Failure of Routine HIV-1 Tests in a Case Involving Transmission With Preseroconversion Blood Components During the Infectious Window Period

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IN THE UNITED STATES, DONATED blood and plasma is tested for antibodies to human immunodeficiency virus types 1 (HIV-1) and 2 (HIV-2) by screening with an enzyme immunoassay (EIA), as well as an HIV-1 p24 antigen EIA.1 Despite a dramatic reduction in risk due to the improved sensitivity of these tests, it is estimated that from 1 in 450,000 to 1 in 660,000 US blood donations may transmit HIV,2,3 with nearly all cases of transfusion-associated HIV infection being caused by donations made during the infectious window period, prior to seroconversion. Contemporary HIV EIAs have an average infectious window period of 25 days.4 The p24 antigen becomes detectable 2 to 3 weeks after HIV infection during the period of high-level viremia associated with the initial burst of virus replication5,6 and, on average, can persist for 4 to 5 days.7

Context Current screening practices for blood donations have been successful in reducing human immunodeficiency virus (HIV) transmission through receipt of contaminated blood products. However, HIV-infected blood donations made prior to seroconversion and before high levels of viral replication occur could test negative using both serologic antigen and antibody tests. Testing based on nucleic acid amplification (NAT) is being implemented to screen for HIV-infected blood donated during this period, yet the issue of single vs minipool donation screening remains unresolved.

Objectives To determine HIV-1 genetic linkage between virus in 2 HIV-1–infected recipients of blood components and virus in the donor, who was HIV antigen and antibody negative at the time of donation; to screen the blood donor’s plasma with HIV NAT assays, including those currently proposed for use in US blood donation screening.

Design and Setting Case study conducted in October 1997 involving the Communicable Disease Centre, Singapore General Hospital, and the Singapore Blood Transfusion Service, Singapore.

Subjects The blood donor and the 2 recipients of donor platelets and red blood cells.

Main Outcome Measures Genetic analysis of the HIV-1 p17 coding region of gag and the C2V5 region of env to determine the genetic relatedness of virus from the donor and recipients; reactivity in quantitative and qualitative assays, and reactivity in donor screening HIV NAT assays in single donation and minipool screening contexts.

Results Direct DNA sequencing demonstrated identical HIV-1 subtype E viral sequences in the donor and recipients. Based on comparisons of a qualitative and quantitative assay for HIV-1 RNA levels, a low level of viremia (range, 5-39 copies/mL in plasma) was estimated to be in the donor’s undiluted blood at the time of donation. Additional testing using donor-screening NAT assays showed consistent detection of HIV RNA in the undiluted donor plasma whereas detection was inconsistent at the 1:16 and 1:24 dilution levels currently used in minipool screening of blood donations in the United States.

Conclusions Transmission of HIV from a blood donor to a platelet recipient and a red blood cell recipient occurred in the preseroconversion infectious window period. The viral load in the implicated donation was estimated to be less than 40 copies/mL of plasma. Current US minipool HIV NAT screening protocols may not be sufficiently sensitive to detect all infectious window-period donations.

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See also pp 229 and 238.
be detected about 6 days before antibody tests become positive. Using a test based on nucleic acid amplification (NAT), it is possible to detect HIV RNA 5 to 10 days prior to p24 antigen detection, which has proven useful in confirming HIV diagnostic serology. Currently, HIV NAT testing is being evaluated in clinical trials regarding its use in routine screening of blood and plasma donations. Ninety-nine percent of plasma collected in the United States is tested with a NAT assay for HIV and hepatitis C virus (HCV).

Currently, NAT for HIV and HCV is performed (under Investigational New Drug applications, approved by the Food and Drug Administration [FDA]) on minipools of 16 to 24 whole blood donations due to the cost and logistical complexity of screening individual donation samples. In recent studies using mathematical modeling, there are estimates of the risk of HIV contamination per 10 million US donations, dropping from 25 with antibody testing only, to 13 or 14 with the addition of minipool HIV NAT testing. There is controversy regarding whether either minipool or single donation NAT screening will be adequately sensitive to interdict all infectious donations.

In this study, we investigated possible linkage between virus from an HIV-infected blood donor and 2 HIV-infected recipients of his blood components who had no other risk for HIV infection. The blood donation and transfusion of blood components occurred in Singapore, where blood donation testing for HIV is similar to US protocols, including anti-HIV-1 and anti–HIV-2 EIAs and an HIV-1 p24 antigen (Coulter Corporation, Miami, Fla). When returning in October 1997, the donor was found to be HIV antibody positive. Two recipients of platelets and red blood cells derived from the June 1997 donation also tested positive for HIV (care of patients and their laboratory testing took place at the Communicable Disease Centre, Singapore General Hospital). The unit of plasma from the window period donation had not been transfused and was available for further testing. Plasma and peripheral blood mononuclear cells (PBMCs) were collected in October 1997 from the platelet recipient and the red blood cell recipient and were available for testing. The preseroconversion plasma was tested for detectable viral load (HIV-1 RNA) using the quantitative Amplicor HIV-1 Monitor assay (see acknowledgment; Roche Molecular Systems, Branchburg, NJ), having a sensitivity limit of 200 copies/mL.

