to the hematology laboratory where complete blood count and manual differential were performed. A second tube was sent to the immunology laboratory where whole blood was incubated with a set of conjugated monoclonal antibodies to specific surface antigens. After incubation, the erythrocytes were lysed and removed. Fluorescein conjugated (green) anti-human leukocyte antibody (HLe-1) and phycoerythrin conjugated (red) anti-monocyte antibody (leu-M3) were used to separate polymorphonuclear cells, lymphocytes and monocytes electronically using flow cytometry. The lymphocytes were then divided by flow cytometry into B and T-lymphocyte subsets using green anti-CD3, red anti-CD4, green anti-CD20, and red anti-CD8 antibodies in the following combinations:

green anti-CD3 and red anti-CD4

cells that are both red and green are CD4+ T cells (helper inducer)

green anti-CD3 and red anti-CD8

cells that are both red and green are CD8+ T cells (suppressor/cytotoxic)

green anti CD20

cells that are green are CD20+ B cells

Lymphocyte subset numbers were quantified by multiplying the percentage of lymphocytes which were positive for each subset against the number of lymphocytes per cubic mm of blood (hence the need for a complete blood count with differential).

ELISA

Needles containing five microliters of HIV+ or HIV- blood were flushed 4-5 times with 50 microliters of normal saline to produce a solution which was concentrated at 10%. Four further dilutions were then prepared, resulting in 1.0%, 0.1%, 0.01%, and 0.001% solutions. Then 10 microliters of each of these five solutions were assayed by ELISA (Abbott Enzyme Immunoassay) using the standard protocol with the exception that the first dilution step was omitted. Ten microliters of these solutions contained 1.0, 0.1, 0.01, 0.001, and 0.0001 microliters of blood respectively. The Abbott ELISA is manufactured by propagating HIV-1 in a T-lymphocyte cell line H9/HTLVIII β . Isolated virus is then disrupted and inactivated with detergent and sonification. Two major viral proteins are purified from the lysate, and a combination of whole lysate and purified viral proteins is coated onto beads. In testing for HIV-1, these beads are then incubated with the sample being tested, and any anti-HIV-1 antibody in the sample binds to the HIV-1 antigens on the solid phase. The beads are then washed to remove unbound material. The beads are then incubated with goat anti-human IgG antibody conjugated with horseradish peroxidase. This antibody binds to human antibody bound to the beads. After washing off unbound enzyme, a hydrogen peroxide containing solution, with o-Phenylenediamine as substrate, is added and a yellow-orange color develops in proportion to the amount of HIV-1 antibody bound to the beads. The absorbence of this solution is read spectrophotometrically and compared to known positive and negative controls to determine positivity.²¹

Western Blots

After performing the ELISA, western blots were done on enough dilutions from each patient to provide a range from positive to negative in order to define the limits of sensitivity of this technique. The Bio-Rad Novopath™ HIV-1 Immunoblot assay was used. This kit is manufactured by propagating HIV-1 in a HUT-78 T-lymphocyte cell line. Partially purified virus is then inactivated and disrupted by treatment with sodium dodecyl sulfate (SDS). Specific HIV-1 proteins are then separated according to molecular weight by gel electrophoresis in the presence of SDS. These separated proteins are then transferred by electroblotting onto a sheet of nitrocellulose which is then washed and cut into strips. Sample is incubated with these strips and then washed. Next a goat anti-human IgG antibody conjugated to alkaline phosphatase (AP) is incubated with the strips and binds to human anti-HIV-1 antibody bound to the strips. Finally, after washing off unbound enzyme, AP substrate solution containing 5-bromo, 4-chloro, 3-indolyl phosphate/ nitro blue tetrazolium is added such that bands appear corresponding to the position of the following HIV-1 proteins (p) or glycoproteins (gp): p18, p24, p32, gp41-43, p51, p55, p65, gp120, and gp160. The pattern of bands was then interpreted as negative, indeterminate or positive. A positive test exhibited reactivity to at least one major product of each of the structural genes: GAG (p55, p24, or p18), ENV (gp160, gp120, or gp41), and POL (p65, p51, or p32). A negative test showed no reactive bands, and an indeterminate test was anything in between.²²

PCR

Preparation

In order to test for the presence of HIV-1 proviral DNA, syringe/needle combinations were first flushed 4-5 times with 100 microliters of a buffer containing 10 mM Tris-HCL, pH 8.3, 50 mM KCL, 2.5 mM MgCl₂, 0.5% Tween 20, 0.5% NP-40, 60 micrograms/ml proteinase K. Four subsequent ten-fold dilutions were then made from this extraction solution. These extracts were digested either for 1 hour at 60 degrees C, inactivated by incubation at 95 degrees C for 15 min, and stored at 4 degrees C. DNA was additionally purified by extraction first with Tris-buffered phenol and then with chloroform/isoamyl alcohol (24:1) and precipitation by addition of Na acetate and ethanol with 5 micrograms of yeast tRNA added as carrier. Precipitates were stored at -20 degrees C and pelleted and dried prior to use in PCR. The samples then tested contained the equivalent of 4.5, 0.45, 0.045, 0.0045, and 0.00045 microliters of blood.

