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Diagnosis of Perinatal HIV-1 Infection by In-House PCR

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SUMMARY **A study on how to apply PCR as a diagnostic test for the infants born to HIV-1 infected mothers is described. All steps including clinical care, blood sampling, specimen processing and PCR analysis were carried out using native facilities and personnel. An open cohort of 130 children was evaluated at birth, 1, 6, 9, 15, and 18 months of age. Definite infection status was assessed by clinical and serological data during an 18 months of follow up period. PCR results were reported as positive or negative when at least 2 concordant data were denoted. This in-house PCR, compared to known infection status, gave 100% sensitivity and 94.4% specificity within 6 months after birth. On the other hand, clinical diagnosis could identify only the infected infants at 9 months of age. The HIV-1 transmission rate from mother to infant was 23.2%. Though this PCR was not at an optimal level of specificity, it was still beneficial to identify uninfected infants in the first year of their lives and avoid unnecessary medical care. Here, we report an in-house PCR that offers good performance at low cost for the diagnosis of HIV-1 vertical transmission.**

Early diagnosis of perinatal HIV infection is a prime step towards appropriate medical care and social management for the family. Usually, a clinical diagnosis of these infants can not be made before the first six months of their lives. Prophylactic strategies for opportunistic pathogens and other medical services may not be as effective if the HIV infection status has not been specified. For laboratory diagnosis, common serological tests fail to discriminate infected ones before 15-18 months after birth.[1] Some new assays like IgM and IgA specific EIA are still not readily available. Virus culture is impractical for diagnostic purposes in many developing countries.

Polymerase chain reaction (PCR), by virtue of its capacity to amplify minute amount of the target DNA, is frequently cited as the most sensitive test for early diagnosis of HIV infection.[2,3,4,5] However, the application of PCR in Thailand is restricted largely to research work in universities and other research units. One remarkable factor affected the test performance is that, these reports occupy the optimal conditions for both specimen handling and analysis which it is intriguing as to whether such systems could be applied for routine service work.

Therefore, we evaluated PCR as a diagnostic test for newborns. We used in-house PCR which had been proven sensitive and specific in native HIV-1 infected people in Thailand.[5,6] The cohort described here was selected from remote areas where specimen collection, transportation and PCR analysis were exactly the same as those done in other special laboratories in Thailand. This report will not only show that PCR diagnosis can be applied for early detection of the new-

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borns, but also demonstrate the effectiveness of our referral system in Thailand.

MATERIALS AND METHODS

Clinical specimens

The cohort comprised 130 children born to HIV-1 seropositive mothers at the Chiang Rai Regional Hospital in northern Thailand during October 1993 to July 1996. All infected mother were positive by ELISA, Western blot and PCR (see below). All mothers had willingly signed their consent forms, without any special incentive, after receiving a thorough explanation of the project. Their children were followed-up, in a Well-Baby Clinic, by a pediatric team. The clinical status was classified by provisional WHO pediatric clinical case definitions of AIDS[7] and symptomatic cases by CDC.[8] Blood specimens were collected from the children at birth, 1, 6, 9, 15 and 18 months after birth. On each visit, person identification, medical care and milk powder, including appropriate counseling were given. The whole blood specimens were transported, on wet ice, by air-freight and were processed within 3 days after sampling. All laboratory analysis and specimen processing were performed in the Virus Research Institute, Department of Medical Sciences, Ministry of Public Health (MOPH), Thailand. Another 20 pairs of seronegative mother and their infants at various ages also followed-up, cross-sectionally, for negative control group.

Serological tests

All plasma from mothers and their infants were tested using a commercial ELISA kit (Genelavia Mixt, Sanofi Pasteur Diagnostic Ltd., France) and a commercial Western blot kit (HIV Blot2.2, Diagnostic Biotechnology Pte, Singapore). Seroreversion was documented at 18 months of age if the maternal antibodies had disappeared from the infants' blood.