Following testing with Amplicor, the US Centers for Disease Control and Prevention became involved and initiated further testing, including the qualitative NucliSens HIV-1 RNA QT assay (Organon Teknika, Durham, NC), which has a lower limit of 80 copies per input volume (in this case, 2 mL), equivalent to 40 copies/mL. Qualitative detection of preseroconversion HIV RNA was performed with the NucliSens HIV-1 RNA QL assay (Organon Teknika), which has a lower level of detection of about 5 copies/mL (manufacturer's estimate) when using 2 mL of plasma. The RNA was extracted by the NucliSens nucleic acid extraction method (first phase of NucliSens QT and QL assays) and amplified in a second qualitative assay (reverse transcription/PCR) using the Promega RT kit (Promega, Madison, Wis) with ED12 primer for complementary DNA (cDNA) production. The cDNA was then used for PCR amplification and DNA sequencing. The donor's preseroconversion plasma was HIV antigen and antibody negative and had previously tested at below level of detection in Australia using the Amplicor assay. Thus, 2 mL of sample was used to obtain the highest level of sensitivity, using the NucliSens QT and NucliSens QL tests. Only 1 mL of sample was used with the NucliSens QT assay (for HIV-1 viral load determination) to test the donor and recipient plasma samples collected 4 months after donation and transfusion (October 1997) as they had already been tested in Singapore and were both HIV-1 antigen and antibody positive. Subsequently, the preseroconversion donor plasma sample was tested with the 2 quantitative HIV RNA (NAT) assays (Roche COBAS AmpliScreen HIV-1 Test, v1.5, Roche Molecular Systems, Pleasanton, Calif; Chiron Procleix TMA HIV-1/HCV Assay, Chiron Corporation, Emeryville, Calif) being evaluated for testing of whole blood donations in the United States. Testing was performed with these assays in accordance with the manufacturer's instructions on undiluted sample and a 1:8 dilution and minipool dilutions (1:16 and 1:24) of donation plasma.

The HIV sequences were derived from the initial donation plasma as well as the donor and recipients' 4-month postdonation/transfusion PBMC samples. Processing of the PBMCs was as described, except DNA was extracted either with Generation Capture column kits (Generation Inc, Minneapolis, Minn) or as described. The PCR amplification of the DNA and cDNA preparations was also as described, except the primary PCR was performed with primers ED5 and ED12.

The PCR-amplified products were purified by Qiagen PCR purification kits (Qiagen Inc, Chatsworth, Calif). Purified DNA was sequenced using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems/Perkin Elmer, Foster City, Calif). Sequencing primers were various combinations of nested PCR primers. The HIV regions sequenced included the C2V5 region (716 nucleotides) of env and the p17 coding region (396 nucleotides) of gag, both regions commonly used for phylogenetic analysis and comparisons of HIV strains in...
transmission cases. The sequence data did not include regions to which NucliSens QT and QL kit primers would anneal. Testing performed at the Centers for Disease Control and Prevention on these specimens did not involve human subjects research.

**RESULTS**

Amplification and direct DNA sequencing of PCR products were performed on the 4 samples (Table 1) involving 396 nucleotides of the p17 region and 716 nucleotides of the C2V5 region. In total, 1112 nucleotides of HIV were compared for virus from each of the 4 samples. Complete identity was observed at all positions; the virus had identical sequences in the p17 and C2V5 regions. Phylogenetic analysis resulted in grouping this virus strain with subtype E HIV-1, which is one of the predominant strains circulating in Singapore (M. L. K., unpublished data, 1996).

Table 1 summarizes viral load testing and sequencing results for the preseroconversion and 4-month postdonation samples from the donor and the 4-month posttransfusion samples from the recipients. The 2 commercial quantitative RNA assays failed to detect HIV RNA during the window period. The HIV RNA was detected in both the qualitative RT-PCR assay (involving a Promega RT kit, NucliSens RNA extraction, and amplification of cDNA as described in “Methods”) and the qualitative NucliSens assay (HIV-1 RNA QL). Using 2 mL of plasma, the qualitative NucliSens assay has a lower limit of detection of about 5 copies/mL, and the quantitative NucliSens QT assay has a lower limit of detection at 40 copies/mL.17 This would predict that the window period preseroconversion plasma sample had a viral load of between 5 to 39 copies/mL. Since both the NucliSens QT and QL assays use the same primers and probes for amplification and detection of HIV RNA, the negative result in the QT assay is due to the higher detection limit for QT and not from HIV sequence heterogeneity. The viral load determinations for the 4-month samples were 16000 copies/mL for the donor and 2800 and 13000 copies/mL for the 2 recipients.