Amplification

A previous series of experiments had shown improved sensitivity of PCR using a double round of amplification with nested primers--fifteen cycles using gag816/1097 (the notation here refers to the 281 base-pair(BP) sequence found between BP number 816 and 1097 on the structural gag gene of HIV-1) followed by 35 cycles using gag856/1046.²³ The primers for the 816/1097 pair consisted of a 21 BP sequence (GAAGGCTTTCAGCCCAGAAGT) at the 816 end and a 21 BP sequence (GGTGGATTATTTGTCATCCAT) at the 1097 end. The primers for the 856/1046 pair were both 20 BP's in length, (TTATCAGAAGGAGCCACCCC) and (CCTGCTATGTCACCTCCCCT) respectively. Using this technique we were able to reproducibly detect HIV-1 proviral DNA in dilutions with as few as two copies of a plasmid containing the HIV-1 genome.²⁴ DNA to be amplified was resuspended in 20 microliters of TE-80 (10 mM Tris-HCL, pH 8.0, 1 mM EDTA), maintained on ice, and

included as part of 100 microliter reactions containing the target DNA, 10 mM Tris-HCL, pH 8.3, 50 mM KCL, 1.5 mM MgCl₂, 100 micrograms/ml gelatin, 1.25 mM of each dNTP, 20 picomoles of the primer pair gag816/1097, and 2 U Taq polymerase. PCR commenced with denaturation of the DNA at 95 degrees C for 2 min directly followed by 15 cycles of amplification in a Biosycler (Bios Corp., New Haven, CT.). A second round of amplification was then performed using 50 picomoles of each of the gag856/1046 primer pair in the same reagents plus 25 microliters of reaction mix from the first round. Following the final cycle, elongation was continued for 6 min. Primers and their associated probes were synthesized in house by the Yale Medical School nucleic acid chemistry facility.

Detection

PCR products were analyzed by electrophoresis and Southern blotting. Aliquots of 20 microliters from each reaction and, as size markers, pBR322/BstNI (New England Biolabs, Beverly, MA) were run on 2.5% agarose gels at 100mA using TAE buffer. DNA in gels was denatured using 0.5 N NaOH, 1.5 M NaCl, neutralized using 0.5 M Tris-HCL, pH 7.2, 1.5 M NaCl, transferred to Hybond-N nylon membranes (Amersham, Arlington Heights, IL), and crosslinked by 5 min shortwave UV exposure. Membranes were prehybridized with a solution containing 6X SSC (20X SSC is 0.3M NaCitrate and 3.0 M NaCl), 5X Denhardt's (0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin), 0.1% SDS, 100 micrograms/ml sheared, denatured salmon sperm DNA for 4 hours at 65 degrees C and, following removal of excess prehybridization buffer, hybridized for 16-20 hours at 70 degrees C using 10⁶ cpm of an appropriate [γ -³²P] ATP-kinased oligomer probe per ml of hybridization buffer (6X SSC, 5X Denhardt's, 100 micrograms/ml denatured salmon sperm DNA).

This probe was picked carefully to have a sequence specific to a region internal to both of the sets of primers used in the amplification so as to add an additional level of specificity to the detection process. It is a 23 BP fragment from gag 927 to 905 with the following sequence: GCATGGCTGCTTGATGTCCCCC. Filters were washed with 6X SSC, 0.1% SDS three times at room temp for 5 min each and twice at 60 degrees C for 15 min each. Amplified DNA was visualized using X-ray film exposed at -70 degrees C in cassettes with intensifying screens.

Results

PCR vs. ELISA

Both ELISA and PCR gave positive signals for the first dilution of all fifteen symptomatic patients and all five asymptomatic patients (sensitivity = 100%). ELISA and PCR gave negative results for all the HIV- patients tested (specificity = 100%) [see Figure 1 a-d in Appendix B for photographs of PCR southern blots].

In every HIV+ patient, symptomatic or asymptomatic, the ELISA was able to detect positive signal at lower dilutions than PCR (see Table 1). In the symptomatic group the mean volume of blood below which ELISA became negative was 0.00086 microliters. This may, in fact, be falsely elevated as samples were not tested below 0.0001 microliters, and four of the fifteen samples were still positive at this dilution. The median was 0.001 microliters.

In the same group, the mean volume of blood below which PCR became negative was 0.067 microliters and the median was 0.045 microliters.

TABLE 1

Minimum Volume of Blood Yielding
Positive Detection of HIV-1 in Symptomatic Patients
(in microliters)

<u>Patient #</u>	<u>CD4+ cells/</u>	<u>ELISA</u>	<u>PCR</u>	<u>Western</u>
	<u>microliter</u>			
1	10	0.001	0.45	1.0
2	800	0.001	0.0045	0.1
3	170	0.0001*	0.45	0.1
4	70	0.01	0.45	1.0
5	30	0.01	0.45	sera
6	420	0.001	0.0045	0.1
7	370	0.0001*	4.5	0.01
8	10	0.01	4.5	sera
9	50	0.001	0.0045	0.1
10	20	0.0001*	0.0045	0.1
11	260	0.001	0.0045	0.1
12	290	0.001	0.045	1.0
13	20	0.001	0.045	0.1
14	730	0.001	0.0045	1.0
15	40	0.0001*	0.045	0.1
Geometric				
Mean:		0.00086	0.062	0.17**
Median:		0.001	0.045	

* Dilutions below this level were not tested.