PCR

HIV-1 proviral DNA was identified in whole blood by an in-house nested PCR using the primers derived from the pol region.[4,5] Briefly, packed cells were lysed twice with a lysis buffer (0.32 M sucrose, 25 mM Tris pH 7.5, 2.5 mM $MgCl_2$ and 1% Triton X-100). The cellular debris was then pelleted and digested in 200 mg/ml Proteinase K in 1x Taq-buffer and 1% NP-40. After completion of digestion at 56°C for 4 hours, Proteinase K was heat inactivated by boiling for 15 minutes. For outer PCR reaction with primer JA17/JA20, 5 µl of the lysate was added to 45 µl of PCR mixture and subjected to be amplified for 30 cycles in DNA thermal cycle-480 (Perkin-Elmer-Cetus). Then, 2 µl of the outer product was amplified further for 25 cycles in a final volume of 20 µl of inner PCR reaction using primer JA18/JA19. The inner product of 130 bp was detected by agarose gel electrophoresis. For each experiment, a positive control (5 copies of 8E5 cells) and a negative control were included to check the system for possible contamination. For checking any possible PCR inhibitors in the sample, a segment of β-globin gene was amplified using primers PC03/PC04.[4] The sequence for the primers and the amplification cycles were illustrated in Table 1.

RESULTS

Follow up of the infants

From October 1993 to July 1996, 598 serial specimens were collected from 130 children. 35 (26.9%) were lost from follow up before 3 visits and their infection status could not be specified. Of those 22 HIV-1 infected infants, 13 cases were symptomatic infected cases (category A and B, CDC criteria) and 1 asymptomatic infected case as defined by persistence of anti-HIV antibody at 18 months of age. Only 3 AIDS cases died during 3-7 months after birth while other 18 infected cases were alive at the end of this study. The remaining 73 children seroreverted and showed no clinical sign of HIV infection, so the maternal-infant transmission rate was 23.2% (22/95).

Raw PCR data at each age of the infants.

All seropositive mothers were positive by PCR (120/120) while seronegative mothers and their infants were negative (40/40). Of the 22 infected children, PCR was positive for 68.2% (15/22) at birth and 100% for all later venipuncture (Table 3).

For 73 uninfected children, PCR was negative for 95.4% (62/65) at birth and 83.3-98.4% at the later 5 sampling ages (Table 3). False positive rate varied from 1.6-16.7% at various ages.

Rating and report PCR result

Definitive PCR results were determined by concordant results for 2 consecutive specimens. Discordant results were rated as PCR

indeterminate and were analyzed again with the next specimens. By this protocol, all definite HIV infected children were reported PCR positive between birth and 6 months of age for infected ones and report negative 94.4% for the same age of infected cases. For those ultimately determined to be HIV negative by 18 months of age, 94.4% tested negative by PCR (Table 4).

Sensitivity and specificity

Using clinical and serological data as the gold standard, sensitivity of PCR was 100% (20/20) and specificity was 94.4% (67/71) at 6 months of age. Among the 4 false positive cases, 2 cases could be clarified as negative PCR by 3 analyses.

DISCUSSION

Maternal to child transmission rate

Twenty-two of 95 children (23.4%) followed from birth to 18

Table 1. The primers and amplification cycle[a]

Primer	Sequences (5' - 3')	Location	Usage
JA17	TAC-AGG-AGC-AGG-TGA-TAC-AG	2431-2450	outer primer
JA20	CCT-GGC-TTT-AAT-TTT-ACT-GG	2678-2697	outer primer
JA18	GGA-AAC-CAA-AAA-TGA-TAG-GG	2481-2500	inner primer
JA19	ATT-ATG-TTG-ACG-GGT-GTA-GG	2591-2610	inner primer
PC03	ACA-CAA-CTG-TGT-TCA-CTA-GC	human β-globin gene	inhibitors check primers
PC04	CAA-CTT-CAT-CCA-CGT-TCA-CC		

Amplification cycle

Outer reaction and β globin: 30 amplification cycles

94°C, 5 min.	55°C, 1 min.	72°C, 1 min.	1 cycle
94°C, 1 min.	55°C, 1 min.	72°C, 1 min.	9 cycles
88°C, 1 min.	55°C, 1 min.	72°C, 1 min.	19 cycles
88°C, 1 min.	55°C, 1 min.	72°C, 7 min.	1 cycle
Soak at 4°C			

Inner reaction: 25 amplification cycles.

85°C, 1 min.	55°C, 1 min.	72°C, 1 min.	24 cycles
85°C, 1 min.	55°C, 1 min.	72°C, 7 min.	1 cycle
Soak at 4°C			

[a]Original sequence data of all primers are according to reference 4. Amplification profiles are according to references 5 and 6.