Table 2 summarizes results of an evaluation of the 2 HIV NAT tests currently in clinical trials for screening blood donations in the United States. A masked panel of plasma specimens that included undiluted and diluted plasma from the Singapore blood donation, as well as negative and positive controls, was prepared. The implicated donation was consistently detected when tested undiluted by both Roche and Chiron assay systems. However, inconsistent reactivity occurred when donor plasma was tested at dilution levels currently used for minipool donation screening (1:16 and 1:24) and even at the 1:8 dilution. Because the Amplicor and the initial NucliSens quantitative tests were both negative and the NAT results at the 1:16 and 1:24 dilution levels were not consistently reactive, it is additionally unlikely that the low viral load result was due to sequence heterogeneity. Also, the AmpliScreen and Chiron assays have different probe and primer sets, which consistently detected virus at undiluted levels. The Amplicor test primers and probes are modified compared with those in the AmpliScreen; thus, the negative result for the Amplicor assay may reflect an unsuccessful primer/probe combination and/or less sensitivity in comparison with AmpliScreen.

From the beginning of this evaluation, we instituted rigorous procedures to ensure that contamination or specimen mix-up did not occur. These included the following: (1) all samples were amplified separately, on different days, in a clean room designated only for primary amplifications; (2) all PBMC samples were reamplified, resequenced, and compared for concordance; and (3) virus from the donor’s preseroconversion blood draw and the 4-month PBMC sample were compared. At no time did we observe discordant sequences that would suggest contamination. Plasma virus from the donor’s and recipients’ 4-month specimens was also sequenced and was found to be consistent with the PBMC sequence findings. Further, during the time...
we were sequencing DNA from these samples, other DNA samples were amplified and sequenced, and none had sequences that were similar to donor or recipient virus, which would have suggested contamination.

**COMMENT**

Blood donor centers in the United States and Singapore rely on multiple approaches to safeguard the blood supply. Current methods of donor risk factor screening and laboratory testing are highly effective in minimizing the risk of transfusion-acquired HIV infection. The blood donor in this investigation falsified answers to the interviewer, thus eliminating one of the screening safeguards. The HIV testing of blood donor samples using an antibody EIA (followed by confirmatory Western blot for a sample with a positive EIA) and an antigen-capture assay (followed by a neutralization procedure for reactive samples) have a sensitivity and specificity exceeding 99%. Plasma RNA detection systems have also been used to confirm p24 antigen EIA reactivity and resolve indeterminate antibody test results, which could reflect early infections. Given the projected low yield and poor cost-effectiveness, there has been substantial controversy over the merits of adding HIV RNA NAT to blood donor screening. Moreover, even the most sensitive NAT assays have a limit of detection for HIV RNA, and hence it is unclear whether NAT will prevent all HIV transmissions in the context of either minipool or single donation testing algorithms. Technically, the category of HIV NAT assays includes commercial kits specifically designed for HIV RNA detection as well as more general nucleic acid–based tests (including RT-PCR), modified in-house to assay HIV. The commercial HIV NAT kits have the advantages of quality control and sensitivity/specificity evaluation. To date, several kits have been developed and tested, but none have been approved by the FDA.

By direct sequencing of virus in samples from the donor and recipients, we found that the p17 and C2V5 sequences were identical at all 1112 positions. Direct sequences represent the most common nucleotide at each position or a consensus sequence of all the viral quasispecies within a person. Since the HIV RT is highly error-prone, incorporating an average of 1 incorrect nucleotide during each round of replication, an identical matching of viral sequences between the donor and the 2 recipients supports a recent common infection source.

We have shown that the donor's stage of infection at the time of blood collection was very early, preceding the time when the p24 antigen or antibody EIA tests could detect HIV infection. Further, the plasma viral load was extremely low, estimated at only 5 to 39 copies/mL. Therefore, it would be expected that the viral population would be very homogeneous, not having undergone as many replication cycles as with a higher level of viremia. This high degree of homogeneity has been observed even early after seroconversion and during early infection. Since the variants in the donor did not have time to evolve away from the "founder" virus, it is consistent that the virus transmitted to the recipients would be identical, along most of their genome, to the variants identified in samples from the donor. Even at 4 months after transfusion, the virus consensus sequence had not had time to evolve away from the founder virus. Since the virus population in early infections is highly homogeneous, it has been argued that during transmission only a single virus may be transmitted and establish infection, or multiple viruses may be transmitted but selection takes place to limit the number of viruses that are able to replicate within the new host. Whatever the case, it would appear that the early replicating virus from this donor was effective in establishing infection, in spite of the low number of virions present.