** Calculated by excluding patients 5 and 8.

When this data is analyzed using a non-parametric two tailed sign test (which asks what is the probability that using two equally sensitive tests one test would be more sensitive than the other 15/15 times) the p-value is <0.0001 . The 95% confidence interval, using this technique is (0.0022 - 0.2222). In other words, one can say with 95% confidence that ELISA is between 5 and 500 times more sensitive than PCR in this group of patients. The same data may be analyzed using a paired, two tailed T test. Functionally, this test uses the null hypothesis that if both detection techniques are equally sensitive then the sum of the differences between the lowest PCR+ dilution and ELISA+ dilution for each subject would equal zero. This number is then calculated and the p-value reflects the credibility of the null hypothesis. Using this test the T value is -5.95, and $p = 0.0000$. Graphically, this improved sensitivity of ELISA over PCR in the symptomatic patients is depicted in Figure 2. The number of patients still testing positive is graphed as a function of decreasing dilutions using a negative logarithmic scale.

In the asymptomatic group the sample size is smaller, and one must be wary of making overly strong conclusions from a limited data pool. Nonetheless, the results suggest a clear trend (see Table 2). In all but one patient, ELISA was positive down to the last dilution while PCR lost signal, on average, at the second dilution. The mean threshold for detection by ELISA was 0.00016 microliters, while for PCR it was 0.45 microliters. Thus, in the asymptomatic group, the ELISA shows improved sensitivity while PCR's sensitivity appears diminished. No matter how strong the differences, it would not be possible to show statistical significance of the results using the sign test (because such a test does not take into account the

Decreasing Sensitivity of ELISA versus PCR as a Function of Dilution

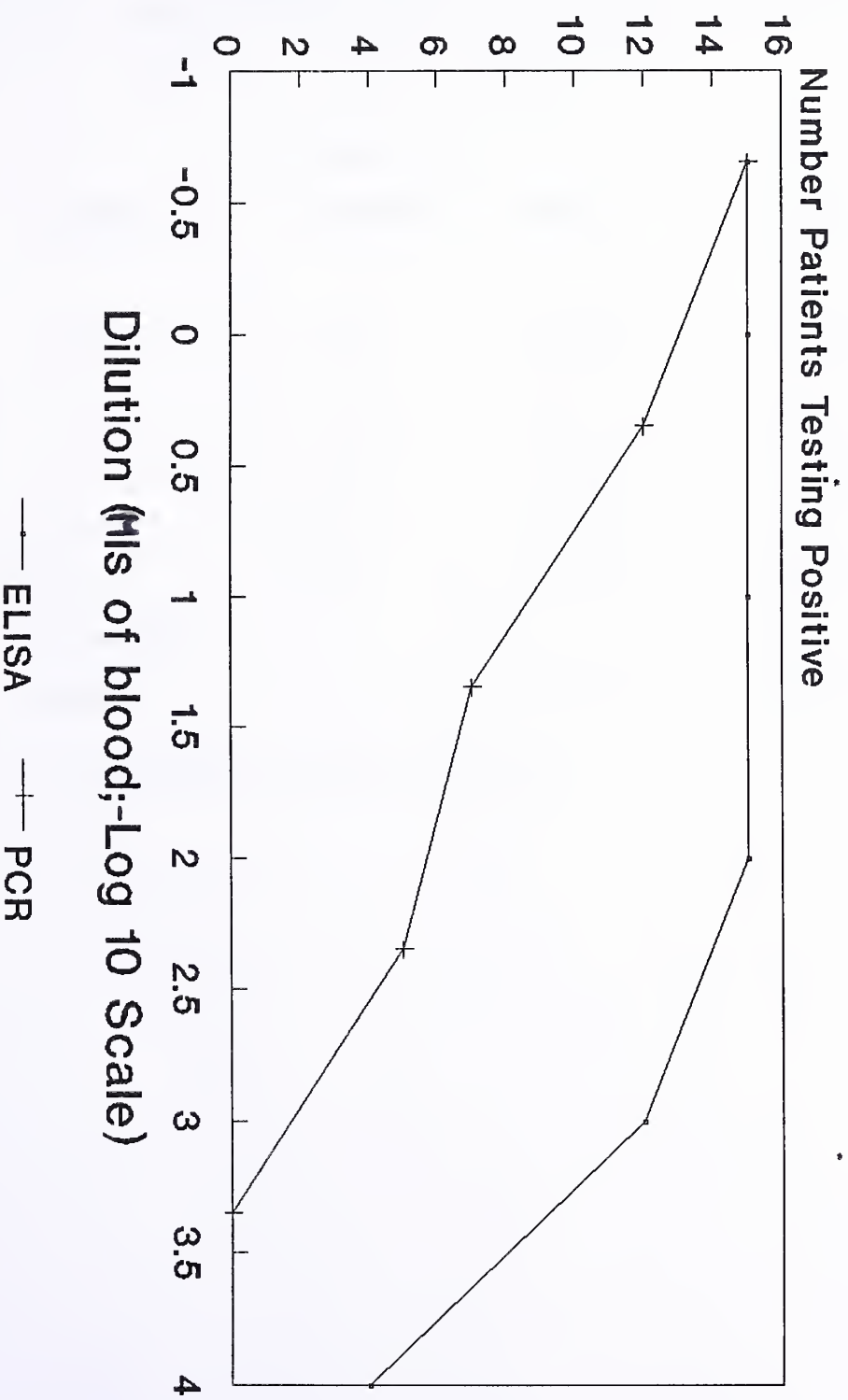


Figure 2

TABLE 2

**Minimum Volume of Blood Yielding
Positive Detection in Asymptomatic Patients
(in microliters)**

<u>Patient #</u>	<u>CD4+ cells/ microliter</u>	<u>ELISA</u>	<u>PCR</u>	<u>Western</u>
20	500	0.0001*	0.45	0.001
21	1090	0.001	0.45	1.0
22	990	0.0001*	0.45	0.01
23	880	0.0001*	0.045	0.01
24	900	0.0001*	4.5	0.001
Geometric				
Mean:		0.00016	0.45	0.01

