

A STUDY TO IMPLEMENT EARLY DIAGNOSIS OF HIV INFECTION IN INFANTS BORN TO INFECTED MOTHERS

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Abstract. A protocol for detecting HIV DNA from specimens collected on filter papers and the effect of storage temperatures on determination of HIV DNA from dried blood spots has been developed and optimized. Blood specimens collected from HIV-1 infected and normal persons were spotted onto blood collection cards (Whatman BFC 180). The HIV DNA was extracted by phenol-chloroform-isoamyl alcohol and was detected for C2V4 of HIV-1 env by nested polymerase chain reaction (nested PCR). One set was stored at -20°C for 14 weeks, another at 37°C for 1 week and then kept at -20°C for 13 weeks and a third set at 25°C for 1 week and then -20°C for 13 weeks. The dried blood spots from each set were detected for the HIV DNA every 2 weeks for 14 weeks. The C2V4 region of HIV env DNA was determined from small amounts of the dried blood collected on the filter papers. The nested PCR procedure could detect as few as 5 copies of HIV proviral DNA, and HIV DNA could be detected from specimens with viral loads of 2×10^4 copies/ml. HIV DNA could be detected from specimens collected at all temperatures tested for at least 14 weeks. Therefore, laboratory diagnosis of HIV infection can be done by PCR on dried blood spots. These techniques will be useful as a tool for studying the epidemiology of HIV infection among populations of interest such as mother to child infection using newborn screening specimens.

INTRODUCTION

Infection with human immunodeficiency virus 1 (HIV-1), a virus that causes AIDS, has increased public health and socioeconomic problems around the world. Moreover, lack of effective treatment and prevention leads to extensive global epidemic. The UNAIDS estimated that newly infected children (0-15 years) with HIV in 2000 were approximately 600,000 (UNAIDS, 2000). Without intervention, 15-30% of babies born to HIV-1 infected mothers are infected either in utero or during labor (Sperling *et al*, 1996), and 10-15% are infected through breastfeeding (Dunn *et al*, 1992).

In Thailand, where subtype E HIV-1 infection is predominant, the national HIV surveillance data by mid 1998 revealed that HIV-1 prevalence among pregnant women was 1.53% in all age groups (Division of Epidemiology, 2000). The perinatal transmission rate for subtype E is 24% (Shaffer *et al*, 1999). Antiretroviral treatments demonstrated that prophylactic administration could prevent HIV transmission. The treatments, however, revealed several adverse effects (Visnegarwala *et al*, 1997; Eastone and Decker, 1997; Brinkman K *et al*, 1998). Direct or indirect effects of antiretroviral drugs on infants have not yet been reported. Owing to the high

cost of the antiretroviral drugs, several trials of short course treatment were studied in developing countries, including Thailand (Shaffer *et al*, 1999a; Guay *et al*, 1999; Wiktor *et al*, 1999). It was clearly shown that a short course of oral zidovudine trial in Thailand could lessen the risk of mother-to-child HIV-1 transmission by half, some infants, however, were still infected (Wiktor *et al*, 1999). In addition, not all children were infected from untreated HIV-positive mothers. Early diagnosis of perinatal HIV infection in infants is important for medical management and family counseling. Several serological techniques such as Enzyme Linked Immunosorbent Assay (ELISA) and Western Blotting have been commonly used to diagnose HIV-infected individuals. However, these serological tests are not useful in identifying infected infants prior to 18 months of age (Jorg *et al*, 1994). A DNA polymerase chain reaction (PCR), which amplifies and detects proviral HIV DNA, is known to be a sensitive and specific method for early diagnosis of HIV perinatal infection. In fact, a number of perinatal transmission studies (Ou *et al*, 1988; Simmonds *et al*, 1990; Young *et al*, 2000) used the PCR techniques to diagnose HIV-infected infants from whole blood. The disadvantage of this technique was the difficulty in collecting enough blood (1-2ml) from infants. Moreover, the storage and transportation were cumbersome. An alternative method

to collect blood from infants was to spot it onto filter papers. Dried blood spot (DBS) has been widely used in neonatal screening programs for metabolic and genetic disorders, including Thailand. The use of PCR as a diagnostic tool for detection of HIV-1 DNA was evaluated and the effect of storage temperatures on determination of HIV DNA from specimens collected as dried blood spots was examined.

MATERIALS AND METHODS

Specimens and preparation of blood spot specimens

Twenty HIV-infected EDTA blood were obtained from diagnosed patients of an anonymous clinic of the AIDS Research Center and The Thai Red Cross Society. Eleven HIV non-infected EDTA blood samples were collected from healthy donors of the National Blood Bank Center, The Thai Red Cross Society. All blood samples were spotted (50 µl for each spot) onto filter paper (Whatman BFC 180). The spots were allowed to dry at room temperature (25°C) for 18 hours.