In another case involving an HIV-infected blood donor in which a red blood cell recipient and a platelet recipient both became infected, the blood donation took place in 1985, before routine HIV screening was initiated (it retrospectively tested HIV seropositive). An early asymptomatic stage was thought to be involved, due to the homogeneity of the V3 env viral sequences. Transmission occurred although the donor's viral load was only 3500 copies/mL. By comparison, the transmission that occurred in our study was from a donor whose viral load was estimated to be at 2 orders of magnitude lower (5-39 copies/mL). In another report, a 1995 case of HIV transmission from a blood donation collected during the window period was described. The donor was found to be HIV antibody negative and p24 antigen negative, but (subsequently) HIV RNA PCR positive. No genetic analysis or viral load data were described for that study.

Considering the current estimates of rates of HIV transmission via blood transfusions, the standard approach using EIA testing and p24 antigen assay combined with Western blot confirmatory testing has served remarkably well in significantly reducing rates of transfusion-associated HIV infection. Nevertheless, the rare occasion of blood donation during the preseroconversion phase of HIV infection has prompted worldwide interest in the use of NAT screening of all blood donations for HIV, hepatitis B virus, HCV, and possibly other bloodborne viral pathogens. A variety of commercial kits, as well as in-house tests, are being evaluated in Europe, where initiation of the evaluation of testing protocols began in 1997. Nucleic acid–based donor testing on an experimental basis is currently being used in the United States. The issues of higher costs for NAT assays and the increased testing time needed are being addressed by initial screening of donated plasma using pooled aliquots. Pooling, however, has a dilution effect, which can substantially decrease assay sensitivity in comparison with that obtained when donated samples are tested individually. A viral concentration step before testing plasma pools can increase sensitivity levels, at the cost of adding another step to the testing procedure. The FDA has proposed a minimum standard for any assay of pooled samples, which is that the assay should have the ability to detect 100 viral copies/mL (5000 viral copies/mL per single donation).

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to assay dilutions of the donor plasma performed better than this standard (consistent reactivity of up to 1.8 dilution of an estimated 5-39 copies/mL specimen), yet failed to unequivocally test positive at the minipool dilution levels currently proposed for batch screening. This indicates that single donation NAT testing may be needed to potentially close the window of infection potential with preseroconversion blood donations.

There is at least 1 animal model (chimpanzee) study implying that the window period extending from infection until antibody detection has a noninfectious incubation (eclipse) phase, followed by an infectious phase when viral nucleic acids can be detected in the peripheral blood. This study showed that HIV NAT assays could detect virus during the entire infectious phase and hence could virtually eliminate transfusion-transmitted HIV infection. Translating the results from this model to human HIV infection may be problematic, however, and points to the need to identify and characterize other HIV transmission cases where initial HIV testing has failed to help address the critical question of the relationship between viral load and infectivity during the early window phase. Finally, although it is clear that nucleic acid testing will likely further minimize the already low risk of HIV infection transmitted via blood transfusion, there is a continual need for improvement in donor risk factor screening and the development of pathogen inactivation procedures, blood substitutes, and more sensitive, technically simple tests.

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Financial Disclosures: Dr Gallarda is director for Blood Screening Development, Roche Molecular Systems; testing performed for this study is for investigational use only (no donors are implied or otherwise involved). Dr Phelps has Chiron stock and stock options and is Divisional Vice President of Blood Testing. In conjunction with Ortho Diagnostics Systems, Chiron manufactures several ELISA kits used for detection of infectious agents, including HIV. In conjunction with Gen-Probe Inc, Chiron is evaluating the Chiron Procleix HIV-1/HCV assay under IND review in the United States and supports this assay commercially in European and Australian markets. Dr Bush received research grants or funding from NHLBI, from the CDC, from Roche Molecular Systems for field testing and assay development of a new kinetic polymerase chain reaction device, and from Chiron Corporation for testing new HCV and HIV nucleic acid technology (all monies go to Blood Centers of the Pacific, San Francisco); received honoraria and/or lecture sponsorship from Ortho Johnson & Johnson, Abbott Diagnostics Systems, Johns Hopkins University, South Florida Blood Systems/University of Southern Florida, Chiron Corporation, and European Plasma Fractionation Association; is a consultant for Haemonetics (Brantree, Mass) and scientific advisor for AC-ROMetrix (Berkeley, Calif); is a member of the DHHS Advisory Committee on Blood Safety and Availability; held positions such as President, Vice-President or CEO, and Research, Biotechnology, Research, Biopharmaceuticals, Pacific, and Vice-President, Research, Blood Systems Inc, Scottsdale, Ariz; and was involved with contributing test data for Chiron assay kits or reagents, Roche Molecular Systems Amplipcr HIV Monitor Kits, and COBAS Amplipcr HCV Monitor Kits.

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