* Dilutions below this level were not tested.

magnitude of the differences between the two tests but only the number of times one test beats the other). However, when analyzed using the paired, two tailed T test the T value is -9.23 and p is < 0.0008. The 95% confidence interval is (0.000032 - 0.0039). Thus, in both the symptomatic and asymptomatic groups, ELISA remains sensitive at significantly lower dilutions than PCR.

Western Blots

The western blots proved uniformly less sensitive than the ELISA at detecting HIV-1 antibody in low concentrations. Of the twenty patients tested, there were no dilutions for which the western was positive and the ELISA negative. The mean threshold for detection by western was 0.17 microliters in the symptomatic group which was greater than both the ELISA and the PCR thresholds. In the asymptomatic group westerns performed somewhat better with a threshold of 0.01 microliters which was lower than the PCR but at least two orders of magnitude less sensitive than the ELISA.

T- Cell Studies

The mean number of CD4 cells required in a solution for a positive signal by PCR was 5.4 among the symptomatic group (see Table 3). The range was from 0.09 to 76.5 with the exception of one outlier at 1,665. In interpreting the data from the two patients with values less than one, it is important to remember that these values represent probabilities. Obviously the true number of CD4 cells in a solution must be a whole number, presumably 1 in these cases. Among the

Table 3

**Threshold Number of CD4 Cells/Dilution Giving Positive
Signal By ELISA and PCR
(Symptomatic Patients)**

Patient #	PCR result*	ELISA result*	CD4 cells/ microliter
1	4.5	0.01	10
2	3.6	0.8	800
3	76.5	0.02	170
4	31.5	0.7	70
5	13.5	0.3	30
6	1.8	0.4	420
7	1665.0	0.04	370
8	45.0	0.1	10
9	0.2	0.05	50
10	0.09	0.002	20
11	1.2	0.3	260
12	13.7	0.3	290
13	0.9	0.02	20
14	3.3	0.7	730
15	1.8	0.004	40
Geometric			
Mean:	5.4	0.08	

* Estimated number of CD4 cells that would be found in the lowest dilution testing positive by these two techniques.

asymptomatic patients, the mean threshold CD4 count for detection by PCR was 379.6. The range was from 39.6 to 4050 CD4+ cells (see Table 4).

Discussion

PCR vs. ELISA

It is difficult to make accurate estimates of the amount of blood remaining in a typical needle/syringe combination returned by a NEP participant. Approximately 30% of needles returned in the New Haven NEP have visible blood which correlates with a volume roughly > 0.5 microliters. In the symptomatic patient, the mean volume of blood required for a positive signal by PCR was 0.045 microliters, about one tenth of this volume. It seems credible, therefore, that within this subpopulation PCR would detect evidence of HIV in the majority of needles returned by HIV+ participants. In a few needles, with volumes below this level, ELISA might prove more sensitive.

The asymptomatic patients pose more of a problem. In this group, the mean threshold below which PCR loses its sensitivity is 0.45 microliters of blood. Because this volume is quite close to the limit of visibility, one can assume that only approximately 30% of returned needles contain this much blood. These data would suggest that in testing needles from this subpopulation, the ELISA assay may offer a distinct advantage over PCR. This is especially important because the majority of HIV+ participants in a NEP are likely to be asymptomatic given the relatively short course from symptomatic AIDS to hospitalization and death compared to the much longer latent phase and the fact that

Table 4

**Threshold Number of CD4 Cells/Dilution Giving Positive
Signal by ELISA and PCR
(Asymptomatic Patients)**

Patient #	PCR result	ELISA result	CD4 count/ microliter
20	225.0	0.05	500
21	490.5	1.1	1090
22	445.5	0.1	990
23	39.6	0.09	880
24	4050.0	0.09	900
Geometric			
Mean	379.6	0.13	

*Estimated number of CD4 cells that would be found in the lowest dilution testing positive by these two techniques.

hospitalized patients do not participate in the NEP. Nonetheless, the sample size from this group is quite small, and the conclusions drawn from it must remain speculative until further data is gathered.