To study the effect of temperatures for determination of HIV DNA from dried blood spots (DBS), the filter papers of each specimen were divided for storage into 3 sets: set 1 kept at -20°C for 14 weeks, set 2 kept at 37°C for 1 week and further stored at -20°C for another 13 weeks and set 3 kept at 25°C for 1 week and further stored at -20°C for another 13 weeks. DNA extraction was performed every 2 weeks after collection.

DNA extraction from dried blood spots

A quarter-inch diameter of each blood spot was punched from the filter paper with a sterile, flamed puncher and was transferred to a microcentrifuge tube. To lyse red blood cells, one milliliter of hemolysis buffer (0.32 M sucrose, 10 mM Tris-HCl pH 7.4, 5 mM MgCl₂, 1% Triton-X 100) was added twice to each tube, rotated at 100 rpm for 30 minutes at room temperature, and centrifuged at 10,000 rpm for 10 minutes. The remaining red blood cells were lysed with sterile distilled water for another 20 minutes. DNA was extracted from the filter paper by 3 different methods.

(a) The DNA was recovered from the DBS by incubation with occasional agitation in 200 µl of 5% chelex-100 solution (Bio-Rad, USA) at 56°C for 20 minutes and then at 100°C for 8 minutes. The eluate were stored at -20°C or immediately used for PCR.

(b) The DBS was digested at 37°C for 18-24 hours in 2.5x buffer (20 mg/ml glycogen, 20% SDS, 0.5 M EDTA,

0.3 M Sodium acetate pH 6.5) containing 2 mg/ml proteinase K. After incubation, the sample was extracted with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1). The DNA was precipitated overnight at -70°C in 3M sodium acetate and 2.5 volumes of 100% ethanol and recovered by centrifugation at 10,000 rpm for 20 minutes. DNA pellets were washed in 70% ethanol, air dried, and reconstituted in 20 µl of sterile deionized distilled water.

(c) The DNA was first extracted by the DBS in 5% chelex-100 solution and then further extracted by adding proteinase K according to the method described in A and B.

DNA amplification

A nested PCR procedure to detect HIV proviral env sequences (C2V4 region) in DBS was performed using 5'ACAGTACAATGTACACATGG 3' and 5' TCACTTCTCCAATTGTCCCTCA 3' as outer primers and 5' CTGTAAATGGCAGTCTAGC 3' and 5' GATGGGAGGGGCATACAT 3' as inner primers (Chen *et al*, 1999). Amplification was performed for 30 cycles on an automated DNA thermocycler (Perkin-Elmer-Cetus, Emeryville, CA, USA). The extracted DNA was denatured at 94°C for 2 min. The thermoprofiles were 95°C, 30s; 60°C 30s; and 72°C 60s. The final extension of the last cycles was at 72°C for 7 min. Amplified product of 525 bp was detected directly by agarose gel electrophoresis (2% SeaKem LE agarose; FMC Bioproducts) and stained with ethidium bromide.

All DBS extracts, negative with env primers, were subjected for β-globin gene amplification using 5' TCCTAAGCCAGTGCCAGAAG 3' and 5' AACTGAGTGGAGTCAAGGCT 3' as primers (Saiki *et al*, 1985) to indicate that the DNA was preserved and no inhibitors were presented in the PCR amplification.

RESULTS

Comparison of methods for DNA extraction

Three different methods were used to extract DNA from DBS of one infected individual. To assess the quality of each DNA eluate, the nested PCR was performed using env primers. Amplified products of 525 bp were observed in all 3 methods of extraction (Fig 1). It was shown that extraction with proteinase K generated less non-specific signals than other methods. The proteinase K method was therefore chosen to extract DNA from other DBS specimens in this experiment.

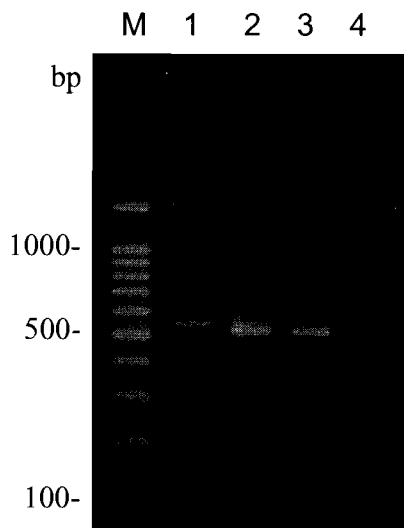


Fig 1. An agarose gel electrophoresis of HIV env amplification products of the same specimen via 3 methods of DNA extraction. Lane M; 100 bp DNA ladder marker, lane 1; 5% Chelex-100 method, lane 2; 5% Chelex-100 + Proteinase K, lane 3; Proteinase K, and lane 4; distilled water (negative control).