Table 2. Follow-up rate for children of HIV infected mothers and seroreversion

	Age (months)					
	0	1	6	9	15	18
Number of children	130	116	102	95	81	74
% follow-up	100	89.2	78.5	73.1	62.3	56.9
% seroreversion	0	0	0	9.5	81.5	85.1

Table 3. PCR results at each age in 22 HIV infected children (upper) and 73 uninfected HIV children (lower)

	Age (month)					
	0	1	6	9	15	18
No. PCR positive	15/22	18/18	15/15	15/15	10/10	8/8
% positive	68.2	100	100	100	100	100

	Age (month)					
	0	1	6	9	15	18
PCR negative	62/65	60/72	61/69	63/68	67/70	61/62
% negative	95.4	83.3	88.4	92.6	95.7	98.4
% false negative	4.6	16.7	11.6	7.4	4.3	1.6

Table 4. Sensitivity and specificity for HIV infected children (upper) and HIV uninfected children (lower) of the PCR assay using 2 concordant results

	Age (month)			
	0, 1, 6	1, 6, 9	6, 9, 15	9, 15, 18
Report PCR positive (3/3 - 2/3 = positive)	20/20	21/21	15/15	11/11
% positive (sensitivity)	100	100	100	100

	Age (month)			
	0, 1, 6	1, 6, 9	6, 9, 15	9, 15, 18
Report PCR negative (3/3 - 2/3 = negative)	67/71	69/71	69/71	69/71
% negative (specificity)	94.4	97.2	95.8	97.2
False positive	4	2	3	2

months of age were identified as HIV infected cases as defined by clinical status, HIV antibody at 18 months, and by PCR. This transmission rate was consistent with the 21-42% previously reported in Thailand[9] and 24.2% reported by the HIV/AIDS Collaboration, Thailand, in Bangkok Collaborative Perinatal HIV Transmission Study.[10]

In this open cohort, lost from follow up rate was 26.9% (35/130) within 34 months. The rate of follow up was compromised due to 6 visits with blood drown every visits during each 18 months intervals and the attendees were not received any special support except milk powder and medical care.

Raw PCR data

Among children ultimately shown to be HIV infected, 7/22 (31.8%) were PCR negative at birth. All subsequent specimens were positive. PCR positive rate at birth (68.2%) was similar to other reports.[11,12,13] Some possible sources of error may be miss-identification of the infants, labeling errors of specimen and the quality of the cold chain for specimens that were stored at 4°C up to 3 days before processing. Some intrinsic limitation of the newborn specimens may include low viral content at delivery and limited volume of blood (Ca. 0.5-1 ml).

Among the 73 uninfected children (Table 3), there were a number of false positive PCR assays at each age (overall 32/406 = 0.79%). Single false positive at 6 analyses was observed in 19/32 (59.4%). Only 4 children had false positive results more than 2 times and such false positive PCR was accounted to 13/32 (40.6%). Main causes of false positive data may come from sample labeling and cross contamination at the collection point or in the laboratory. Another possible explanation of multiple persistence false positive PCR may be late viral clearance as described in some reports.[14,15,16]

Protocol for rating PCR results

Definitive PCR results were derived from 2 consecutive concordant data from 3 consecutive specimens (Table 4). By this protocol, PCR gave 100% sensitivity and 94.4% specificity during birth to 6 months of age. This protocol improved both sensitivity and specificity, similar to the guidelines used of PCR by CDC.[8] There is no difference between primer pair concordance, both sensitivity and specificity, as described earlier.[17] Sensitivity and specificity were 100% as evaluated in Thai specimens.[5,6]

Application of PCR for diagnosis of vertical HIV infection in Thailand portends a great advantage for the noninfected infants since 75-85% of the children born to HIV seropositive mothers are treated as those that are infected. An established PCR should be thoroughly studied before implementation. Firstly, optimal conditions for laboratory analysis and sample management must be assessed. Secondly, the expense of PCR should be affordable . By our in-house PCR, we reduced the expenses 20 fold compared to that of a commercial HIV-PCR (2.3 by 44.4 US dollars a test). Finally, the quality control and standardization of PCR are extremely important. From our data, false diagnosis was still a problem and thus, we emphasize the need to monitor HIV-PCR test by reliable laboratories.

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