One possibility that must also be considered is that the impressive sensitivity of the ELISA is not due to specific detection of anti-HIV-1 antibody but to non-specific binding of some other cross-reactive antibody. There have been reports of rare cases of false positive results with ELISA when this technique is used to screen large populations.²⁵ One could postulate that IVDU's who are injecting foreign material and sharing needles may be exposed to a heavy non-self antigen load and therefore produce a large number of uncommon antibodies. It would be interesting, for this reason, to test the blood of known HIV- IVDU's by ELISA to see if there is any incidence of false positives. However, such an experiment was not within the scope of this study. A second way to evaluate this hypothesis is to compare the ELISA results to those from the western blots. Because western blot testing requires specific binding to at least three different HIV-1 antigens to be considered positive, it would presumably be less vulnerable to such non-specific cross-reacting antibodies. If, therefore, the ELISA were testing positive secondary to such contamination, one would not expect to see a similar trend in the western blots. However, in these experiments, there is a close relation between ELISA and western. In those patients in whom the ELISA is most sensitive, the western is most sensitive, and the inverse is also true. This is suggestive evidence, then, that a cross-reacting antibody is not playing a major role. It is important to note that the inferior sensitivity of PCR is not secondary to technical imperfections but to the biological principles upon which it is based. For a positive signal by PCR, there must be at least one molecule of HIV-1 proviral DNA. Controls were run with experimental

samples which routinely showed strong positives when only three copies of the HIV-1 genome were present. Presumably the dilutions below which PCR is negative but ELISA remains positive represent samples where there are no further infected CD4 cells present but there is still adequate antibody for detection by ELISA.

Western Blot

In both the symptomatic and asymptomatic patients, the western blot is approximately two orders of magnitude less sensitive than the ELISA. Given this disparity, western blots could not be used effectively to confirm ELISA in testing needles. While this lack of confirmation would be unacceptable in making individual diagnoses, it is less of a problem for this application. In testing needles, one follows trends of HIV positivity over "populations" of returned needles. While conceivably there may be some error at the level of individual tests, these errors should cancel out when large numbers of needles are compared at different time intervals.

T-Cell Studies

It has been previously discussed that positive signal by PCR depends on two factors: the concentration of HIV-1 proviral DNA within the CD4 population and the number of CD4 cells in a sample. It appears that among the symptomatic patients, PCR's sensitivity is limited by this second factor with a mean threshold of detection of 5.4 cells. In the asymptomatic group, however, the number of CD4 cells fall in a range from 39.6 to 4,050, and the concentration of proviral DNA must be the limiting factor. The decreased

sensitivity of PCR in this population would suggest that the effect of lower copy numbers of proviral DNA outweighs the effect of concentration of CD4 cells/microliter. Overall, the PCR results show an HIV-1 proviral copy number of approximately 1 per 1-75 CD4 cells (with the exception of one outlier from patient 7) in symptomatic patients and 1 per 40-4,000 in asymptomatic patients (again, the sample size here is quite small). These numbers are consistent with the copy numbers previously cited from the literature.

Conclusion

ELISA is a more sensitive technique than PCR for detection of HIV-1 infection in small amounts of blood from needle/syringe combinations. This effect appears especially pronounced when testing needles from asymptomatic patients in whom the antibody concentration is high but the concentration of HIV-1 proviral DNA in the CD4 population is relatively low. The inferior sensitivity of PCR in this application is not secondary to technical imperfections but to the biological principles upon which it operates.

Since the early 1980's, NEP's have been set up all over the world. To date there has been no proof that such programs diminish transmission of HIV-1 among IVDU's. To generate such proof requires documentation of decreasing infectedness of needles used by participants which, in turn, requires testing returned needles for evidence of HIV-1. This experiment suggests that both PCR and ELISA may be effective ways to do such testing. Paradoxically, it shows improved sensitivity using an ELISA. This is fortunate because it is a cheaper, easier, less technically demanding, and more

expeditious assay than is PCR. Its use should allow other programs around the world to evaluate their programs directly and make informed conclusions about their effectiveness.

APPENDIX A

Patient #	Volume of blood in dilution (in micro- liters)	Symptomatic (S) vs. asymptoma- tic (A)	PCR result	ELISA result	Western result	CD4+ cells per dilution
1	sera	S		+		
1	4.5	S	+			45
1	1.0	S		+	+	10
1	.45	S	+			4.5
1	0.1	S		+	+ / -	1.0
1	.045	S	-			0.5
1	.01	S		+	-	0.1
1	.0045	S	-			.05
1	.001	S		+	-	.01
1	.00045	S	-			.005
1	.0001	S		-		.001
2	sera	S		+		
2	4.5	S	+			3600
2	1.0	S		+		800
2	.45	S	+			360

2	.1	S		+	+	80
2	.045	S	+			36
2	.01	S		+	+ / -	8
2	.0045	S	+			3.6
2	.001	S		+	-	.8
2	.00045	S	-			.4
2	.0001	S		-		.08
3	sera	S		+		
3	4.5	S	+			765
3	1.0	S		+		170
3	.45	S	+			76.5
3	.1	S		+	+	17
3	.045	S	-			7.7
3	.01	S		+	-	1.7
3	.0045	S	-			.8
3	.001	S		+	+ / -	.2
3	.00045	S	-			.08
3	.0001	S		+		.02
4	sera	S		+		
4	4.5	S	+			315
4	1.0	S		+	+	70
4	.45	S	+			31.5
4	.1	S		+	-	7
4	.045	S	-			3.2
4	.01	S		+	-	.7