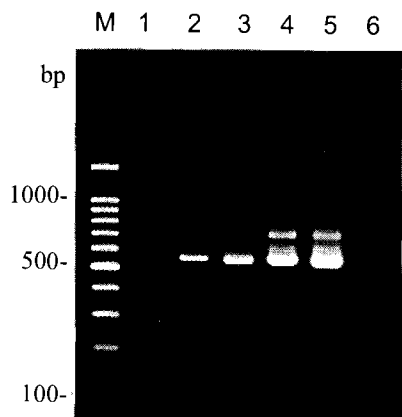


Fig 2. Sensitivity of the nested PCR amplification. Lane M; 100 bp DNA ladder marker, lane 1; DBS with no ACH-2 cells, lane 2; DBS with 5 ACH-2 cells, lane 3; DBS with 10 ACH-2 cells, lane 4; DBS with 50 ACH-2 cells, lane 5; DBS with 100 copies ACH-2 cells and lane 6; distilled water.

Sensitivity of HIV-PCR detection

The sensitivity of the method for HIV detection was assessed using dried blood spots spiked with varying numbers of ACH-2 cells, containing 1 HIV DNA copy per cell (Clouse *et al*, 1989), in EDTA blood of an HIV-seronegative donor. Assuming random distribution of the infected cells, the blood was applied to the filter paper. Thereafter, 0, 5, 10, 50, and 100 copies per spot were tested. The PCR method was able to detect at least 5 copies of proviral HIV DNA per PCR (Fig 2).

Detection of HIV DNA from clinical samples

The nested PCR was used to detect HIV DNA on DBS from four infected individuals with known viral loads. Positive DBS PCR products were found in some specimens (Fig 3) whereas all specimens gave positive products of b-globin genes (Fig 4). It was shown that positive PCR products could be detected from specimens with viral loads $> 2 \times 10^4$ copies/ml.

PCR amplification using DNA extracted from whole blood of the same infected individuals was also performed. The results were similar to the findings using DNA extracted from DBS (data not shown).

Determination of HIV DNA from dried blood spots stored at various temperatures

DNA was extracted every 2 weeks from the filter papers kept at 3 different temperatures. The nested PCR was done to amplify C2V4 region of HIV-1 env. It was shown that the PCR products of 525 bp were detected from all HIV-infected specimens kept at all temperatures upto 14 weeks (Fig 5, shown only specimen numbers 16-20). The amplification products from DBS of HIV non-infected patients were not detected (data not shown).

DISCUSSION

Increases in HIV prevalence among pregnant women in Thailand are resulting in a sharp increase in the number of children-born-to-infected mothers. Vertical transmission mostly occurs during late pregnancy. Women who seroconverted after routine antenatal HIV testing would not normally be identified. Early determination of infant infection status is extremely useful for future care. DNA polymerase chain reaction has been used for several years to diagnose perinatal HIV-1 infection, from either whole blood or dried blood spots, with high sensitivity and specificity (Cassol *et al*, 1991; Cassol *et al*, 1994; Biggar *et al*, 1997). However, it can also be determined by commercial kits that are too expensive for developing countries to afford as a diagnostic tool.

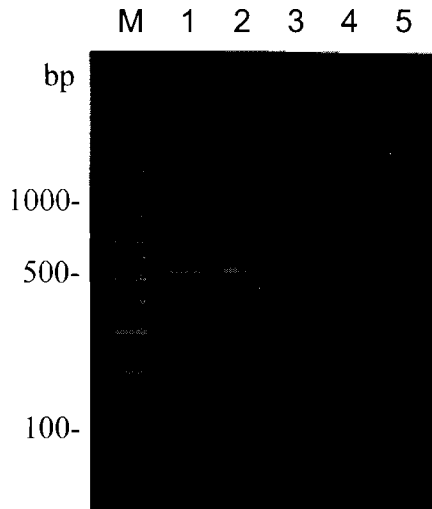


Fig 3. Detection of env amplification product of DBS from HIV-infected individuals. Lane M; 100 bp DNA ladder marker, lane 1; sample 98958, lane 2; sample 02561, lane 3; sample 52518, lane 4; sample 98573 and lane 5; distilled water.

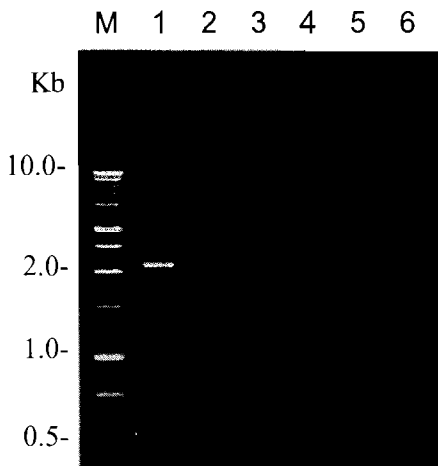


Fig 4. Detection of β -globin PCR product of DBS from HIV-infected individuals. Lane M; 10 kb DNA marker, lane 1; positive control (normal blood), lane 2; sample 98958, lane 3; sample 02561, lane 4; sample 52518, lane 5; sample 98573 and lane 6; distilled water.