4	.0045	S	-			.3
4	.001	S		-	-	.07
4	.00045	S	-			.03
5	sera	S		+	+	
5	4.5	S	+			135
5	1.0	S		+	+ / -	30
5	.45	S	+			13.5
5	.1	S		+	-	3
5	.045	S	-			1.4
5	.01	S		+	-	.3
5	.0045	S	-			.1
5	.001	S		-	-	.03
5	.00045	S	-			.01
6	sera	S		+		
6	4.5	S	+			1790
6	1.0	S		+		420
6	.45	S	+			179
6	.1	S		+	+	42
6	.045	S	+			17.9
6	.01	S		+	+ / -	4.2
6	.0045	S	+			1.8
6	.001	S		+	-	.4
6	.00045	S	-			.2
6	.0001	S		-		.04

7	sera	S		+		
7	4.5	S	+			1665
7	1.0	S		+		370
7	.45	S	-			166.5
7	.1	S		+	+	37
7	.045	S	-			16.7
7	.01	S		+	+	3.7
7	.0045	S	-			1.7
7	.001	S		+	+ / -	.4
7	.00045	S	-			.2
7	.0001	S		+		.04
8	sera	S		+	+	
8	4.5	S	+			45
8	1.0	S		+	+ / -	10
8	.45	S	-			4.5
8	.1	S		+	+ / -	1.0
8	.045	S	-			.5
8	.01	S		+	-	.1
8	.0045	S	-			.05
8	.001	S		-	-	.01
8	.00045	S	-			.005
9	sera	S		+		
9	4.5	S	+			225
9	1.0	S		+		50
9	.45	S	+			22.5

9	.1	S		+	+	5
9	.045	S	+			2.3
9	.01	S		+	-	.5
9	.0045	S	+			.2
9	.001	S		+	-	.05
9	.00045	S	-			.02
9	.0001	S		-		.005
10	sera	S		+		
10	4.5	S	+			90
10	1.0	S		+	+	20
10	.45	S	+			9
10	.1	S		+	+	2
10	.045	S	+			.9
10	.01	S		+	+ / -	.2
10	.0045	S	+			.09
10	.001	S		+	-	.02
10	.00045	S	-			.009
10	.0001	S		+		.002
11	sera	S		+		
11	4.5	S	+			1170
11	1.0	S		+	+	260
11	.45	S	+			117
11	.1	S		+	+	26
11	.045	S	+			11.7
11	.01	S		+	-	2.6

1 1	.0045	S	+			1.2
1 1	.001	S		+	-	.3
1 1	.00045	S	-			.1
1 1	.0001	S		-		.03
1 2	sera	S		+		
1 2	4.5	S	+			1365
1 2	1.0	S		+	+	290
1 2	.45	S	+			136.5
1 2	.1	S		+	+ / -	29
1 2	.045	S	+			13.7
1 2	.01	S		+	+ / -	2.9
1 2	.0045	S	-			1.4
1 2	.001	S		+	-	.3
1 2	.00045	S	-			.1
1 2	.0001	S		-		.03
1 3	sera	S		+		
1 3	4.5	S	+			90
1 3	1.0	S		+	+	20
1 3	.45	S	+			9
1 3	.1	S		+	+	2
1 3	.045	S	+			.9
1 3	.01	S		+	-	.2
1 3	.0045	S	-			.09
1 3	.001	S		+	-	.02
1 3	.00045	S	-			.009

13	.0001	S		-		.002
14	sera	S		+		
14	4.5	S	+			3285
14	1.0	S		+	+	730
14	.45	S	+			328.5
14	.1	S		+	+ / -	73
14	.045	S	+			32.9
14	.01	S		+	-	7.3
14	.0045	S	+			3.3
14	.001	S		+	-	.7
14	.00045	S	-			.3
14	.0001	S		-		.07
15	sera	S		+		
15	4.5	S	+			180
15	1.0	S		+	+	40
15	.45	S	+			18
15	.1	S		+	+	4
15	.045	S	+			1.8
15	.01	S		+	+ / -	.4
15	.0045	S	-			.2
15	.001	S		+	-	.04
15	.00045	S	-			.02
15	.0001	S		+		.004
20	sera	A		+		

20	4.5	A	+			2250
20	1.0	A		+	+	500
20	.45	A	+			225
20	.1	A		+	+	50
20	.045	A	-			22.5
20	.01	A		+	+	5
20	.0045	A	-			2.3
20	.001	A		+	+	.5
20	.00045	A	-			.2
20	.0001	A		+	+ / -	.05
21	sera	A		+		
21	4.5	A	+			4905
21	1.0	A		+	+	1090
21	.45	A	+			490.5
21	.1	A		+	+ / -	109
21	.045	A	-			49.1
21	.01	A		+	+ / -	10.9
21	.0045	A	-			4.9
21	.001	A		+	-	1.1
21	.00045	A	-			.5
21	.0001	A		-	-	.1
22	sera	A		+		
22	4.5	A	+			4455
22	1.0	A		+	+	990
22	.45	A	+			445.5

2 2	. 1	A		+	+	9 9
2 2	. 0 4 5	A	-			4 4 . 6
2 2	. 0 1	A		+	+	9 . 9
2 2	. 0 0 4 5	A	-			4 . 5
2 2	. 0 0 1	A		+	+ / -	1 . 0
2 2	. 0 0 0 4 5	A	-			. 5
2 2	. 0 0 0 1	A		+	-	. 1
2 3	sera	A		+		
2 3	4 . 5	A	+			3 9 6 0
2 3	1 . 0	A		+	+	8 8 0
2 3	. 4 5	A	+			3 9 6
2 3	. 1	A		+	+	8 8
2 3	. 0 4 5	A	+			3 9 . 6
2 3	. 0 1	A		+	+	8 . 8
2 3	. 0 0 4 5	A	-			4 . 0
2 3	. 0 0 1	A		+	+ / -	. 9
2 3	. 0 0 0 4 5	A	-			. 4
2 3	. 0 0 0 1	A		+	+ / -	. 0 9
2 4	sera	A		+		
2 4	4 . 5	A	+			4 0 5 0
2 4	1 . 0	A		+	+	9 0 0
2 4	. 4 5	A	-			4 0 5
2 4	. 1	A		+	+	9 0
2 4	. 0 4 5	A	-			4 0 . 5
2 4	. 0 1	A		+	+	9

24	.0045	A	-			4.1
24	.001	A		+	+	.9
24	.00045	A	-			.4
24	.0001	A		+	+ / -	..09

Data from HIV- Patients

Patient #	Volume of blood in		PCR result	ELISA result
	dilution (in microliters)			
16	sera			-
16	4.5		-	
16	1.0			-
17	sera			-
17	4.5		-	
17	1.0			-
18	sera			-
18	4.5		-	
18	1.0			-

Appendix B

(Figures 1a-d)

Figures 1a-d show photographs of the southern blots showing PCR results for patients 1-24. Where there are patient numbers bracketing five rows, each row represents one of the five tested dilutions in decreasing order. The letter M stands for markers (kinase labelled pBR 322 digested with BstN1) used to identify whether the bands are appearing in the expected locations for sequences of their molecular weight. The negative controls include lanes with no DNA, K562 (a human erythroleukemia cell-line), HT 1080 (a human epithelioid carcinoma), C₆ (a mouse astrocytoma), and human white blood cell DNA from HIV- volunteers. These negative controls were run through the identical amplification and detection steps as the patient samples. pBH10 is a plasmid containing one copy of the HIV-1 genome. The letters A, B, and C correspond to 3.5, 35, and 350 copies of this plasmid in the dilution tested. In Figure 1b, lane D corresponds to 2 microliters of white blood cell DNA extracted from an HIV+ patient. This volume would contain approximately 5,000-6,000 cell equivalents. In Figure 1d, lane D is the same as in Figure 1b, and lane E is a tenfold dilution of lane D (containing 500-600 cell equivalents). Finally, in Figure 1b, the lanes marked HIV- needles correspond to patients 16, 17, and 18, the HIV- volunteers.