A sensitive method for detection of HIV DNA from small amount of dried blood collected in the filter papers, is currently being studied. Our nested PCR procedure was capable of detecting as few as 5 copies of HIV proviral DNA. HIV DNA could be detected from specimens with viral loads of 2×10^4 copies/ml.

In the preliminary study, PCR products could not be detected in some DBS collected from anti-HIV seropositive persons. Possible reasons of PCR negative results may have been; a low viral titer, the presence of inhibitors, storage system ((Innis *et al*, 1990) and/or genetic variation. Repeated PCR amplification was necessary to ensure results, using specimens with different times of collection. Because of a small number of DBS tested in this study, there were no false-positive results. However, these might have occurred if more samples had been tested. False-positive results might be due to specimen contamination, carryover of PCR product or non-specific amplification of genomic sequences.

The stability test was performed to simulate actual collection and transportation systems occurring in rural areas. There was no difference in diagnosis of HIV infection from specimens kept at any of the tested temperatures. This suggested that DNA was stable in specimens collected as dried blood spots.

In conclusion, the blood samples collected on the filter paper appeared to be a reliable source for HIV-detection by PCR. Large numbers of DBS samples could be easily collected and stored for retrospective analysis or shipped to other laboratories for testing. Further studies on accuracy, precision, specificity and limitation of our PCR system would be necessary in order to implement this detected system. DNA extracted from DBS could also be kept for genetic studies related to the Thai population.

ACKNOWLEDGEMENTS

We are grateful to Professor Praphan Phanuphak, Ms Sunee Sirivichayakul, Mr Somboon Nookhai of AIDS Research Center, The Thai Red Cross Society for providing HIV-infected blood, and the staff of the National Blood Bank Center, The Thai Red Cross Society for providing blood from their donors. We also thank Ms Weena Treesangsrri, Ms Walailuk Wanawichet and the staff of the radioisotope units, National Institute of Health, Thailand for their assistance with laboratories.

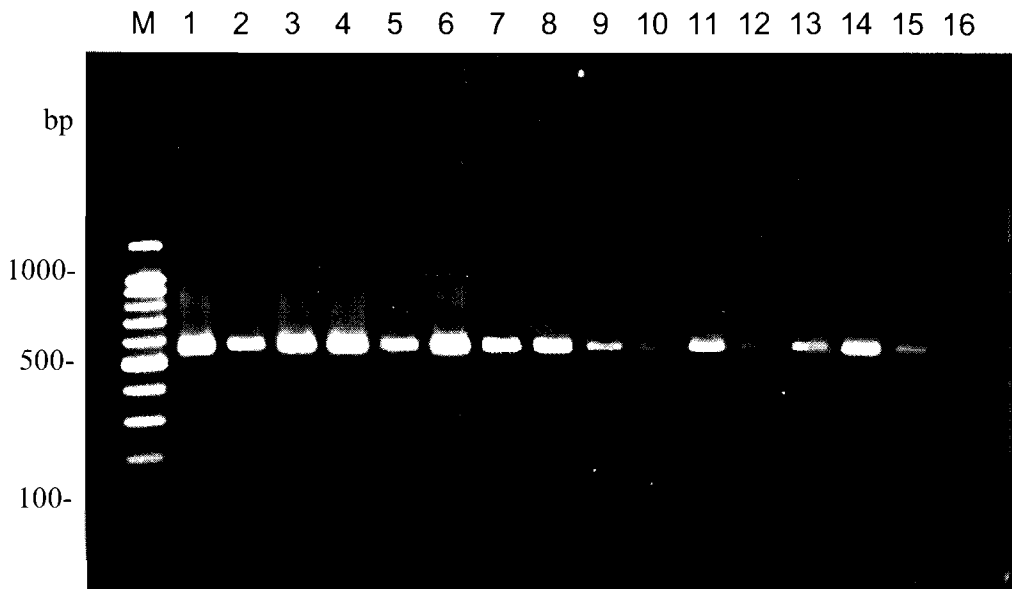


Fig 5. Detection of HIV env PCR products after collection at various temperatures for 14 weeks. Lane M; 100 bp DNA ladder marker, lane 1-5 samples 16-20 kept at -20°C, lane 6-10; samples 16-20 kept at 37°C, lane 11-15; samples 16-20 kept at 25°C and lane 16; distilled water.

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