01

02

03

04

M

C

05

M

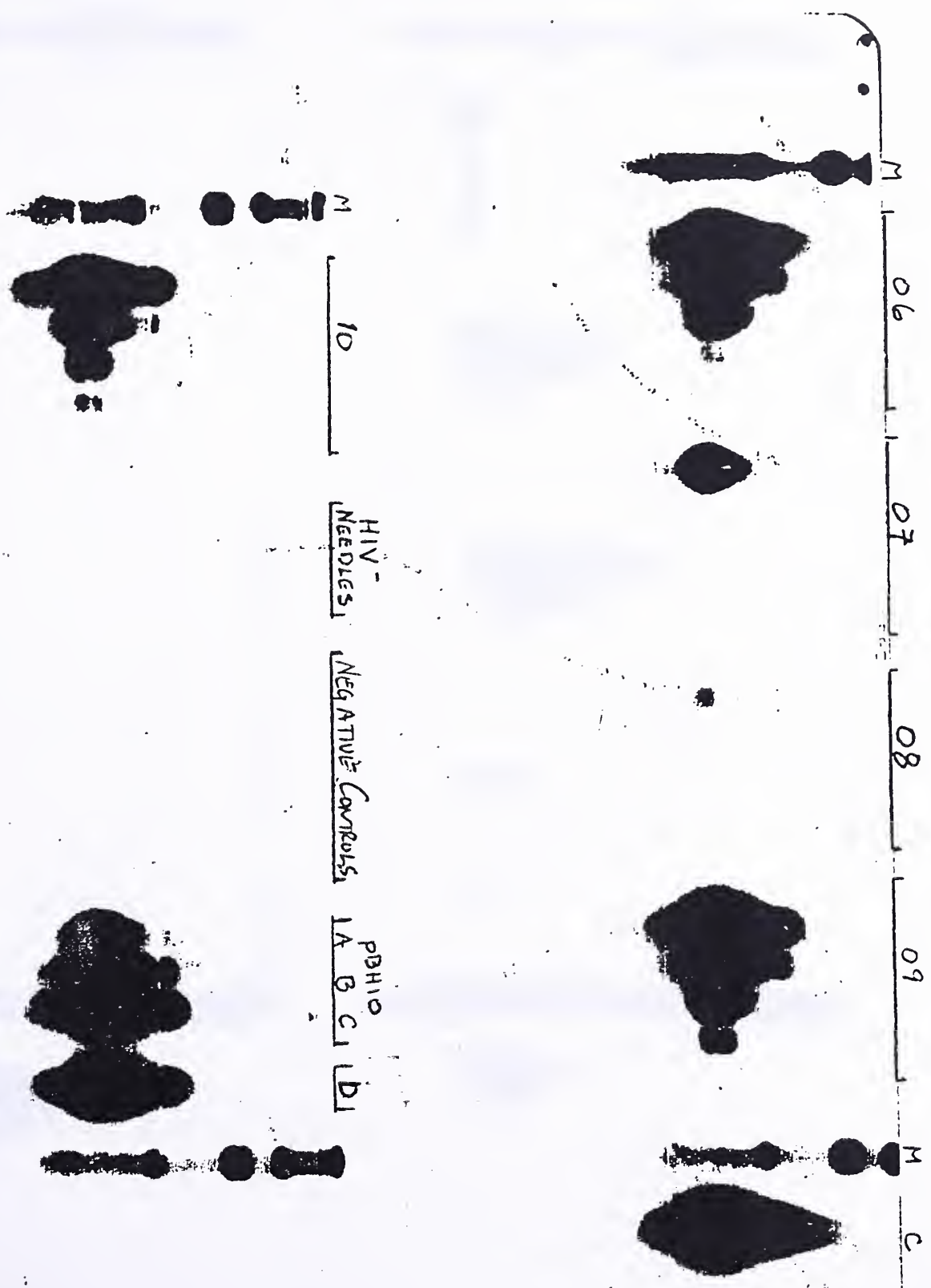
NEGATIVE CONTROLS

P B H I O
A B C

M

a ①

Figure 1a



HIV-1
NEEDLES

NEGATIVE CONTROLS

PB110
A B C 1 D 1

M

06

07

08

09

M

C

M

10

HIV-1
NEEDLES

NEGATIVE CONTROLS

PB110
A B C 1 D 1

M



Figure 1c

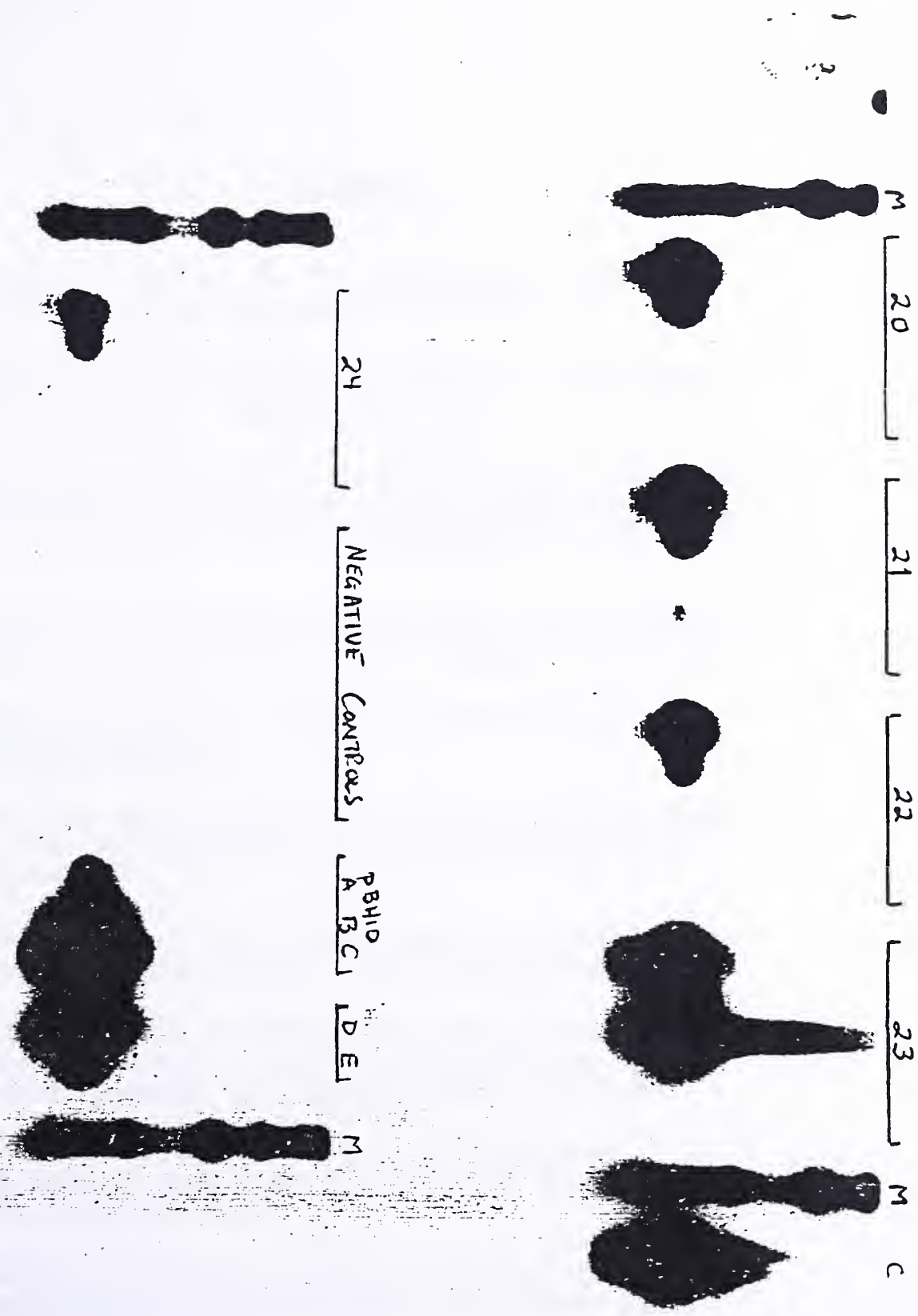


Figure 1d

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